Enzyme-aided recovery of protein and protein hydrolyzates from rapeseed cold-press cake

Katariina Rommi

VTT SCIENCE 130

Enzyme-aided recovery of protein and protein hydrolyzates from rapeseed cold-press cake

Katariina Rommi

Thesis for the degree of Doctor of Philosophy to be presented, with due permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in auditorium 2041, Biocenter 2 at the University of Helsinki, on June 10th 2016 at 12 noon.

ISBN 978-951-38-8433-8 (Soft back ed.)
ISBN 978-951-38-8434-5 (URL: http://www.vttresearch.com/impact/publications)

VTT Science 130

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8434-5

Copyright © VTT 2016

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

Cover image: Dr. Ulla Holopainen-Mantila and Dr. Juhani Sibakov

Juvenes Print, Tampere 2016

Preface

This study was performed at VTT Technical Research Centre of Finland Ltd during the years 2012–2016. The research received funding from the European Community's Seventh Framework Programme FP7/2007–2013 under grant agreement number 289170–APROPOS, and from the Jenny and Antti Wihuri Foundation. Funding was kindly provided by the Doctoral Programme in Food Chain and Health for participation in conferences and courses.

I wish to address my deepest thanks to my thesis supervisors for their invaluable guidance during the PhD work. I am grateful to Professor Kaisa Poutanen for her expert advice in compiling the results into publications and in writing the thesis. I wish to thank Dr. Raija Lantto and Dr. Terhi Hakala for entrusting me with this interesting research topic and for encouraging and supporting me to take responsibilities in the APROPOS project. I am thankful to my supervisor and team leader, Dr. Emilia Nordlund, for her dedication and critical advice in interpretation of the results of the experimental work.

I am sincerely thankful to my co-authors and colleagues, Dr. Ulla Holopainen-Mantila and Dr. Dilek Ercili-Cura, for their invaluable assistance in interpretation of the microscopy and protein analysis results, and to Sari Pohjola for her notable contribution in the experimental work of Publication III. My co-authors at Universitat Politècnica de Catalunya, Diana Rivera, Dr. Margarida Fernades and Professor Tzanko Tzanov are warmly acknowledged for fruitful collaboration.

Further thanks go to our project team in APROPOS, particularly Petteri Kangas and Marja Nappa, who taught me to perform and interpret techno-economic calculations. I am grateful to Dr. Lee Anne Murphy for organizing my three-month research visit to the University of Manitoba, Canada, and to Professor Peter Eck for supervising me during my visit.

I am sincerely thankful to Dr. Anne Pihlanto and Professor Birthe Møller Jespersen for preliminary examination of this thesis and for the encouraging feedback discussions. Professors Marko Virta and Annele Hatakka are acknowledged for supervising my doctoral studies and for guiding me in the graduation process on behalf of the University of Helsinki. I wish to also thank Professor Kati Katina for acting as a member of the thesis advisory committee.

VTT provides an excellent opportunity for young researchers to develop their scientific expertise and at the same time establish skills in multidisciplinary net-

working, project management and customer collaboration. I am thankful to Vice President Anu Kaukovirta-Norja for providing excellent research facilities for the work. I wish to thank my former team leader, Dr. Tiina Liitiä, and my former and current team members for a positive and inspiring work environment where support for the juniors is always available. I am grateful to VTT's skilled and helpful technical staff, particularly Heljä Heikkinen, Eero Mattila, Nina Vihersola and the staff of laboratory 251, microscopy laboratory and HPLC laboratory, for contributing to the experimental work and teaching me a number of analysis methods. I wish to thank Dr. Juhani Sibakov for his kind and skillful advice in milling and dry fractionation, and Atte Mikkelson for the carbohydrate analytics.

Especially warm thanks go to my peers, Katariina Kemppainen, Ronny Wahlström, Piritta Niemi, Miriam Kellock, Saara Hanhikoski, Lauri Reuter, Helena Henno, Outi Mäkinen, Ilkka Kajala, Outi Mattila, Kirsi Kiiveri, Jenni Rahikainen and many others for the knowledge sharing, reviving lunch breaks and many of the most cheerful moments of the PhD work.

My friends and family have regularly refuelled me with positive energy during the years of PhD work. I am grateful to my dear family, Leena, Vesa and Mikko, for their endless care and appreciation. My beloved fiancé, Anttipekka, I am sincerely thankful for your empowering love, patience and the happiness that you bring to my life.

Helsinki, April 2016

Katariina Rommi

Academic dissertation

Supervisors Professor Kaisa Poutanen
VTT Technical Research Centre of Finland Ltd

Dr. Raija Lantto
VTT Technical Research Centre of Finland Ltd

Dr. Emilia Nordlund
VTT Technical Research Centre of Finland Ltd

Dr. Terhi Hakala
VTT Technical Research Centre of Finland Ltd

Member of the thesis advisory committee
Professor Kati Katina
Department of Food and Environmental Sciences
University of Helsinki, Finland

Pre-examiners Dr. Anne Pihlanto
Natural Resources Institute Finland (Luke)

Professor Birthe Møller Jespersen
Department of Food Science
University of Copenhagen, Denmark

Opponent Professor Susan Arntfield
Department of Food Science
University of Manitoba, Canada

Custos Professor Marko Virta
Department of Food and Environmental Sciences
University of Helsinki, Finland

List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV.

I **Rommi K, Hakala TK, Holopainen-Mantila U, Nordlund E, Poutanen K, Lantto R.** 2014. Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (*Brassica rapa* L. and *Brassica napus* L.) press cakes. *Journal of Agricultural and Food Chemistry* 62: 7989−7997.

II **Rommi K, Ercili-Cura D, Hakala TK, Nordlund E, Poutanen K, Lantto R.** 2015. Impact of total solid content and extraction pH on enzyme-aided recovery of protein from defatted rapeseed (*Brassica rapa* L.) press cake and physicochemical properties of the protein fractions. *Journal of Agricultural and Food Chemistry* 63: 2997–3003.

III **Rommi K, Holopainen U, Pohjola S, Hakala TK, Lantto R, Poutanen K, Nordlund E.** 2015. Impact of particle size reduction and carbohydrate-hydrolyzing enzyme treatment on protein recovery from rapeseed (*Brassica rapa* L.) press cake. *Food and Bioprocess Technology* 8: 2392–2399.

IV **Rivera D, Rommi K, Fernandes MM, Lantto R, Tzanov T.** 2015. Biocompounds from rapeseed oil industry co-stream as active ingredients for skin care applications. *International Journal of Cosmetic Science* 37: 496–505.

The publications are reproduced with kind permission from the publishers. In addition, unpublished results related to Publications II and IV and a techno-economic evaluation of rapeseed protein extraction are presented in the summary.

Author's contributions

I Katariina Rommi was responsible for planning the study with Terhi Hakala and Raija Lantto, and for execution of the experimental work. She performed the enzyme activity assays and part of the composition and protein analytics. She had the main responsibility for data analysis, interpretation of results and writing of the publication together with all co-authors.

II Katariina Rommi was responsible for planning the study with Terhi Hakala and Raija Lantto, and for execution of the experimental work, statistical analysis and gel electrophoresis. She had the main responsibility for data analysis, result interpretation and writing the publication in collaboration with all co-authors.

III Katariina Rommi designed and coordinated the experiments together with Emilia Nordlund. She supervised the research trainee, BSc. Sari Pohjola in execution of the experimental work and analytics for saline and alkaline extraction, and performed the experimental work for water extraction. She performed the statistical analyses, interpreted the results and wrote the publication in collaboration with all co-authors.

IV Katariina Rommi was responsible for production of protein hydrolyzates from dry-fractionated rapeseed press cake and analysis of hydrolyzates by gel electrophoresis. Katariina Rommi participated in interpretation of the results and writing the publication with MSc. Diana Rivera and Dr. Margarida Fernandes.

Contents

Preface ... 3

Academic dissertation ... 5

List of publications ... 6

Author's contributions ... 7

List of abbreviations ... 11

1. Introduction ... 12

1.1 Rapeseed production and processing ... 13
1.1.1 Varieties, production volumes and markets ... 13
1.1.2 Seed structure, composition and processing ... 14
1.1.3 Oil extraction ... 16
1.2 Rapeseed oil pressing residue ... 17
1.2.1 Proteins ... 18
1.2.2 Carbohydrates ... 19
1.2.3 Oil ... 20
1.2.4 Phenolic compounds ... 20
1.3 Human use perspectives of rapeseed protein ... 21
1.3.1 Nutritional quality ... 21
1.3.2 Technical functionality ... 22
1.3.3 Bioactive properties ... 24
1.3.4 Compounds limiting nutritional, technical and sensory quality ... 25
1.3.5 Potential applications ... 26
1.4 Fractionation technologies for rapeseed protein recovery ... 27
1.4.1 Defatting of press cake ... 27
1.4.2 Dry fractionation ... 28
1.4.3 Wet fractionation ... 29
1.4.4 Downstream processing of protein extracts ... 34
1.4.5 Use of enzymes in rapeseed protein extraction and hydrolysis ... 36
1.5 Prospects for market entry of rapeseed protein ingredients ... 40

2. **Aims of the study** 43

3. **Materials and methods** 44

3.1 Raw materials and enzymes 44
3.2 Pretreatment and dry fractionation 45
3.3 Carbohydrate-hydrolyzing enzyme treatment 46
3.4 Protein extraction and isoelectric precipitation 47
3.5 Production of protein hydrolyzates 49
3.6 Analytical methods 49
3.6.1 Analysis of structure and composition 49
3.6.2 Analysis of protein solubility and thermal properties 50
3.6.3 Analysis of bioactive properties 50
3.6.4 Statistical analysis 51
3.7 Techno-economic evaluation of production costs 51

4. **Results** 55

4.1 Composition and structure of raw materials 55
4.1.1 Influence of processing on the microstructure of rapeseed press cake 57
4.2 Enzyme-aided protein extraction 58
4.2.1 Effects of raw material pretreatments on protein extractability 59
4.2.2 Effect of carbohydrate-hydrolyzing enzyme treatment on protein extractability 60
4.2.3 Effects of extraction parameters on protein extractability 61
4.3 Composition and solution behavior of protein fractions 62
4.4 Techno-economic feasibility of rapeseed protein extraction schemes 63
4.5 Production of rapeseed protein hydrolyzates 65
4.5.1 Composition, degree of hydrolysis and molecular weight range of rapeseed protein hydrolyzates 65
4.5.2 Antioxidativity and enzyme inhibitory activity of rapeseed protein hydrolyzates 67

5. **Discussion** 68

5.1 Carbohydrate composition of raw materials 69
5.2 Factors influencing rapeseed protein extractability and recovery 70
5.2.1 Pretreatment 70
5.2.2 Carbohydrate-hydrolyzing enzyme treatment 73
5.2.3 Extraction conditions 75
5.2.4 Techno-economic feasibility of enzyme utilization in rapeseed protein extraction 77
5.3 Factors influencing the functional properties of rapeseed protein fractions 78
5.3.1 Technical functionality 78
5.3.2 Bioactivity 79
5.4 Limitations of the study 82
5.4.1 Design of experiments and data analysis 82

5.4.2 Analytics 83
5.5 Future prospects 85
6. Conclusions 87
References 89
Publications I–IV
Abstract
Tiivistelmä

List of abbreviations

ACE	angiotensin converting enzyme
ANOVA	analysis of variance
BCA	bicinchoninic acid
CAA	cellular antioxidative activity
d.m.	dry matter; the mass of dry material
DPPH	2,2-diphenyl-1-picrylhydrazyl
EBC	European Brewery Convention
EDTA	ethylenediaminetetraacetic acid
FAN	free amino nitrogen
FAOSTAT	Food and Agriculture Organization of the United Nations Statistics Division
HNE	human neutrophil elastase
kDa	kilodalton
LC	liquid chromatography
MPO	myeloperoxidase
ORAC	oxygen radical absorbance capacity
pI	isoelectric point; the pH at which the net charge of a protein is zero
RPI	rapeseed protein isolate
SC-CO_2	supercritical carbon dioxide
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Introduction

Growing global demand for food and feed protein drives the search for new, sustainable protein sources based on plants, insects and co-streams of the food industry. The environmental burden of meat and dairy production, along with health problems linked to excess meat consumption, have increased consumers' interest towards plant-based alternatives to animal-based foods. Plant proteins have potential to complement or replace animal proteins in various food applications, with successful examples based on e.g. soy and wheat proteins already on the market. However, the negative image of soy production and increasing demand for gluten-free products also open up a clear market potential for other plant protein sources. Particularly legumes, oilseeds, tuberous crops and their processing co-streams have been recognized as promising raw materials for sustainable production of new protein-rich food ingredients.

The present work focuses on enzyme-aided valorization of rapeseed cold-press cake into protein-rich fractions targeted for human use. Rapeseed is an economically important oilseed crop that represents the third largest source of vegetable oil after soybean and oil palm. After oil extraction approximately 60% of the seed mass is left as a protein- and carbohydrate-rich co-stream which is mainly used as animal feed. Depending on the extraction method this material can be designated as press cake, which remains after physical pressing, or meal, which is obtained when pressing is followed by solvent extraction.

The growth of rapeseed oil production, driven by its widely recognized health benefits and the global demand for liquid biofuel (biodiesel), has led to increased generation of oil pressing co-streams. In particular, consumer trends towards unrefined virgin rapeseed oil and concerns about the environmental and health risks of current extraction solvents are expected to drive new investments in cold pressing. Rapeseed cold-press cake (32–36% protein) is particularly interesting as a potential food protein source due to better conservation of protein quality during cold pressing than in the industrial hot pressing and solvent extraction process. Rapeseed proteins not only possess favorable nutritional properties, but they also contain bioactive peptides with potential health benefits. Production of protein and peptide ingredients for food and cosmetics would generate added value from rapeseed co-streams, and contribute to the required increase of plant-based food protein supply. A number of technologies have been developed for protein en-

richment from rapeseed meal, but their commercialization has faced challenges in part due to limited cost efficiency. Enzymatic methods provide a gentle means of biomass processing, and are investigated here as a way to improve the feasibility of protein and peptide extraction from rapeseed cold-press cake.

1.1 Rapeseed production and processing

1.1.1 Varieties, production volumes and markets

Rapeseed is a dicotyledonous plant belonging to the *Brassica* genus of the mustard family (*Brassicaceae*). It is related to common *Brassica* food crops such as mustard, broccoli, cauliflower, turnip, rutabaga, cabbage and brussels sprout (Downey, 1983; Bell, 1984). Rapeseed crops are considered to include the three closely related species, oilseed rape (*B. napus*), turnip rape (*B. rapa*, previously called *B. campestris*), and leaf mustard (*B. juncea*). The world's commerce is largely supplied by *B. napus* and *B. rapa*. *B. napus* is the major rapeseed crop in North America, most of Europe and China. *B. rapa* is cultivated in the northern parts of Europe and China, and is currently the dominant oilseed crop in Finland due to favorable climate conditions and fewer production risks. *B. juncea* is mainly cultivated in India, where the seeds are commonly used for spices and condiments. All crops contain spring and winter varieties.

Until the 20th century, rapeseed oil in Europe was mainly used for non-food purposes as the early rapeseed varieties were considered inedible due to high concentrations of potentially antinutritive erucic acid and glucosinotates in the seed (Bell, 1984). In the 1970s, major achievements in traditional plant breeding led to substantial reduction of these antinutritional factors in new *B. napus* and *B. rapa* varieties which have been adapted as the predominant cultivars in e.g. Canada, Europe, Australia and the United States. Canola refers to all Canadian "double-low" rapeseed cultivars containing less than 30 µmol / g of alkenyl glucosinolates in the oil-free seed, and in which erucic acid constitutes less than 2% of the total fatty acids in the oil (Bell, 1993).

Rapeseed is globally the second most abundant oilseed crop after soybean, with a 14% share of the overall oilseed cultivation (FAO, 2015), and the major oilseed produced in Europe. In 2013, rapeseed cultivation reached 73 Mt worldwide, 21 Mt in the European Union (EU 27), and 80 000 t in Finland. EU is by far the largest rapeseed producer, whereas the major individual production countries are Canada, China, India, Germany, France and Australia (Table 1). The development of rapeseed market from 1991 to 2012 was reviewed by Carré and Pouzet (2014). Global production of oilseeds (soybean, rapeseed, cotton seed, sunflower seed, linseed, copra and castor seed) has grown remarkably during the past 25 years; from ca. 190 Mt in 1985 to over 450 Mt in 2010. In Europe the clear increase of rapeseed cultivation has been linked to biodiesel production. Oilseed prices have been at historically high levels since 2010: rapeseed prices ranged between 280 and 500 €/t in 2010–2013, showing a marked increase from the

average price (286 €/t) of years 2004–2013. Since 2013 the seed prices have followed a downward trend together with the decreasing vegetable oil prices.

Table 1. Major production countries and production volumes of rapeseed.

Country	Production (Mt)
Canada	17.9
China	14.5
India	7.8
Germany	5.8
France	4.4
Australia	4.1
Poland	2.7
Ukraine	2.4
United Kingdom	2.1
Czech Republic	1.4
Russian Federation	1.4
United States of America	0.9
Denmark	0.7
Belarus	0.7
Romania	0.7

FAO (2015)

1.1.2 Seed structure, composition and processing

Plant seeds generally consist of three major components: embryo, endosperm and seed coat. As rape seeds mature the endosperm degenerates and the seed coat enwraps the embryo (Hu et al., 2013). Therefore in mature rape seeds, only two main seed components can be distinguished: 1) embryo (i.e. kernel), consisting of cotyledons and embryonic axis, and 2) seed coat (i.e. hull), consisting of epidermis, palisade and one line of aleuronic cells (Figure 1) (Hu et al., 2013). The embryo acts as a storage site for oil and protein which are concentrated in vacuoles inside the embryonic cells. Oil and protein bodies are also found in the thin aleuronic layer of rape seeds (Figure 1).

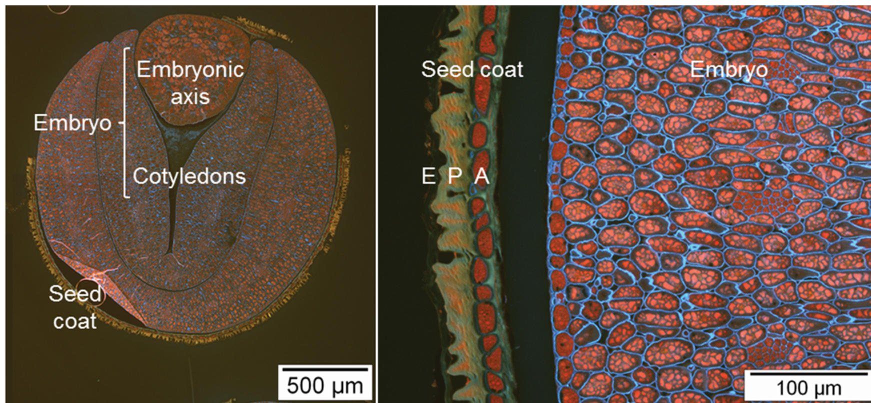

Figure 1. Micrographs of cross sections of turnip rape (*B. rapa*) seed. Seeds were sectioned and stained with Calcofluor White and Acid Fuchsin, showing cell wall glucans as blue and proteins as red, respectively. E) epidermis, P) palisade, A) aleurone layer. (Courtesy of Dr. Ulla Holopainen-Mantila, VTT.)

Seeds of the species *B. rapa* and *B. napus* are very similar in their structure and chemical composition: whole seeds of both species have been reported to contain approximately 44–46% crude fat, 21–23% crude protein, 12–13% carbohydrates, 4–6% acid-insoluble (Klason) lignin and 4% ash (Eriksson et al., 1994). More compositional variation occurs between cultivars with dark or yellow hulls. Hulls of the traditional dark-colored varieties comprise about 16% of rapeseed dry weight and contain cellulose (32%), pentosans (15%), protein (12–16%), pectin (15%), sugars (4%) lignin (12–28%) and other phenolics (6–12%) such as condensed tannins (1.5%) (Aspinall and Kuo-Shii, 1974; Bell, 1984; Eriksson et al., 1994). In addition, yellow-seeded “triple-low” cultivars containing thinner hulls with lower polyphenol content were bred in the 1970s to improve the nutritive value of rape-seed meal as animal feed (Theander et al., 1977).

Rapeseed is primarily cultivated for its oil which is separated by pressing and/or solvent extraction mainly for food or biodiesel. Although the seeds are commonly pressed without prior fractionation, a few small enterprises in Germany (Teuto-burger Ölmühle GmbH and Kroppenstedter Ölmühle Walter Döpelheuer GmbH) produce food oil from partially dehulled seeds. Dehulling partially removes the polyphenolic-rich hulls from rape seeds, which enables production of light colored, mild-tasting kernel oil by cold pressing (Rass and Schein, 2013). On the other hand, dehulling may reduce the oil yield during pressing and hinder the oxidative stability of cold-pressed oils due to lower tocopherol and phytosterol contents when compared to oils from whole seeds (Yang et al., 2011). It is notable that removal of the cellulose and polyphenolics-containing hulls also increases protein concentration and affects the carbohydrate composition of the press cake.

1.1.3 Oil extraction

Rapeseed oil is generally produced using hot pressing followed by solvent extraction, or using only physical hot or cold pressing (Figure 2). Cold pressing is a gentle oil extraction method in which oil is merely pressed from the seeds, commonly using a screw press. Cold pressing is favored at small production sites as an approach to produce virgin rapeseed oil with natural flavor (Pekkarinen et al., 1998; Yang et al., 2011). A major advantage of cold pressing is that protein solubility in the residual seed mass (i.e. press cake) is better retained than after hot pressing or solvent extraction due to the avoidance of high temperatures, although friction during pressing can cause temperatures to increase to about 50–60°C. Hot (pre-expeller) pressing is another physical method in which the seeds are heated before pressing to facilitate oil recovery (Pekkarinen et al., 1998).

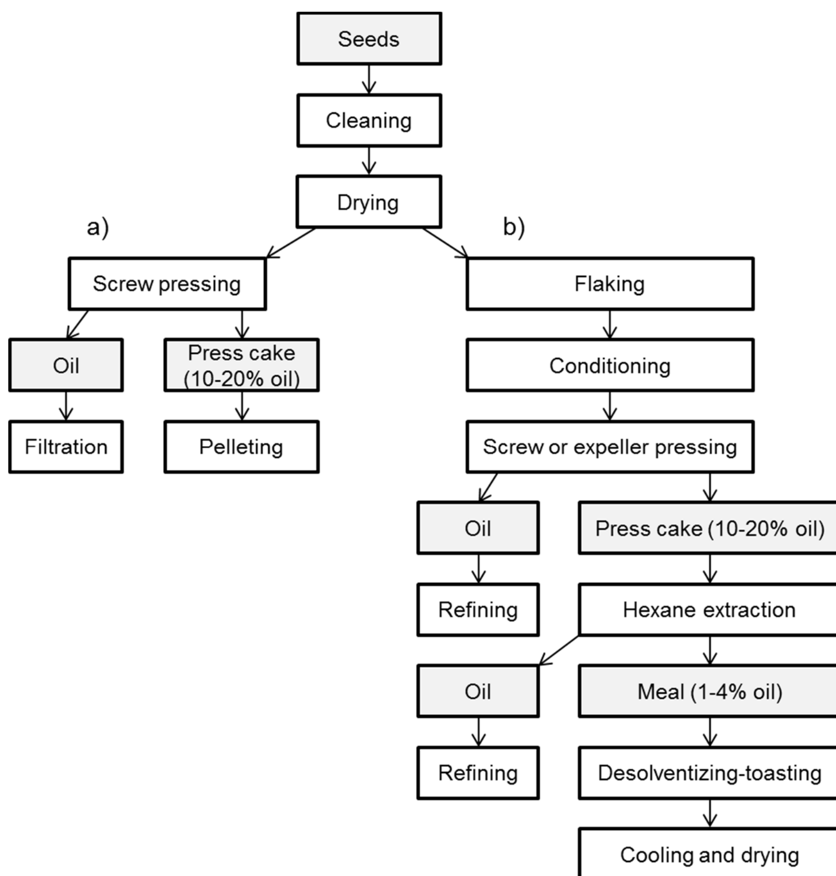

Figure 2. Production of rapeseed oil by a) cold pressing or b) hot pressing followed by solvent extraction.

In Finland the largest oil mill (>100 000 seed t/a) uses hot pressing whereas all smaller enterprises rely on cold pressing (Heinonen, 2001). Most of the rapeseed oil sold worldwide for food or biodiesel is produced by a combination of pressing and solvent extraction due to the high oil yield obtained by this technology. The process consists of several steps, in which the seeds are flaked using roller mills, conditioned at 80–100°C, and pre-pressed before removal of the remaining oil by hexane extraction. The residual seed mass (i.e. meal) is desolventized by heating (e.g. 30 min at 95–115°C) to produce a meal which contains less than 1% oil on a dry matter basis.

1.2 Rapeseed oil pressing residue

The residual seed mass from rapeseed oil extraction constitutes approximately 55-60% of the seed dry weight when calculated on the basis of the crude fat content of the seed (Eriksson et al., 1994). The residual seed mass after physical pressing is typically designated as press cake, and the defatted material after solvent extraction is designated as meal. Rapeseed cold-press cakes typically have a moisture content around 9%, and contain approximately 11–18% residual oil, 32–36% protein, 44% crude carbohydrates and 6% ash on a dry matter basis (Table 2) (Schöne et al., 1996; Spragg and Mailer, 2007). Cold pressing partially disrupts the cell structure of rapeseed along with disintegration of the oil bodies (Niu et al., 2012). Since seeds are generally pressed without dehulling, hull particles constitute approximately 30% of rapeseed meal.

Table 2. Composition of rapeseed cold-press cake.[a]

Component	Concentration (% d.m.)
Crude protein	32.1
Crude fat	18.0
Crude fiber	10.2
Crude carbohydrates [b]	43.6
Sugars	10.5
Pentosans	3.6
β-glucan	0.1
Starch	N.d.
Acid detergent lignin	8.1
Crude ash	6.3

[a] Schöne et al. (1996). [b] calculated as crude fiber + nitrogen-free extract. N.d.) not detectable.

Rapeseed press cake and meal are primarily used in feed formulations for dairy cattle, pigs, poultry and farmed fish. As reviewed by Carré and Pouzet (2014), meal prices ranged between 150 and 300 €/t in 2010–2012, corresponding to approximately 30–60% of the seed price and 55% of the price of soybean meal. Although rapeseed meal has only a slightly lower energy value than soybean meal, it is considered as a secondary feed due to its higher fiber and phenolic content. In terms of essential amino acids for nutrition, rapeseed meal is lower in lysine (similarly to cereals) but higher in the sulfur-containing amino acids, cysteine and methionine, than soybean meal, which makes it an interesting protein source also for human consumption. However, utilization of rapeseed press cake and meal as food is limited by several factors, including their high content of fiber and polyphenolics which reduce the digestibility of rapeseed meal, the presence of potential antinutrients such as glucosinolates and phytates, and their dark color and astringent taste resulting from the presence and oxidation of phenolic compounds, as reviewed by Xu and Diosady (2012).

1.2.1 Proteins

A major proportion of rapeseed proteins are globular storage proteins. Their composition and properties have been widely studied, as reviewed by Wanasundara (2011), Aider and Barbana (2011) and Tan et al. (2011). The main storage proteins, cruciferin and napin, constitute ca. 60% and 20% of the total protein, respectively, although the ratio can vary considerably between cultivars (Höglund et al., 1992). Cruciferin (12S globulin) is a 300 kDa protein related to soybean glycinin (Table 3). The protein is salt-soluble and has an isoelectric point (pI) in the range of pH 4–7 (Schwenke et al., 1983). Rapeseed cruciferin typically exists as a hexamer of 50 kDa polypeptides, each consisting of an acidic heavy chain (30 kDa) and a basic light chain (20 kDa) linked with a single disulfide bond (Wu and Muir, 2008). The hexameric form dominates at high ionic strength and in mildly alkaline conditions, but changes in pH and ionic strength can promote reversible dissociation of the polypeptides (Schwenke et al., 1983).

Table 3. Properties of major rapeseed proteins.

Protein group	Type	Solubility	Molecular weight (kDa)	pI
Cruciferin [a]	globulin	saline	300	4-7
Napin [a]	albumin	water	14.5	11
Oleosin [b]	prolamin	ethanol	15-26	>7
LTP [c]	albumin	water	7-9	9-10

[a] Schwenke et al. (1983), Monsalve and Rodriguez (1990), Wu and Muir (2008), [b] Jolivet et al. (2011), [c] Ostergaard et al. (1995).

Napin (2S albumin) is a water-soluble, basic protein with a pI of ca. 11 (Monsalve and Rodriguez, 1990). It is composed of two disulfide-linked polypeptides of ca. 4.5 and 10 kDa (Wu and Muir, 2008). Especially the different size and solubility of cruciferin and napin complicate their recovery in a single fraction (Salleh et al., 2002). In addition to the two main storage proteins, lipid-soluble oleosins and small water-soluble proteins such as trypsin inhibitors and lipid transfer proteins (LTP) have been identified in rapeseed (Ostergaard et al., 1995; Bérot et al., 2005; Jolivet et al., 2011). The prolamin-type oleosins represent 2–8% of the total protein and associate with oil bodies (Jolivet et al., 2011). Other minor oil-associated proteins include caleosins and steroleosins.

1.2.2 Carbohydrates

Rapeseed carbohydrates have been widely studied due to their effect on the digestibility of rapeseed meal as feed. The overall monosaccharide profile includes glucose (31–40%), arabinose (18–22%), uronic acids (15–27%), galactose (8–10%), xylose (8–10%), mannose (2–6%) and rhamnose (1–2%) (Simbaya et al., 1996; Meng et al., 2005; Pustjens et al., 2013). Glucose originates mainly (87%) from cellulose which is present in the cell walls (Slominski and Campbell, 1990). Other cell wall polysaccharides include pectins, xyloglucan, arabinan and arabinogalactan (Ghosh et al., 2004; Pustjens et al., 2013).

Xyloglucans consist of a cellulosic backbone branched with xylose side chains. In fucoamyloids, which are typical for rapeseed, the xylose side chains are substituted with fucose and galactose residues (Siddiqui and Wood, 1977). The higher relative xylose concentration in dehulled seeds than in hulls suggests that xyloglucans are mainly localized in the embryo (Eriksson et al., 1994). Pectins are a group of polysaccharides with a backbone containing galacturonic acid. Rapeseed kernel and hull differ in their pectin composition: pectin in the kernel is claimed to contain only ca. 30% galacturonic acid and an unusually high proportion of neutral sugars such as arabinose and rhamnose, suggesting the presence of rhamnogalacturonan I (Siddiqui and Wood, 1977; Pustjens et al., 2013). Hull pectin, on the other hand, is claimed to contain ca. 80–90% galacturonic acid, indicating the presence of homogalacturonan.

Up to 27% of the carbohydrates in rapeseed press cake and meal are soluble in water (Pustjens et al., 2013). The water-soluble carbohydrates are mainly mono- and oligosaccharides such as sucrose, glucose, fructose, galactose, raffinose and stachyose which act as reserve carbohydrates in rapeseed (Siddiqui and Wood, 1977; Meng et al., 2005; Pustjens et al., 2013) and constitute around 10–11% of the press cake (Spragg and Mailer, 2007). As well as sugars, 14–18% of rapeseed polysaccharides are also water-soluble (Slominski and Campbell, 1990). For example, water-soluble pectin, branched arabinan, (Eriksson et al., 1996) and arabinogalactan (Siddiqui and Wood, 1972) have been extracted from rapeseed kernels. In contrast to cereal grains, the starch content in mature rapeseed is negligible (Bell, 1984; Eriksson et al., 1994).

1.2.3 Oil

Hexane extraction effectively removes over 90% of the oil from rapeseed, but after cold pressing a substantial amount of oil (ca. 10–30% depending on process parameters) remains in the press cake. Rapeseed oil is one of the healthiest commercially marketed vegetable oils: The fatty acid composition is ideal for human consumption due to the high proportion of mono- and polyunsaturated fatty acids. Oleic acid (18:1n-9) constitutes 57%, linoleic acid (18:2n-6) 22% and α-linolenic acid (18:3n-3) 13% of the total fatty acids in cold-pressed rapeseed oil (Palomäki et al., 2010). Linoleic acid and α-linolenic acid have an approximately 2:1 ratio, which is considered favorable for human health (Simopoulos, 2002).

Apart from potential health benefits, another important aspect of rapeseed oil is its possible influence on the oxidative stability of rapeseed press cake or protein concentrates during processing or storage. High-fat rapeseed press cake (32% oil) showed much lower oxidative stability than peanut (40% oil) and soybean press cakes (17% oil) over an 8-week storage period at 23°C or 65°C (Stephens et al., 1997). Lipid oxidation was indicated by the rapidly increasing peroxide value of oil extracted from rapeseed press cake, with more severe oxidation observed during storage at an elevated temperature.

1.2.4 Phenolic compounds

When compared to other oilseeds, rapeseed is exceptionally rich in phenolic compounds which contribute to the characteristic dark color, bitter taste and astringency of rapeseed meal. Many of these plant secondary metabolites exert antioxidative activity while certain polyphenolics such as condensed tannins may limit the digestibility and nutrient availability. The main phenolic compound in rapeseed is sinapic acid which constitutes up to 80% of the phenolic acids in rapeseed meal. Most of the sinapic acid is present in esterified form called sinapine and located in the embryo, which may contain 2.9% of sinapine per defatted dry mass (Blair and Reichert, 1984). Minor phenolic acids in rapeseed meal include ferulic, caffeic, vanillic, gallic, protocatechuic, p-coumaric and p-hydroxybenzoic acids (Krygier et al., 1982).

Despite several early studies (Theander et al., 1977; Slominski and Campbell, 1990; Simbaya et al., 1996), there is uncertainty in the content and composition of lignin and other polyphenolics in rapeseed. Klason lignin, which is commonly used as the measure of acid-insoluble lignin in wood materials, may also contain non-lignin compounds such as other phenolics, protein, ash and cutin (Bunzel et al., 2011). Oxidative characterization of rapeseed hull polyphenolics indicated that the hulls contain more other polyphenolics than lignin (Theander et al., 1977). Especially the hulls of dark colored varieties were rich in non-lignin polyphenolics, whereas the contents of lignin in dark and yellow hulls were similar. Major rapeseed non-lignin polyphenolics include condensed tannins, which are mainly localized in the hulls. Rapeseed meals have been reported to contain 0.7–0.8% con-

densed tannins (Shahidi and Naczk, 1989). By comparison, the content of hydrolysable tannins has been reported to be negligible (Theander et al., 1977).

1.3 Human use perspectives of rapeseed protein

1.3.1 Nutritional quality

The well-balanced amino acid composition of rapeseed proteins makes them an interesting plant-based protein source for human nutrition (Bell and Keith, 1991; Newkirk et al., 2003). Rapeseed has an especially high proportion of the essential sulfur amino acids (cysteine and methionine) when compared to legumes such as soy and pea, as reviewed by Day (2013). The content of another essential amino acid, lysine, is somewhat lower in rapeseed than in soybean, but high enough to meet the recommendations for daily intake.

There is still a shortage of human studies on the nutritional value and bioavailability of *B. rapa* and *B. napus* proteins for humans. Based on the work of (Bos et al., 2007), digestibility of rapeseed proteins is limited in humans, but this is compensated by their high postprandial biological value (fraction of the absorbed nitrogen which becomes incorporated in the proteins of an individual). The protein digestibility corrected amino acid score (PDCAAS), which is widely used for evaluation of the nutritional quality of proteins from 0 to 1.00, was reported to be 0.86 for a rapeseed protein isolate and 0.84 for a soy protein isolate (Fleddermann et al., 2013). The PDCAAS score of a rapeseed protein hydrolyzate was reported to be 1.00, corresponding to that of egg and milk proteins. The Results from *in vitro* gastro-intestinal digestion models indicate that some rapeseed proteins, especially napin, resist pepsin digestion (Wanasundara, 2011). Partial hydrolysis of the proteins by exogenous enzymes is a potential way to improve digestibility. Rapeseed napins have been reported to possess allergenicity in children (Puumalainen et al., 2006), which may additionally limit their food use.

Generally in the production of protein ingredients, the influence of processing conditions on the amino acid composition and digestibility of protein should be considered in order to retain good nutritional value in the product. Exposure to excess heat, such as during desolventizing and toasting of solvent-extracted rapeseed meal, has been connected with reduced nitrogen solubility, amino acid (especially lysine) bioavailability and feed value of the meal (Mustafa et al., 2000; Newkirk et al., 2003; Khattab and Arntfield, 2009).

1.3.2 Technical functionality

Besides nutritional value, the quality of food proteins is often determined by their techno-functional properties such as thermal stability and solubility, dispersion, gelation, emulsion, and foaming properties, which affect their behavior during food processing and in end products. These attributes can exceed the importance of nutritional value when the proteins are targeted to function as performance ingredients in foods. The functional properties of rapeseed proteins have been studied extensively, and a number of reviews are available (Moure et al., 2006; Aider and Barbana, 2011; Tan et al., 2011; Wanasundara, 2011; Aachary and Thiyam, 2012). The technical functionality of rapeseed protein fractions is greatly affected by the processes used for oil and protein extraction, which influence the folding state of protein and the composition of protein extracts (Karaca et al., 2011; Manamperi et al., 2011). Cruciferin and napin show distinct techno-functional properties related to their different size and chemistry, and the ratio of these proteins as well as the presence of non-protein compounds may substantially influence the performance of rapeseed protein products.

Thermal stability of proteins is influenced by their secondary structure and the surrounding environment. Rapeseed napin shows a denaturation peak at 110°C and is thermally more stable than cruciferin, which is denatured already at 91°C under excess water conditions (Wu and Muir, 2008). In napin, the four intra- and inter-chain disulfide bonds and compact structure give the protein high structural stability over a wide range of temperature and pH (Pantoja-Uceda et al., 2004). In cruciferin, non-covalent interactions are expected to be more important than disulfide bonds in stabilizing the protein conformation (Aachary and Thiyam, 2012).

Solubility and dispersion stability of proteins are important factors influencing several other techno-functional properties. Proteins are polyelectrolytes which carry a positive net charge at a pH below their isoelectric point (pI), and a negative charge at pH > pI. The solubility of a protein is lowest at a pH close to its isoelectric point, where proteins have a low or neutral net charge. In these conditions, the lack of repulsive electrostatic forces between protein molecules exposes them to aggregation. Proteins are generally most soluble in their native state, whereas denaturation by heat, shear or chemicals may promote aggregation due to the changed surface properties of proteins in unfolded state. Rapeseed napin is readily soluble in water in its native form, whereas for cruciferin the solubility depends largely on the surrounding pH and ionic strength (Wu and Muir, 2008). Native cruciferin tends to be soluble at acidic or alkaline pH far from its isoelectric range (pH 4-7), or in the presence of electrolytes such as NaCl.

Even when proteins are not fully soluble, they may form stable dispersions as colloidal particles. Colloidal dispersions include e.g. gels, foams and emulsions in which one phase exists as droplets or particles in a continuous phase. Certain proteins can act as effective emulsifiers by stabilizing lipid-water interfaces. Reports on the emulsifying activity of rapeseed protein isolates are highly variable (Vioque et al., 2000; Yoshie-Stark et al., 2008; Dong et al., 2011; Manamperi et al., 2011), most probably due to different protein extraction procedures causing

variation in the cruciferin to napin ratio and the state of the proteins (Karaca et al., 2011). Rapeseed napin is suggested to be more surface active in stabilizing oil-water interfaces than cruciferin (Krause and Schwenke, 2001), whereas other reports emphasize the emulsion stability of cruciferin over napin (Wu and Muir, 2008). The emulsifying capacity of rapeseed proteins is a desired property in terms of various food applications, but is considered as a challenge in the aqueous processing of rape seeds or high-fat press cakes, as the formation of protein-oil emulsions tends to hinder separation of the protein and oil fractions (Zhang et al., 2007; Manamperi et al., 2011; Von Der Haar et al., 2014).

The foaming capacity of protein isolates is mostly determined by their molecular flexibility and ability to reduce surface tension. Rapeseed protein fractions have been reported to possess slightly better foaming capacity than soy protein fractions, which could be related to the lower surface viscosity of rapeseed proteins (Dong et al., 2011). Another important attribute of proteins utilized in the food industry is their gelation ability. Several factors, most importantly protein concentration and pH, influence the network formation and quality, as reviewed by Aachary and Thiyam (2012). Comparison of the gelation behavior of rapeseed proteins showed that cruciferins or mixed protein isolates produced stronger gels than napins, which alone had limited gelling ability (Krause and Schwenke, 2001).

Techno-functional properties of protein dispersions are greatly influenced by the presence of non-protein compounds. Charged polysaccharides can particularly enhance the solubility and interfacial properties of rapeseed protein isolates by complexing with oppositely charged proteins. For example, addition of guar gum and *κ*-carrageenan into rapeseed protein solutions was found to enhance the emulsifying properties and improve the strength of rapeseed protein gels (Uruakpa and Arntfield, 2005, 2006), whereas formation of soluble complexes between pectin and rapeseed napin was connected with increased foam stability (Schmidt et al., 2010). On the other hand, protein functionality may be hindered by the presence of secondary metabolites and detergent residues. Phytic acid, sodium salts and denaturants can weaken the surface activity and emulsion stability of rapeseed protein (Krause and Schwenke, 2001; Uruakpa and Arntfield, 2005), and sinapic acid and its derivatives were found to impair the characteristics of heat-induced canola protein gels (Rubino et al., 1996).

One way to tune the techno-functional properties of proteins is through chemical or enzymatic modification such as acylation, phosphorylation succinylation, hydrolysis and cross-linking, as reviewed by Wanasundara (2011). For example, foaming properties of rapeseed protein isolates can be enhanced by limited proteolysis (Larré et al., 2006), acetylation or phosphorylation (Krause et al., 2002). Furthermore, a combination of limited hydrolysis and cross-linking of rapeseed protein using protease and transglutaminase enzymes was found to result in improved gel strength (Pinterits and Arntfield, 2007).

1.3.3 Bioactive properties

In addition to their roles as nutrients or performance proteins, food proteins can exhibit various bioactive properties with potential to evoke positive health effects in humans or improve the shelf life of products. Napin-like proteins have been found to possess biological functions besides their role as nitrogen storage: for example, rapeseed napins have been suggested to exhibit substantial antifungal activity (Terras et al., 1993). Various food proteins carry bioactive peptides in their primary structure. Partial proteolysis, either by human digestive enzymes or by commercial proteases, is needed to expose these bioactive sequences. Bioactivity of rapeseed protein hydrolyzates is a growing area of research, and particularly antihypertensive and antioxidative activities have been recognized in a number of studies (Yoshie-Stark et al., 2006, 2008; Cumby et al., 2008; Wu et al., 2008; Zhang et al., 2008; He et al., 2013). Additionally, antidiabetic, anorexigenic, hypocholesterolemic, anticancer and antiviral properties have been identified in rapeseed protein hydrolyzates, as reviewed by Aachary and Thiyam (2012).

Certain rapeseed peptides (such as the di- and tripeptides phenylalanine-leucine and valine-serine-valine) have been found to exhibit antihypertensive properties by inhibiting the activity of angiotensin converting enzyme (ACE), an enzyme with a central role in blood pressure regulation (Wu et al., 2008). ACE inhibitory peptides have been identified from several plant and animal proteins, and are being extensively studied as potential natural ACE inhibitors to replace the current inhibitory drugs, as reviewed by Udenigwe and Aluko (2012). In addition to blood pressure regulation, ACE-inhibitory peptides have application potential as cosmeceuticals in skin care. A French cosmetic active ingredients supplier, Sederma, markets a face care ingredient (Eyeliss) containing rapeseed dipeptide 2 (valine-tryptophan). As reviewed by Zhang and Falla (2009), this ACE-inhibitory peptide is targeted to reduce puffiness under the eyes by promoting lymph drainage.

Antioxidative compounds limit unfavorable chemical or enzymatic oxidation reactions in the human body or in various products such as foods, cosmetic formulations and chemicals. Antioxidant action can be exerted by several different mechanisms, as reviewed by Huang et al. (2005). For example, antioxidativity of protein and peptides may result from the abundance of exposed amino acid residues which can transfer electrons or hydrogen atoms to free radicals (radical scavenging), or from the ability of proteins and peptides to chelate transition metals. Rapeseed protein hydrolyzates have been reported to exhibit both types of antioxidative properties *in vitro* (He et al., 2013). In addition, rapeseed protein hydrolyzates have been demonstrated to inhibit lipid peroxidation and linoleic acid oxidation (Mäkinen et al., 2012; He et al., 2013).

1.3.4 Compounds limiting nutritional, technical and sensory quality

Concerns related to antinutritional factors such as glucosinolates, phytates and phenolics in rapeseed may limit consumer acceptability of rapeseed meal or press cake as a food protein source (Table 4). Glucosinolates play a role in plant defense response and are mainly located in rapeseed embryo (Fang et al., 2012). The current double-low rapeseed varieties contain significantly reduced amounts of glucosinolates (<30 µmol / g oil-free dry matter), but possible enrichment of these compounds or their breakdown products during processing of the meal or press cake could have negative effects on the food quality of rapeseed protein products. When rapeseed cells are broken, the endogenous thioglucosidase enzyme, myrosinase, starts to hydrolyze glucosinolates into nitriles, hydroxynitrites, isothiocyanates and thiocyanates (Bell, 1984). These degradation products can contribute to undesirable organoleptic properties in rapeseed products and change the properties of rapeseed proteins, as reviewed by Pudel (2012).

Table 4. Compounds limiting the nutritional and sensory quality of rapeseed press cake and meal.

Compound	Concentration in rapeseed meal	Sensory and nutritional effects [e]	Detoxification approaches [e]
Glucosinolates and breakdown products	0.2–1.3% [a]	bitter taste, potential toxicity or anticancer activity	heat inactivation of myrosinase, solvent extraction
Phytic acid	3–4% [b]	chelation of proteins and minerals	phytase treatment
Phenolic acids	0.6–1.7% [c]	bitter taste, dark color	solvent extraction, membrane processing
Tannins	0.7–0.8% [d]	astringency, dark color, chelation of proteins	solvent extraction

[a] Bell and Keith (1991), average molecular weight of glucosinolates assumed as 420 g/mol, [b] Akbari and Wu (2015), [c] Vuorela et al. (2003), Sun et al. (2008), [d] Shahidi and Naczk (1989), [e] modified from Pudel (2012).

The effects of glucosinolates on human health are controversial: reports on the interference of thyroid activity in production animals have raised concerns relating to the safety of these substances in human use (Bell, 1984), but there is no proof of similar effects in humans. By contrast, the potential anticancer activity of glucosinolates is increasingly recognized (Cartea and Velasco, 2008). The conventional solvent-based oil extraction process involves several heating steps which are suggested to help inactivation of myrosinase in rapeseed meal, as reviewed by Pudel (2012). In the case of cold-press cakes, short heat inactivation of myrosinase (5 min) has occasionally been performed to prevent the formation of undesirable glucosinolate hydrolysis products (Niu et al., 2012).

Phytic acid and its salts, phytates, are phosphorus storage compounds found in several grains and oilseeds. Rapeseed meal contains around 3–4% phytic acid (Blaicher et al., 1983; Akbari and Wu, 2015). Dehulling has been found to cause a minor increase in the phytic acid content (Ghodsvali et al., 2005), suggesting that the compound is slightly more prevalent in rapeseed embryo. Phytic acid has a strong tendency to form electrostatic interactions with rapeseed proteins at pH values below their isoelectric points, resulting in the formation of insoluble complexes (Schwenke et al., 1987). By chelating proteins and minerals such as Zn and Fe, phytic acid can reduce the bioavailability of these nutrients in animals and humans. Phenolic compounds, particularly tannins, can also complex with proteins, thereby lowering their nutritional value. Tannins are known for their ability to cause unpleasant astringency, a drying sensation in the mouth due to precipitation of salivary proteins (Bate-Smith, 1973). A low-concentration antinutrient in rapeseed, erucic acid, is a monounsaturated fatty acid which used to be abundant in early rapeseed varieties (Bell, 1993). In today's double-low rapeseed cultivars, erucic acid content has been reduced to an acceptable level (less than 2% of total fatty acids in oil) by traditional breeding.

1.3.5 Potential applications

Incorporation of rapeseed proteins in foods has been widely investigated, although the brown color of the protein isolates produced by current methods is considered as a major limitation to their applicability (Von Der Haar et al. 2014, Yoshie-Stark et al. 2006). Rapeseed protein concentrates and isolates have been studied as animal protein or egg replacers in emulsified meat and bakery products. The high fat absorption and water holding capacities of rapeseed proteins are considered to be favorable for the cooking stability of meat products, as reviewed by Wanasundara (2011). In a study by Yoshie-Stark et al. (2006), partial (2%) replacement of animal protein with rapeseed protein concentrate resulted in improved taste and aroma, but poorer color and texture when compared to control sausages containing casein. Von Der Haar et al. (2014) investigated high-pressure homogenization (HPH) as a means to improve the techno-functional properties of rapeseed protein concentrates in meat and bakery products. Sausages containing 2% HPH-treated rapeseed protein concentrates had similar water and fat separation to control sausages, but darker color and strawy and oily taste. Substitution of egg proteins by HPH-treated rapeseed protein concentrates in pound cakes resulted in similar volume expansion to that of the reference, but again slightly darker color.

Protein isolates (81–91% protein) from dehulled rapeseed meal have been reported to be suitable for partial substitution of egg in mayonnaises and bakery products (Von Der Haar et al., 2014). In the most successful baking experiments, rapeseed protein isolates have been added to wheat flour at up to 18% protein concentration without detrimental effects on dough and loaf quality in wheat bread (Mansour et al., 1999). Hydrolyzed rapeseed proteins may also have suitable attributes for food products: rapeseed protein hydrolyzates have been shown to

increase the water holding capacity and cooking yield of sausages (Cumby et al., 2008). However, when incorporating protein hydrolyzates in foods, the taste profile of peptides must be considered, as several peptides have been connected to bitter taste (Kim and Li-Chan, 2006). On the other hand, thermal reaction of rapeseed protein hydrolyzates with xylose and cysteine has been successfully utilized to generate meat-like flavor substances (Guo et al., 2010).

Production of rapeseed protein-based edible films has been increasingly studied in the past few years. Jang et al. (2011) found that protein extracts alone did not exhibit film forming properties, but produced films with suitable mechanical properties for packaging applications when blended with gelatin and small amounts of emulsifiers and plasticizers. Chang and Nickerson (2014) reported that films prepared from rapeseed protein isolate had similar or better tensile strength and water barrier properties than corresponding films from soy or egg protein, but considered the non-transparent nature of the films as a limitation in some applications. Other non-food applications proposed for rapeseed proteins include surfactants (Sánchez-Vioque et al., 2004), plastics (Manamperi et al., 2010), wood adhesives (Li et al., 2012) and paper additives, as reviewed by Pudel (2012). Lomascolo et al. (2012) reviewed the potential of rapeseed meal as a nutrient source in fermentative processes.

1.4 Fractionation technologies for rapeseed protein recovery

Before application of rapeseed proteins as food ingredients, they must be extracted or otherwise enriched from the seeds, press cake or meal due to their limited digestibility and techno-functional properties, undesirable taste and color, and antinutritive compounds present in the raw materials. Food protein products are usually classified on the basis of their protein content as concentrates (>65% protein) or isolates (>90% protein). A traditional way of producing soy and rapeseed protein concentrates involves washing of the meal with solvents (e.g. aqueous isopropanol or methanol/ammonia/water) to remove non-protein compounds such as low molecular weight carbohydrates, glucosinolates and phenolics, while retaining most of the protein in the washed solids (Jones and Holme, 1979; Diosady et al., 1989). Such processing increases both the protein content and the digestibility of the material to some extent. The drawbacks of this approach are that several washing steps with solvents may be needed, and protein solubility is reduced due to partial denaturation during intensive alcohol treatment (Xu and Diosady, 2012; Von Der Haar et al., 2014).

1.4.1 Defatting of press cake

Reduction of the oil content of rapeseed press cake has been considered to facilitate protein recovery by wet and dry fractionation (Sun et al., 2008; Sari et al., 2013; Barakat et al., 2015). Oil has been identified as one of the major biomass components limiting plant protein extraction (Sari et al., 2015), particularly in alka-

line extraction in which saponification of lipids and formation of lipid-protein complexes hinder protein recovery (Manamperi et al., 2011). The industrially used hexane extraction efficiently removes oil from rapeseed, but high temperatures applied during solvent removal have unfavorable effects on protein quality. In order to better retain protein solubility and simultaneously reach low oil content in the meal, alternative defatting and solvent removal methods have been sought. Gentle solvent removal procedures which have been studied include fluidized-bed, air, and flash desolventizing, as reviewed by Pudel (2012).

A gentle defatting method, supercritical carbon dioxide (SC-CO_2) extraction, has been proposed as a future 'green' technology for rapeseed oil extraction, as reviewed by Rempel and Scanlon (2012). Above a critical temperature (31°C) and pressure (74 bar), CO_2 becomes a 'supercritical' fluid, which has the characteristics of both a gas and a fluid, and in which the main rapeseed oil constituents including triglycerides, triacylglycerols and free fatty acids are readily dissolved. Supercritical CO_2 has several advantages over conventional solvents such as hexane, including easier solvent recovery and recyclability, improved operational safety, and better oil quality (Rempel and Scanlon, 2012). The selectivity and efficiency of SC-CO_2 extraction can be controlled by modification of temperature, pressure and time, or by the use of co-solvents such as ethanol, which enhances the recovery of phospholipids. The method may be applied at several stages of oilseed processing, either for crushed rape seeds or for additional defatting of rapeseed press cake. The technology is in commercial use for production of bioactive phytochemicals and specialty oils, and may potentially be taken into use in food oil production once the operation costs have been reduced to an economical level (Pudel, 2012).

1.4.2 Dry fractionation

Dry processing offers a sustainable and economical way to produce plant protein concentrates and to pretreat raw materials for enhanced protein isolation in aqueous conditions, as reviewed by Schutyser and van der Goot (2011) and Schutyser et al. (2015). Energy consumption of milling and air classification has been estimated to be substantially lower than that of wet fractionation processes in wheat gluten production (Schutyser and van der Goot, 2011).

Cellulose in the plant cell walls has been identified as on average the most important limiting factor for protein extraction from various feedstocks such as oilseed meals and cereal by-products (Sari et al., 2015). Cell walls may form a physical barrier for protein release, and therefore, milling has been suggested to facilitate protein recovery from cellulosic raw materials (Sari et al., 2015). For example, particle size reduction has been reported by Russin et al. (2007) to improve protein extraction from soybean flour. In many of the published wet fractionation procedures, rapeseed meals have been ground (Aluko and McIntosh, 2001; Ghodsvali et al., 2005; Wu and Muir, 2008; Manamperi et al., 2011; Cheung et al., 2014) and passed through a 0.2–0.5 mm screen (Blaicher et al., 1983; Akbari and Wu, 2015) or flaked with a roll mill (Dong et al., 2011) before dispersion in an

aqueous solution, whereas few studies have involved more extensive milling before protein extraction (Yoshie-Stark et al., 2008; Helling et al., 2010).

When rapeseed proteins are produced for food applications, removal of the hulls is recommended to reduce fiber and antinutrient content (Naczk and Amarowicz, 1998). Dehulling of rapeseeds is generally not in industrial use due to limited feasibility and reduced oil yields, but as an alternative strategy, hull removal from defatted, fine-milled oilseed meals by air classification or electrostatic separation has been investigated (King and Dietz, 1987; Barakat et al., 2015). By ultrafine milling and electrostatic classification, Barakat et al. (2015) were able to recover 93% of the proteins from sunflower cake (31% protein) in a fine fraction which contained 49% protein. However, higher protein concentrations than this may be difficult to obtain by dry fractionation of oilseed materials. The efficiency of air classification depends greatly on the feedstock: it is most successfully applied to pulses and some cereals, whereas it is less effective for oilseeds such as lupin and soybean (Schutyser and van der Goot, 2011). The distinct composition and microstructure of oilseeds in comparison to cereals and pulses influence their fragmentation behavior, and may limit the efficiency of air classification. Dry fractionation of oilseeds and their co-streams is also challenged by their high oil content, which can hinder free dispersion of the milled particles during air classification unless fat is removed by solvent or SC-CO_2 extraction, as reviewed by Schutyser and van der Goot (2011).

1.4.3 Wet fractionation

Process development for production of rapeseed protein isolates started already in the 1970s, around the time when the first rapeseed varieties low in glucosinolates and erucid acid were introduced, as reviewed by Xu and Diosady (2012). The early wet fractionation processes were based on soy protein technology, consisting of aqueous extraction and precipitation. Later on, extraction procedures have been tailored on the basis of rapeseed protein solubility, which is influenced by parameters such as pH, ionic strength and temperature. Whereas soy protein is comprised mainly of globulins (pI 4–7.5), rapeseed contains two major proteins with different size and solubility (Salleh et al., 2002). The fact that rapeseed napin solubilization is favored at acidic pH and cruciferin solubilization at alkaline pH complicates their recovery in a single fraction. Rapeseed meal has a natural pH of 5–6.2 when dispersed in water (Barker et al., 2002). At this pH, rapeseed cruciferin has limited solubility, and therefore, additives such as alkali, acid or salt are commonly used to generate more favorable aqueous extraction conditions. The most commonly used wet processing schemes for rapeseed protein isolation include alkaline or saline extraction followed by concentration of the extracted proteins by precipitation or ultrafiltration (Figure 3). A number of reviews are available on wet fractionation technologies developed for rapeseed meal and press cake (Aider and Barbana, 2011; Tan et al., 2011; Wanasundara, 2011; Xu and Diosady, 2012).

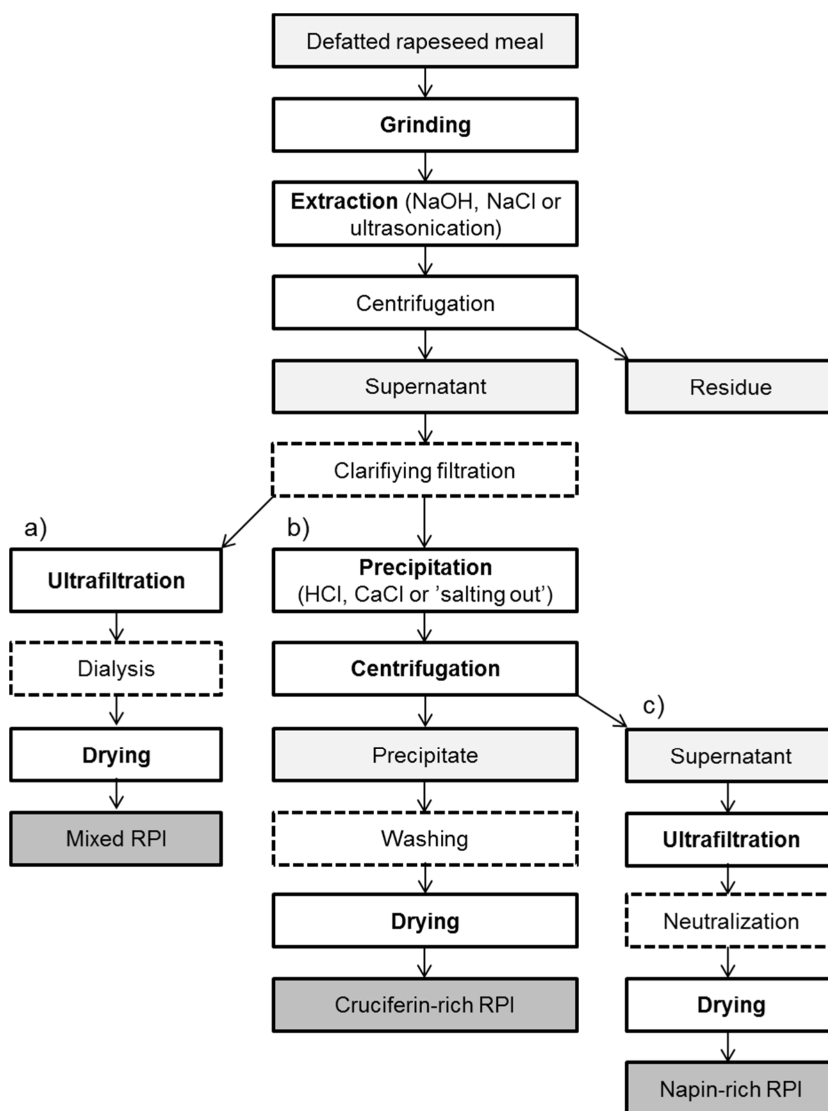

Figure 3. Common rapeseed protein isolation methods consisting of a) alkaline or saline extraction and membrane filtration to produce a mixed rapeseed protein isolate (RPI) (Dong et al., 2011; Karaca et al., 2011), or b) alkaline or saline extraction and isoelectric precipitation (Aluko and McIntosh, 2001) or 'salting out' precipitation (Cheung et al., 2014) to produce a cruciferin-rich RPI, and c) membrane filtration of the supernatant after precipitation to obtain a napin-rich RPI (Yoshie-Stark et al., 2008).

Alkaline extraction is the most studied method for production of rapeseed protein isolates, with examples of the process parameters presented in Table 5. As both cruciferin and napin are soluble at pH 11–12 (Manamperi et al., 2011), high yields of over 90% of the total protein have been extracted from low-temperature treated rapeseed meal at >pH 11 (Blaicher et al., 1983). Rapeseed meal is usually mixed with water at a meal to solvent ratio of 1:30 to 1:5 (corresponding to approximately 3–17% total solid content) and the desired pH is obtained using NaOH. Nearly all extraction studies are based on defatted rapeseed meal since separation of oil and protein is difficult in alkaline extraction conditions (Manamperi et al., 2011). Particularly meals defatted under mild thermal conditions are preferred because of their better protein solubility (Salleh et al., 2002). As an exception, Helling et al. (2010) patented a procedure with which 86% of the protein can be recovered from high-fat (20% oil) rapeseed press cake into an alkaline extract containing protein and oil.

Advantages of alkaline extraction include high protein extraction yield and reduced interactions between phytic acid and protein (Ghodsvali et al., 2005). On the other hand, alkaline conditions may expose proteins to denaturation, thereby impairing their solubility and related techno-functional properties (Ma et al., 1990). Extremely high pH (>12) should also be avoided as it could lead to protein degradation (Manamperi et al., 2011) or reduction of protein digestibility through lysinoalanine formation (Deng et al., 1990). Another drawback of alkaline extraction procedures is the dark brown color of the resulting protein extracts due to co-extraction and polymerization of polyphenolics, as reviewed by Xu and Diosady (2012) and Wanasundara (2011).

To improve the yield and color of protein isolates, additives such as polyacids (Gillberg and Tornell, 1976), sodium hexametaphosphate (SHMP) (Thompson et al., 1976), sodium sulfite (Keshavarz et al., 1977; Akbari and Wu, 2015) and sodium bisulfite (Blaicher et al., 1983) have been applied in alkaline extraction. Sodium dodecyl sulfate (SDS, 0–2 g/100 g) has been reported to increase protein solubility but to reduce the selectivity of alkaline extraction for protein (Akbari and Wu, 2015). Additionally, ultrasonication has been successfully used to facilitate alkaline extraction of rapeseed protein (Dong et al., 2011). Recently, Das Purkayastha and Mahanta (2015) used response surface methodology to optimize an alkaline extraction process in terms of yield, color, phytate level reduction and functional properties of the resulting rapeseed protein isolates. The influence of extraction time (1–5 h), meal to solvent ratio (1:10–1:30) and addition of NaCl or sodium sulfite was investigated. Meal to solvent ratio was reported to have the highest impact on protein yield and phytate content of the protein isolates: when processing was performed at elevated solid content, protein yield was reduced and phytate content was increased. The optimal parameters were reported to be 1.5 h extraction time, a meal to solvent ratio of 1:27, and the presence of 0.03 M NaCl and 0.4% sodium sulfite.

Table 5. Examples of alkaline extraction and isoelectric precipitation parameters used for rapeseed protein recovery.

Raw material	Extraction pH	Precipitation pH	T (°C)	t (min)	Meal to solvent ratio (g : 100 ml)	Max. protein extraction yield (%)	Max. protein yield in precipitate (%)	Reference
LRM	8–11	3.6–6	30	20	3	79	56	Blaicher et al., 1983
LRM	12–13	4–5	RT	20	5	94	63	Zhou et al., 1990
RM	12	3.5	RT	60	5	99	88	Klockeman et al., 1997
LRM	-	4	23	20	10	-	-	Aluko and McIntosh, 2001
dehulled LRM	9.5–12	3.5–7.5	RT	15	5.6	70	45	Ghodsvali et al., 2005
dehulled LRM	7.4	5.8	30	45	20	69	37	Yoshie-Stark et al., 2008
RPC	11–12.5	2–3.5	-	-	-	86	54	Helling et al., 2010
RPC	11–12	3.5–5.5	-	60	25	-	>28	Manamperi et al., 2011
LRM	11.5	3.6–5.8	35	45	4	71	-	Dong et al., 2011
LRM	-	4	RT	20	10	-	-	Karaca et al., 2011
RM	10	-	25	1440	10	60	-	Sari et al., 2015
LRM	11	-	25	90	3.7	33	-	Das Purkayastha and Mahanta, 2015
LRM	12.5	4	RT	60	10	56	39	Akbari and Wu, 2015

RM) rapeseed meal, LRM) low-temperature defatted rapeseed meal, RPC) rapeseed press cake. RT) room temperature. -) not available.

Saline extraction with NaCl (Table 6) is the second popular approach that has been studied for rapeseed protein extraction, and takes advantage of the salt solubility of cruciferin. Burcon NutraScience Corporation has an extensive patent portfolio on salt extraction of rapeseed protein: in the general procedure, preferably low-temperature defatted rapeseed meal is mixed with 0.15 M NaCl solution at a meal to solvent ratio of 1:10 to 1.5:10, and agitated for 30 min, resulting in ca. 10–25% protein extraction yields according to examples in the patent publications (Barker et al., 2002; Segall et al., 2010). Bérot et al. (2005) combined saline and alkaline conditions in rapeseed protein extraction. In the procedure, which was demonstrated in both analytical and preparative scale (3.5 kg raw material), rapeseed meal was suspended in 0.05 M Tris-HCl buffer, pH 8.5, containing 0.75 M NaCl, 5 mM EDTA and 0.3% sodium bisulfite. The extraction was performed for 60 min at a meal to solvent ratio of 1:10 (analytical) or 1:30 (preparative). These extraction conditions resulted in good protein yields of 77% and 63% in the analytical and preparative scale, respectively, and prevented oxidation of rapeseed phenolics. A drawback of the process was the co-extraction of a large amount of pigments and salts, nearly all of which could however be removed by nanofiltration.

Table 6. Examples of saline extraction parameters used for rapeseed protein recovery.

Raw material	Extraction pH	NaCl (M)	T (°C)	t (min)	Meal to solvent ratio (g : 100 ml)	Max. protein extraction yield (%)	Reference
dehulled RM	-	0.7	24	180	0.2	-	Siy and Talbot, 1981
RM	5–6.8	0.5	8	240	10	-	Murray, 1999
RM	5.3–6.2	0.15	RT	30	15	10	Barker et al., 2002
dehulled LRM	8.5	0.75	-	60	4–10	77	Bérot et al., 2005
LRM	5–6.8	0.15	RT	30	10	25	Segall et al., 2010
LRM	7	0.1	RT	120	10	-	Karaca et al., 2011
dehulled LRM	5.8–6.3	0.2	RT	90	10	-	Cheung et al., 2014

RM) rapeseed meal, LRM) low-temperature defatted rapeseed meal. RT) room temperature. -) not available.

Saline extraction typically enables lower rapeseed protein yields than alkaline extraction, but may be beneficial in terms of techno-functional properties of the resulting protein fractions: protein isolates obtained by salt extraction have been reported to possess higher solubility and interfacial activity compared to those produced by alkaline extraction (Karaca et al., 2011). Rapeseed protein extraction in mildly alkaline and saline conditions (pH 8.5, 0.75 M NaCl) has been shown to be feasible (Bérot et al., 2005), whereas addition of salt to extraction at high pH (12.5) has been reported to reduce protein yields, possibly due to the salting out effect of NaCl in highly alkaline conditions (Akbari and Wu, 2015).

Alkaline or saline extractions are generally not very selective for rapeseed cruciferin or napin. Recently, the different techno-functional properties of these proteins and allergenicity concerns related to napin have generated interest in procedures allowing the extraction of napin and cruciferin in separate stages. Wanasundara and McIntosh (2008) patented a method in which napin is extracted from rapeseed meal at pH 3–5, followed by alkaline extraction of cruciferin at pH 8-10. However, in acidic conditions such as those described for napin extraction, the co-extraction of phytic acid is also increased (Blaicher et al., 1983). Co-extractability of napin and phytic acid in acidic conditions was evident in a recent study, in which washing of rapeseed meal with HCl solution (pH 2.5-5) before alkaline extraction extracted a substantial proportion of the napin (25–45% yield), phytic acid (28–47% yield) and phenolic compounds (33–40% yield) (Akbari and Wu, 2015).

1.4.4 Downstream processing of protein extracts

Aqueous extraction of proteins in saline or alkaline conditions yields a liquid product stream, which is often dilute due to the low meal to solvent ratios used in extraction procedures. To avoid the high energy costs of extensive drying, precipitation or membrane separation steps are commonly applied as a means to concentrate the protein extracts. These methods also allow partial removal of non-protein compounds such as sugars, phenolics and phytates, which are easily co-extracted with proteins, or sodium ions (from NaCl or NaOH) introduced during the extraction process. The concentrated protein fractions are finally spray- or vacuum-dried to yield protein isolates (>90% protein) or concentrates (>65% protein).

Isoelectric precipitation by pH adjustment is the most commonly used approach to recover protein from rapeseed alkaline extracts. It is based on the tendency of cruciferin to aggregate at pH values close to its isoelectric point (Gillberg and Tornell, 1976). Precipitation of alkali-extracted rapeseed proteins has been reported to be most effective at pH 4-5.5, although the optimal precipitation pH may vary depending on the seed variety (Zhou et al., 1990; Ghodsvali et al., 2005). Addition of $CaCl_2$ to alkaline extracts before isoelectric precipitation has been used occasionally to facilitate dissociation of protein-phytate complexes (Aluko and McIntosh, 2001; Ghodsvali et al., 2005). In a study by Aluko and McIntosh (2001), $CaCl_2$-assisted isoelectric precipitation produced rapeseed protein precipitates with lower foaming capacity but better emulsifying activity when compared to acid-

precipitated protein isolates. Isoelectric precipitation generally results in limited yields of precipitated protein (50–70% of extracted protein) due to the retention of a large proportion of napin in the soluble fraction. In this way it represents an approach to partly separate the major proteins into a cruciferin-rich protein precipitate and a napin-rich soluble protein isolate (Akbari and Wu, 2015). Another method for precipitation of especially salt-extracted proteins ("salting out") involves reduction of ionic strength and temperature by addition of cold water, which results in the formation of a protein micellar mass (PMM). This method has been used in conjunction with saline extraction (Barker et al., 2002; Cheung et al., 2014), and produces a protein isolate consisting mainly of cruciferin (Cheung et al., 2014).

Membrane technologies for concentration of rapeseed protein extracts have been reviewed by Xu and Diosady (2012). Concentration and desalting of protein extracts by ultrafiltration and diafiltration have certain advantages over isoelectric precipitation in terms of the technological functionality of protein. An alkali-extracted, ultrafiltered protein isolate was found to possess higher solubility at pH 2–8 and better oil adsorption, emulsifying and foaming capacity than corresponding isoelectric precipitates (Dong et al., 2011), most probably due to differences in the protein composition of these fractions. Drawbacks of ultrafiltration and particularly of diafiltration include their water and energy intensiveness and dilute waste streams, which limit the sustainability and cost-efficiency of these technologies.

Membrane separation has proven efficient for the removal of antinutrients and other undesirable compounds from protein extracts or soluble protein fractions remaining after isoelectric precipitation. Rapeseed phenolic acids and condensed tannins are easily co-extracted with proteins in alkaline conditions due to non-covalent and covalent interactions (Xu and Diosady, 2000), and the presence of these compounds leads to deterioration of organoleptic, functional and nutritional properties in rapeseed protein isolates and concentrates. In the study by Xu and Diosady (2000), diafiltration removed a considerable fraction of the phenolics from rapeseed alkaline extracts. Addition of 0.05M NaCl and 0.1% SDS was suggested to further enhance the dissociation of proteins from phenolic acids and condensed tannins by weakening ionic bonds (Xu and Diosady, 2002). Membrane technologies have also been effective in reducing phytic acid levels of rapeseed extracts when high salt concentrations (0.7–0.9 M NaCl) reduce the electrostatic interaction between phytate and protein (Thompson, 1990). A third group of undesirable components, glucosinolates, have been reported to remain below their detection limits in alkali-extracted, acid-precipitated protein isolates from double-low rapeseed meal, suggesting no substantial enrichment of these compounds during the protein isolation process (Blaicher et al., 1983).

Ultrafiltration and diafiltration are used in the process of Burcon Nutrascience Corporation for production of high-quality rapeseed protein products (Puratein® and Supertein®) with phenolic acid contents of ≥0.3% and glucosinolate contents of ≥0.8 µmol/g (Mejia et al., 2009a, 2009b). When pure protein fractions are targeted for special applications or research purposes, liquid chromatography can be used to separate proteins based on their size or surface charge (Dong et al.,

2011). Chromatography has also been demonstrated for pigment removal from rapeseed protein isolates in analytical and preparative scale (Bérot et al., 2005).

1.4.5 Use of enzymes in rapeseed protein extraction and hydrolysis

Although most rapeseed protein extraction methods are based on chemical treatments, several enzyme-aided approaches have also been documented. Enzymes are increasingly used in food processing (including dairy, bakery, meat and juice products), and a variety of industrial food-grade enzymes are commercially available. In rapeseed meal valorization, protease enzymes have been studied most intensively due to their ability to hydrolyze protein into soluble, bioactive peptides. In addition, phytase (Rozenszain and Beye, 2012), myrosinase (Woyewoda et al., 1978) and polyphenolc oxidase enzymes (Lacki and Duvnjak, 1998) have been studied for antinutrient removal from rapeseed meal and its protein fractions. Carbohydrate hydrolases have been applied to facilitate aqueous protein and oil extraction from rape seeds (Zhang et al., 2007; Latif et al., 2008; Von Der Haar et al., 2014) and press cakes (Kvist et al., 2005; Niu et al., 2012), or to increase the protein content of rapeseed meal through solubilization of carbohydrates (Rodrigues et al., 2014).

Carbohydrate-hydrolyzing enzyme treatment has been widely applied to improve the digestibility of fiber-rich feedstuffs. Along with a comprehensive understanding of rapeseed carbohydrate composition and cell wall structure, the documented work on feed quality improvement of rapeseed meal provides a useful basis for selection of enzymes active on rapeseed carbohydrates (Meng et al., 2005; Kiarie et al., 2008; de Vries et al., 2014; Pustjens et al., 2014). Commercial preparations with pectinase (polygalacturonase and pectin methyl esterase), cellulase, *β*-glucanase, xylanase and other hemicellulase activities have commonly been utilized (Table 7), in correspondence with the presence of pectin, cellulose, xyloglucan, arabinogalactan and arabinan in rapeseed cell walls. Pustjens et al. (2012) studied the efficacy of commercial pectinolytic enzyme preparations (Pectinex Ultra SP from Novozymes and Multifect Pectinase PE from Genencor) in hydrolyzing carbohydrates during *in vitro* pepsin digestion of untreated and pretreated rapeseed meals. Pectinase treatment in combination with mild acid pretreatment solubilized 68% of the carbohydrates and substantially reduced the viscosity and water holding capacity of the meal.

Enzymatic hydrolysis of carbohydrates has also been applied to increase the protein content of solvent-extracted rapeseed meal as a solvent-free alternative to alcohol washing (Rodrigues et al., 2014). Out of three commercial enzyme preparations (Pectinex Ultra SP-L, Celluclast 1.5L and Viscozyme L from Novozymes), a fungal multi-enzyme complex containing cellulase, *β*-glucanase and various hemicellulase activities (Viscozyme L) was reported as the most effective in hydrolyzing carbohydrates during a 24 h treatment at pH 3.5. As a result, protein content of the meal increased from 41 to 68%, corresponding to the requirements of a protein concentrate.

Table 7. Examples of enzymatic treatment parameters used for processing of rapeseed materials.

Raw material	Main activities of enzyme preparations	Enzyme dosage	pH	T (°C)	t (h)	Meal/seed to solvent ratio (g : 100 ml)	Max. carbohydrate extraction yield (%)	Max. protein extraction yield (%)	Reference
RM	pectinase, cellulase, mannanase, xylanase, glucanase, galactanase	0.01-0.04 g/g	5.2	45	16	1.6	-	-	Meng et al., 2005
RM	pectinase, *β*-glucanase	50 µl/g	3.5	40	1.25	-	50	-	Pustjens et al., 2012
RM	cellulase, *β*-glucanase, hemicellulase, pectinase	0-192 U/g	3.5-4	35-55	24	5-15	80	-	Rodrigues et al., 2014
RPC	*β*-glucanase, pentosanase, hemicellulase, pectinase	1000 IU/g	-	-	3	20	-	71	Kvist et al., 2005
dehulled RS	1) pectinase, cellulase, *β*-glucanase, xylanase	25 µl/g	5	48	2	20	-	83	Zhang et al., 2007
	2) endoprotease	15 µl/g	9	60	3	20	-	-	
RS	pectinase, cellulase, *β*-glucanase, xylanase, phytase, endoprotease	-	-	45	2	17	-	-	Latif et al., 2008
dehulled RPC	hemicellulase, cellulase, pectinase, endoprotease	10 mg/g	4.5-9	-	1.25	20	-	83	Niu et al., 2012
RM	endoproteases	13 µl/g	2.5-11	50-60	3	15	-	80	Sari et al., 2013

RS) rape seed, RPC) rapeseed press cake, RM) rapeseed meal. -) not available.

Treatment with carbohydrate hydrolases, often in combination with proteases, has been reported to improve protein recovery from rape seeds, meals and press cakes (Table 7). Up to 83% of the total protein has been extracted from dehulled, cold-pressed rapeseed press cake using hemicellulases, pectinases and cellulases in combination with protease treatment (Niu et al., 2012). Kvist et al. (2005) patented a hybrid technology based on sequential carbohydrate hydrolysis and wet milling for protein and oil extraction from expeller-extracted rapeseed press cake. In the procedure, rapeseed press cake (31% protein and 24% oil) is treated with an enzyme mixture containing *β*-glucanase, pentosanase, hemicellulase and pectinase activities. The slurry (19% solid content) is intermittently wet-milled at 1 h intervals for 3 h to facilitate enzyme access into the substrate matrix and dispersion of hydrolysis products. Hydrolysis is halted by heating the slurry to 95°C, followed by centrifugation to recover an extract (71% protein yield) and an emulsified oil layer (73% oil and 20% protein). An earlier patent by Neumuller (2000) describes a similar enzyme-aided extraction procedure applied to several oilseed meals, followed by isoelectric precipitation of the extracted protein. Furthermore, a patent by Wanasundara and McIntosh (2008) covers an optional process route in which the residual rapeseed meal after napin extraction is treated with cell wall-degrading enzymes (cellulase, hemicellulase and xylanase) at pH 3–4.5 to recover a soluble sugar fraction and a solid cruciferin-rich product.

Apart from whole protein extraction, production of rapeseed protein hydrolyzates has been increasingly studied in the 2000s. Enzymatic hydrolysis of rapeseed proteins has several advantages: the treatment may facilitate rapeseed protein solubilization, and when favorably controlled, yield peptides with improved techno-functional and/or bioactive properties. Protease treatment has been reported to intensify the effects of carbohydrate-hydrolyzing enzymes in the extraction of proteins from dehulled, wet-milled rape seeds (Zhang et al., 2007) and dehulled cold-press cake (Niu et al., 2012), enabling the highest reported protein yields (83%) by enzyme-aided extraction (Niu et al., 2012). In most studies, however, protein hydrolysis has been performed after the protein isolates have first been recovered by alkaline or saline extraction (Table 8). By limited proteolysis, Vioque et al. (2000) were able to improve the emulsifying and foaming activities and water and oil absorption capacities of rapeseed protein isolates, with optimal results obtained at a degree of hydrolysis of 3.3%.

Table 8. Examples of enzymatic treatment parameters used for hydrolysis of rapeseed protein isolates into functional peptides.

Enzyme preparations	pH	T (°C)	t (h)	DH (%)	Obtained functional properties	Reference
1) Alcalase 2.4L	8	50	1	60	extensive hydrolyzate, limited techno-functional properties	Vioque et al., 1999
2) Flavourzyme	7		2			
Alcalase 2.4L	8	RT	1	3.1-7.7	emulsifying foaming, water and oil absorption	Vioque et al., 2000
Alcalase 2.4L	9	60	5	28	-	Tessier et al., 2006
1) pepsin	2	37	2	2.5-4.6	ACE inhibition, radical scavenging	Yoshie-Stark et al., 2006, 2008
2) pancreatin	7	37	3	7.7-9.1		
Alcalase 2.4L	8.5	60	2	20.7	ACE inhibition	Wu and Muir, 2008
Alcalase	8	50	1	20.6	radical scavenging, reducing power	Cumby et al., 2008
Flavourzyme	7	50	2	6.3		
Alcalase	7	55	3	-	ACE inhibition, inhibition of lipid peroxidation	Mäkinen et al., 2012
trypsin	7	40	3	-		
pepsin	2	40	3	-		
Subtilisin	8	60	3	-		
Thermolysin	8	60	3	-		
Alcalase	8	50	4	-	antioxidativity (radical scavenging, metal chelation, reducing power, inhibition of linoleic acid oxidation)	He et al., 2013
Proteinase K	7.5	37	4	-		
1) pepsin	2	37	4	-		
2) pancreatin	7.5	37	4	-		
Thermolysin	8	50	4	-		
Flavourzyme	6.5	50	4	-		

DH) degree of hydrolysis. -) not available.

Many of the techno-functional and bioactive properties of protein hydrolyzates are strongly influenced by the degree of hydrolysis and the specificity of enzymes towards different cleavage sites on the polypeptide chain. Extensive hydrolysis is usually not considered favorable for production of food protein ingredients, as it may weaken some of the techno-functional properties of proteins (Vioque et al., 1999) and yield small peptides with undesirable bitter taste (Kim and Li-Chan, 2006). On the other hand, small peptide size may be needed for attainment of specific bioactivities. Several *in vivo* bioactivities such as ACE inhibition are suggested to be mediated by di- and tripeptides which can remain intact in the gastrointestinal tract (Wu and Muir, 2008). In order to maximize the yield of such peptides, endoproteases which cleave peptide chains in the middle to produce polypeptides are more frequently used than exopeptidases which release free amino acids from the ends of polypeptide chains.

Alcalase 2.4L (Novozymes), a *Bacillus licheniformis* serine endopeptidase with an alkaline pH optimum, is the most studied enzyme product for rapeseed protein hydrolysis, probably due to its commercial availability, food grade status and high protease activity. Additionally, gastrointestinal proteases (pepsin, pancreatin) have been used (Yoshie-Stark et al., 2006). In studies in which different proteases were compared, Alcalase produced hydrolyzates with the highest ACE inhibition and lipid peroxide inhibition activity (Mäkinen et al., 2012). By contrast, an extensive hydrolyzate from sequential pepsin and pancreatin treatment showed the strongest DPPH radical scavenging activity (He et al., 2013).

1.5 Prospects for market entry of rapeseed protein ingredients

The growing need for sustainable and plant-based protein sources opens wide potential for rapeseed protein concentrates, isolates and hydrolyzates in food, cosmetic and other non-food applications. In addition to the well-established feed use, rapeseed could become a marked alternative to soybean as a food protein source in Europe. Whereas soybean and wheat gluten currently dominate the global plant protein market, new food protein products from a range of plant sources are being developed and introduced to consumers. Alternative plant protein products commercialized or under commercialization include those from rice, corn, pea and potatom as reviewed by Day (2013). The development of plant-based alternatives to animal proteins is facilitated by the increasing availability of industrially feasible technologies to extract plant proteins and enhance their flavor and texture in e.g. meat analogues. On the other hand, potential sensory and technological challenges and insufficient comprehensive human studies on the allergenicity, digestibility and toxicity of the new plant protein products can hinder the market entry of new products. Safety, legislation, and nutritional and technological aspects of plant protein sources in relation to food use have been reviewed by Boland et al. (2013), Day (2013) and van der Spiegel et al. (2013).

Several market studies on the growth potential of plant protein products are available. Revenue of the global protein ingredients market for is projected to reach $14 109.7 million in 2017, at a compound annual growth rate (CAGR) of 4.6% (Frost & Sullivan, 2013). Increased health awareness of consumers and increasing wealth in developing countries are driving forward the market of functional foods and beverages. Plant proteins currently cover 40% of the total protein ingredient market which is still led by dairy proteins, and are expected to surpass dairy proteins in certain applications due to their cost effectiveness (Frost & Sullivan, 2013). There is still shortage of comprehensive reviews on the prices of plant protein products, but according to a price comparison between soy and dairy protein products by Frost & Sullivan (2013), the prices of soy protein products represent about 60-80% of whey protein prices (Table 9). Having similar characteristics in terms of amino acid composition and techno-functional properties, standard rapeseed protein isolates could reach a price level comparable to that of the current soy and pea protein products on the market. However, this will require feasible technologies which limit the content of undesirable or antinutritive factors such as phenolics, phytates and glucosinolates in rapeseed protein products.

Table 9. Examples of price estimates for plant- and animal-based protein ingredients in 2012.

Source	Product	Price ($/kg)
Soy	Highly functional soy protein isolate [a]	9
	Soy protein isolate [b, c]	3–8
	Soy protein hydrolyzate [b]	6
	Potato protein isolate [c]	10
Pea	Pea protein isolate [a, c]	4–7
Wheat	Gluten [a]	0.5–1.5
Milk	Whey protein isolate [b]	10–12
	Whey protein hydrolyzate [b]	9
	Whey protein concentrate (80% protein) [b]	8
	Whey protein concentrate (34% protein) [b]	4
Milk	Milk protein concentrate / isolate (>80% protein) [b]	11–13
	Milk protein concentrate (70% protein) [b]	7
	Milk protein concentrate (42% protein) [b]	3
Egg	Spray-dried egg white [a]	13
Animal skin/bone	Gelatin [a]	2–5

[a] Burcon NutraScience (2015), [b] Frost & Sullivan (2013), Solanic (2013).

Rapeseed proteins are currently on the verge of commercialization: several technologies for rapeseed protein production are available, and prototypes for food and non-food applications have been demonstrated. Rapeseed protein products were recently approved for food use in several countries: Burcon's canola protein products received GRAS status in 2008, and in 2014, *B. napus* protein isolates were approved for marketing in Europe as a novel food ingredient (European Commission, 2014). Unprocessed rapeseed meal has not received GRAS status to date and has thus not been approved safe for human consumption.

Despite considerable advancements, the value chain of rapeseed protein production still suffers from lack of manufacturers and major end users of the protein fractions. Two Canadian enterprises, Burcon NutraScience Corporation and former BioExx Specialty Proteins Ltd, have commercialized production processes for rapeseed protein isolates but have faced challenges, in part related to high processing costs and insufficient product quality in comparison to soy protein products (Xu and Diosady, 2012). The current wet fractionation technologies for production of plant protein ingredients use large amounts of energy, water and chemicals during the multiple processing steps. Recently, production of protein isolates from lupin seeds was reported to require over 80 kg water, 22.4 kg hexane, 40 g NaOH and 40 g HCl per kg of protein isolate (Berghout et al., 2015), which well demonstrates the development needs related to reduction of water and chemicals consumption.

2. Aims of the study

The main aim of this work was to develop sustainable enzyme-aided processing schemes for extraction of protein or protein hydrolyzates from rapeseed cold pressing residue, and to elucidate factors influencing protein extractability and the properties of the obtained protein-rich fractions.

The specific aims were:

1. to identify effects of hybrid treatment (particle size reduction and enzymatic hydrolysis of polysaccharides) and extraction parameters on the chemical composition and microstructure of rapeseed press cake in relation to protein recovery,
2. to develop enzyme-aided procedures suitable for enrichment of protein from rapeseed press cake at increased solid content and without chemicals,
3. to evaluate the techno-economic feasibility of enzyme-aided rapeseed protein extraction compared to alkaline extraction and isoelectric precipitation, and
4. to produce rapeseed extracts enriched in bioactive peptides using proteolytic enzyme treatment.

3. Materials and methods

3.1 Raw materials and enzymes

Rapeseed press cakes from two origins (*Brassica rapa* and *Brassica napus*) were used as raw materials for protein enrichment in Publications I–IV (Table 10). Two batches of non-dehulled (i.e. whole-seed) *B. rapa* cold-press cake were obtained from Kankaisten Öljykasvit Oy (I, II, III, IV). The batches were produced in different years, presumably explaining the compositional variation. Additionally, one batch of *B. napus* press cake was received from Kroppenstedter Ölmühle Walter Doepelheuer GmbH after cold pressing of partially dehulled seeds (I). Commercial *B. napus* protein isolate, Isolexx from BioExx Specialty Proteins Ltd. (>90% protein on a dry matter basis), was used as a reference raw material for rapeseed protein hydrolysis into peptides (IV).

Table 10. Raw materials used for extraction of rapeseed protein or protein hydrolyzates.

Raw material	Supplier	Year	Publication
B. rapa press cake (batch 1)	Kankaisten Öljykasvit Oy, Finland	2012	I, II, IV
B. rapa press cake (batch 2)	Kankaisten Öljykasvit Oy, Finland	2014	III
B. napus press cake from partially dehulled seeds	Kroppenstedter Ölmühle Walter Doepelheuer GmbH, Germany	2012	I
Isolexx Canola Protein isolate	BioExx Specialty Proteins Ltd., Canada	2012	IV

Carbohydrate hydrolase and protease preparations used for enzymatic treatment of rapeseed press cake and commercial rapeseed protein isolate are listed in Table 11. Protein concentration in the preparations was quantified by the Lowry method (Lowry et al., 1951). Enzyme activity profiles, including endo-1,3(4)-*β*-glucanase, polygalacturonase (Bailey and Pessa, 1990), endo-1,4-*β*-xylanase (Bailey et al., 1992), *β*-glucosidase (Bailey and Nevalainen, 1981) and protease activity (Lowry et al., 1951) were determined using colorimetric enzyme assays (I).

Table 11. Enzyme preparations used for bioprocessing of rapeseed press cake and commercial rapeseed protein isolate.

Enzyme preparation	Manufacturer	Source organism	Main measured activity	Main activity (nkat/mg protein)	Measured side activities of >100 nkat/g	Publication
Pectinex Ultra SP-L	Novozymes	*Aspergillus aculeatus*	polygalacturonase (EC 3.2.1.15)	2876	endo-1,4(3)-*β*-glucanase (EC 3.2.1.4)	I, II, III
Depol 740 L	Biocatalysts	*Trichoderma sp., Humicola sp.*	endo-1,4- *β*-xylanase (EC 3.2.1.8)	288	endo-1,4(3)-*β*-glucanase (EC 3.2.1.4)	I
Celluclast 1.5L	Novozymes	*Trichoderma reesei*	endo-1,4(3)-*β*-glucanase (EC 3.2.1.4)	158	N.d.	I
Protex 6L	Genencor	*Bacillus licheniformis*	serine endopeptidase (EC 3.4.21)	163	N.a.	IV
Protamex	Novozymes	*B. licheniformis, B. amyloliquefaciens*	serine endopeptidase (EC 3.4.21), metalloendopeptidase (EC 3.4.24)	205	N.a.	IV
Corolase 7089	AB Enzymes	*Bacillus subtilis*	serine endopeptidase (EC 3.4.21)	214	N.a.	IV
Alcalase 2.4L FG	Novozymes	*Bacillus licheniformis*	serine endopeptidase, glutamyl endopeptidase (EC 3.4.21)	72	N.a.	IV

N.d.) not detected. N.a.) not analyzed.

3.2 Pretreatment and dry fractionation

Pelletized *B. rapa* press cake was ground using a cutting mill and optionally defatted by SC-CO_2 extraction under 300 bar pressure at 40–42°C (II, III, IV) (Table 12). Particle size of the ground rapeseed press cakes was further reduced by dry milling, primarily using a pin disc mill operated at 17800 rpm (I, II, IV). Alternatively, defatted *B. rapa* press cake was milled in a pin disc mill (8900 rpm) or air-flow mill to 164 or 7 µm median particle size, respectively (III). Defatted, fine-milled (17800 rpm ×2) *B. rapa* press cake was additionally air-classified at 15000 rpm to separate fine kernel fragments from coarse hull particles, yielding a kernel-rich fraction of rapeseed press cake which was used as a raw material for protein hydrolyzate production (IV).

Table 12. Pretreatment schemes applied in different publications.

Raw material	Pretreatment	Median particle size	Publication
B. rapa press cake	1) grinding 2) pin disc milling, 17800 rpm	54 µm [a]	I
B. napus press cake from partially de-hulled seeds	1) grinding 2) pin disc milling, 17800 rpm	N.a.	
B. rapa press cake	1) grinding 2) SC-CO_2 extraction 3) pin disc milling, 17800 rpm ×2	35 µm [a]	II
B. rapa press cake	1) grinding 2) SC-CO_2 extraction	600 µm [b]	III
	1) grinding 2) SC-CO_2 extraction 3) pin disc milling, 8900 rpm	164 µm [b]	
	1) grinding 2) SC-CO_2 extraction 3) pin disc milling, 17800 rpm	21 µm [b]	
	3) air-flow milling	7 µm [b]	
B. rapa press cake	1) grinding 2) SC-CO_2 extraction 3) pin disc milling, 17800 rpm ×2 4) kernel-rich fraction of air classi-fication	16 µm [a]	IV

[a] particle size analysis in water. [b] particle size analysis in ethanol. N.a.) not analyzed.

3.3 Carbohydrate-hydrolyzing enzyme treatment

Treatments with carbohydrate-hydrolyzing enzymes were performed with various conditions and substrate to enzyme to water ratios, as summarized in Table 13. All treatments were performed in Milli-Q water without pH adjustment, i.e. at the natural pH of the substrate (pH 5.8–6.0). First, three commercial enzyme preparations, Pectinex Ultra SP-L, Depol 470 L and Celluclast 1.5L, were compared for their ability to facilitate protein recovery from *B. rapa* and partially dehulled *B. napus* press cakes at 10% (w/v) total solid content (e.g. 2.5 g dry substrate in a total volume of 25 g) (I). Enzymes were dosed at 10 mg total protein / g dry substrate. Treatment temperatures were selected as 50°C for Depol and Celluclast and 30 or 40°C for Pectinex on the basis of the temperature optima reported by the manufacturers. After horizontal agitation of tubes for 48 h with sodium azide as an antimicrobial agent, protein extracts were recovered by centrifugation at 4°C, 3220 × *g*. On the basis of the results, Pectinex (containing polygalacturonase and endo-1,4(3)-*β*-glucanase as its main activities) was selected as a suitable enzyme prep-

aration for enzyme-aided rapeseed protein extraction, and 40°C was identified as a favorable temperature for Pectinex.

Subsequently, total solid content, treatment time, temperature and enzyme dosage were varied during pectinase treatments which were carried out as a separate step before protein extraction (II, III). The treatments were performed at an increased total solid content of 20 or 40% (w/v). The pectinase preparation (Pectinex) was dosed at 5 or 10 mg total protein / g dry substrate, and treatments were carried out for 2 or 4 h in a closed 800 ml stirred tank reactor. After treatment, the suspensions were aliquoted either directly (II) or after lyophilization and gentle grinding (III) into tubes for protein extraction.

3.4 Protein extraction and isoelectric precipitation

Protein recovery by enzyme-aided water extraction was compared with alkaline extraction and isoelectric precipitation or saline extraction as the state-of-art protein extraction methods. Protein was extracted from rapeseed press cake into Milli-Q water (II, III), alkaline solution (II), saline buffer (III) or alkaline buffer (III) as summarized in Table 13. Water extraction was performed without pH adjustment at the pH of the substrate (pH 5.5 after enzyme treatment and pH 5.8–6.0 without enzyme treatment). Alkaline extractions were performed in water solution in which the pH was adjusted to 10 using NaOH (II), or in 0.2 M sodium phosphate buffer, pH 12 (III). Saline extractions were performed in 0.2 M sodium phosphate buffer, pH 6 (III). After horizontal agitation at 25–30°C, protein extracts were separated by centrifugation at 4°C, 3220 × *g* (II) or 11963 × *g* (III). Alkaline protein extracts (pH 10) were optionally subjected to isoelectric precipitation (II). The extracts were adjusted to pH 4 using HCl, and precipitates were separated by centrifugation. Water extracts and isoelectric precipitates were lyophilized for analysis of solubility, ζ-potential and particle size (II).

Mass balances during protein extraction and precipitation were determined on the basis of the dry weight of residual solids or extracts after centrifugation. Protein yields (protein recovery or total protein solubilization) were calculated on the basis of total nitrogen analysis using two approaches: Protein recovery was determined as the percentage of total nitrogen removed from the solids (I, II) or recovered in the extracts (III), calculated by dividing the amount of protein in the residual solids or extract by the amount of total protein in the press cake. Total protein solubilization was determined as the percentage of total nitrogen present in the liquid phase, calculated by multiplying the protein concentration of extracts by the total liquid volume during extraction and dividing by the amount of total protein. Protein recovery in the precipitates was expressed as the percentage of total nitrogen or alkali-extracted nitrogen which was recovered in the isoelectric precipitate.

Table 13. Studied enzyme treatment and extraction schemes for differently pre-treated rapeseed press cake samples.

Raw material	Enzyme treatment (±)			Protein extraction		Pub-lica-tion
	Enzyme; dosage	**Time**	**Solid content**	**solvent; pH**	**Solid content**	
B. rapa press cake, milled	Pectinex, Depol, Celluclast; 10 mg/g	48 h	10%	no separate extraction step		I
B. napus press cake from partially dehulled seeds, milled	Pectinex, Depol, Celluclast; 10 mg/g	48 h	10%	no separate extraction step		I
B. rapa press cake, defatted, milled	Pectinex; 5 mg/g	2 h	20%	NaOH solution; pH 10	10%	II
			40%		10%	
			40%		20%	
			20%	water; pH 5.5-6.0	10%	
			40%		10%	
			40%		20%	
B. rapa press cake, defatted, milled (600 µm)	Pectinex; 10 mg/g	4 h	40%	NaH_2PO_4 buffer; pH 6	5%	III
				water; pH 5.5-6.0	20%	
B. rapa press cake, defatted, milled (164 µm)	Pectinex; 10 mg/g	4 h	40%	NaH_2PO_4 buffer; pH 6	5%	
B. rapa press cake, defatted, milled (21 µm)	Pectinex; 10 mg/g	4 h	40%	NaH_2PO_4 buffer; pH 6	5%	
				Na_3PO_4 buffer; pH 12	5%	
				water; pH 5.5-6.0	20%	
B. rapa press cake, defatted, milled (7 µm)	Pectinex; 10 mg/g	4 h	40%	NaH_2PO_4 buffer; pH 6	5%	
				Na_3PO_4 buffer; pH 12	5%	
kernel-rich fraction of defatted, milled *B. rapa* press cake	Alcalase, Corolase, Protamex, Protex; 10 mg/g	2 or 4 h	10%	no separate extraction step		IV

3.5 Production of protein hydrolyzates

The kernel-rich fraction obtained from dry fractionation of defatted *B. rapa* press cake and the commercial *B. napus* protein isolate were treated with commercial endoprotease preparations, Alcalase 2.4L FG, Corolase 7089, Protamex and Protex 6L to obtain crude protein hydrolyzates (IV). In addition to single treatments, two-stage experiments in which Protex treatment was followed by another protease treatment were performed as a means to produce hydrolyzates with a higher degree of hydrolysis. The treatments were performed at 10% total solid content in Milli-Q water, at the natural pH of the substrates (pH 6.3–7.3). Each enzyme was dosed at 10 mg total protein / g dry substrate. Substrate-water suspensions were heated to 100°C before protease addition to inactivate endogenous enzymes, and after each enzyme treatment to inactivate the added enzymes. Hydrolyzates were separated by centrifugation and lyophilized for bioactivity assays.

3.6 Analytical methods

3.6.1 Analysis of structure and composition

Microstructure and particle size. Press cake samples were prepared for microscopy according to Holopainen-Mantila et al. (2013) (I, III). Thin sections of the samples were stained with Acid Fuchsin and Calcofluor White to visualize proteins as red and *β*-linked glucans as blue during examination under a fluorescence microscope. Additional sections were stained with Ruthenium Red which shows pectins as red in bright-field illumination (Hanke and Northcote, 1975; Hou et al., 1999). Particle size distribution in press cake samples was determined by laser light (750 nm) diffraction (III). Samples were dispersed and run in water (I, II, IV) or ethanol (III).

Chemical composition. Chemical composition of press cakes, protein extracts, residual solids or protein precipitates was analyzed in order to assess raw material and product quality and to estimate material balances. Total nitrogen content was analyzed by the Kjeldahl method as described in Kane (1986). Crude protein content was calculated from the total nitrogen using a conversion factor of 6.25. Ash content was quantified gravimetrically as the inorganic residue after combustion at 550°C. Total sinapic and ferulic acid were quantified by liquid chromatography (LC) according to Vuorela et al. (2003) (IV). Crude fat content was determined by heptane extraction of lipids and weighing of the extracted dry mass.

Comprehensive carbohydrate analytics were utilized in order to elucidate raw material and product composition and the function of the carbohydrate hydrolyzing enzymes (I). Overall carbohydrate content was determined by total hydrolysis of heptane-defatted samples with sulfuric acid (Sluiter et al., 2008) and analysis of the released neutral monosaccharides by LC (Tenkanen and Siika-Aho, 2000). Acid-soluble lignin was measured spectrophotometrically from the acid hydrolyzate (Goldschmid, 1971), and acid-insoluble material (Klason lignin) was determined

as the weight of the acid hydrolysis residue. Uronic acids originating from pectin and hemicellulose were determined by gas chromatography after acid methanolysis according to Willför et al. (2009). Additionally, water-soluble carbohydrates were determined by water extraction of heptane-defatted samples at 60°C. Free neutral monosaccharides, sucrose, and uronic acids in the water extracts and previously described protein or peptide extracts were analyzed by LC. Total water-soluble carbohydrates were determined by LC after acid hydrolysis of the extracts. Insoluble non-cellulosic polysaccharides were determined by dilute acid hydrolysis of the water extraction residues followed by LC analysis of neutral monosaccharides (I). Digestible starch and *β*-glucan were determined enzymatically according to McCleary et al. (1997) and Munck et al. (1989), respectively (I).

Protein and peptide analysis. Molecular weight distribution of proteins was visualized by gel electrophoresis (SDS-PAGE) in reducing (I, II) or non-reducing conditions (II). Protein contents of hydrolyzates and unhydrolyzed extracts were determined by the Lowry method (IV). Release of peptides and amino acids during protein hydrolysis was analyzed on the basis of free amino nitrogen (FAN) concentration according to the Analytica EBC (1997) method (IV). Degree of hydrolysis was determined as the proportion FAN in the hydrolyzates out of total raw material nitrogen. Molecular weight distribution of peptides was visualized by reducing SDS-PAGE (IV).

3.6.2 Analysis of protein solubility and thermal properties

Protein solubility of water extracts and isoelectric precipitates was determined at pH 4 and 8 (II). The samples were dispersed in water to 2% (w/w) protein concentration based on the total nitrogen content, adjusted to pH 4 or 8, stirred for 2 h and centrifuged to separate the supernatants. Protein concentration of the supernatant was measured by the Lowry method to determine the proportion of soluble protein in relation to the total nitrogen content of the dispersion. Particle size distribution and *ζ*-potential of the supernatants were analyzed by dynamic and electrophoretic light scattering, respectively (II). Denaturation temperature and enthalpy of proteins in milled rapeseed press cakes were determined by differential scanning calorimetry (III).

3.6.3 Analysis of bioactive properties

Biocompatibility. Biocompatibility of rapeseed protein hydrolyzates was determined on the basis of the viability of human foreskin fibroblast cells in the presence of extracts (0.2 or 2 mg/ml) (IV). Cell viability was monitored using a colorimetric AlamarBlue assay with resazurin as an active compound to assess the reducing power of living cells. The cell viability percentage was determined on the basis of the proportionality of resorufin formation to the number of viable cells.

Cellular antioxidativity and radical scavenging activity. Antioxidativity of hydrolyzates was assessed on the basis of their ability to suppress cellular oxidative stress and scavenge radicals (IV). Cellular antioxidant activity (CAA) was determined using the assay developed by Wolfe and Liu (2007), which measures the ability of compounds to prevent oxidation of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to dichlorofluorescein (DCF). DPPH radical scavenging activity of hydrolyzates was determined using a method modified from Mahdi-Pour et al. (2012), based on the ability of compounds to reduce an organic nitrogen radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). Oxygen radical absorbance capacity (ORAC) of hydrolyzates was determined according to Alashi et al. (2014) with modifications (IV).

Anti-inflammatory and anti-wrinkle activity. Anti-inflammatory activity of hydrolyzates (0.2 mg/ml) was determined on the basis of their effect on myeloperoxidase (MPO) activity, which was detected spectrophotometrically using guaiacol as a substrate (IV). Anti-wrinkle activity of hydrolyzates was determined on the basis of their ability to inhibit human neutrophil elastase (HNE) activity according to the method described by Vasconcelos et al. (2011) with modifications (IV). N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide was used as a substrate for HNE.

3.6.4 Statistical analysis

Statistical analysis of the protein extraction and precipitation yields from 3–4 replicate experiments was carried out by general linear model multivariate analysis using SPSS Statistics software (II, III). Level of significance among means was set at $p < 0.05$ and assessed by Tukey's post hoc test. Data from the bioactivity assays was analyzed by one-way analysis of variance (ANOVA) using Graph Pad Prism statistical software. Significant differences among means were determined by Dunnett's post hoc test with a significance level set to $p < 0.05$ (IV).

3.7 Techno-economic evaluation of production costs

An early-stage light techno-economic assessment of the production costs of protein-enriched fractions from defatted rapeseed cold-press cake by a) alkaline extraction and isoelectric precipitation, b) enzyme treatment, alkaline extraction and isoelectric precipitation, c) water extraction, or d) enzyme treatment and water extraction was carried out. For each process scheme the extraction step was performed at 10% or 20% solid content (II), and therefore, a total of eight process schemes were evaluated. Extraction and precipitation yields and doses for enzymes, alkali and acid were calculated on the basis of laboratory-scale experiments (II), as presented in Table 14. The production capacity was selected as 10 000 tons of defatted press cake per year.

Table 14. Key parameters of rapeseed protein production schemes selected for techno-economic feasibility analysis.

	Enzyme treatment: 40% d.m. Extraction: 10% d.m.				Enzyme treatment: 40% d.m. Extraction: 20% d.m.			
	alkali	**enzyme/ alkali**	**water**	**enzyme/ water**	**alkali**	**enzyme/ alkali**	**water**	**enzyme/ water**
Total solid content in hydrolysis (%)	40	40	40	40	40	40	40	40
Enzyme dose (ml/g dry press cake)	0	0.08	0	0.08	0	0.08	0	0.08
Total solid content in extraction (%)	10	10	10	10	20	20	20	20
NaOH dose (mg/g dry press cake)	23	30	0	0	25	29	0	0
Protein yield of extraction (% of total protein)	46	53	29	40	47	48	25	32
Non-protein yield of extraction (% of total non-protein)	28	42	24	41	20	27	15	28
Acid dose (mg/g dry press cake)	13	15	0	0	12	15	0	0
Protein yield of isoelectric precipitation (% of extracted protein)	60	42	0	0	64	53	0	0
Non-protein yield of isoelectric precipitation (% of extracted non-protein)	19	16	0	0	51	41	0	0
Solid content of the residue after centrifugation (%)	30	30	30	30	30	30	30	30
Solid content of water extract after evaporation (%)	20	20	20	20	20	20	20	20
Thermal efficiency of evaporator (%)	90	90	90	90	90	90	90	90
Solid content of the water extract, isoelectric precipitate or residue after drying (%)	95	95	95	95	95	95	95	95
Thermal efficiency of dryers (%)	50	50	50	50	50	50	50	50

For calculation of energy costs, electricity consumption of the process was estimated as 100 kWh/t liquid, which is similar to the electricity consumption of milk processing in Europe (European Commission, 2006). For calculation of the heat needed for evaporation and/or spray-drying of the products and residue to 95% total solid content, solid content of the residue after centrifugation was estimated as 30%. Thermal efficiencies of the evaporator and dryers were estimated as 90% and 50%, respectively. Price information for chemicals, water and energy was obtained from standard databases such as ICIS (2006) (Table 15). The prices of electricity, heat and process water were estimated as 100 €/MWh, 10 €/GJ and 1 €/m^3, respectively. The feed price of rapeseed press cake was estimated as 200€/t on the basis of a review by Carré and Pouzet (2014). Defatting of the cold-pressed press cake by SC-CO_2 or hexane extraction was not included in the cost analysis. When outsourced, the price of SC-CO_2 extraction is approximately 2.40–2.80 € / kg press cake for a batch size of 50 t, and 1.80–2.00 € / kg for a 200 t batch (NATECO2, 2014).

Table 15. Estimated prices of raw materials, chemicals, water and energy for the techno-economic feasibility analysis.

Product	**Price**
Defatted rapeseed press cake [a]	200 €/t
Enzyme [b]	25000 €/t
NaOH [c]	370 €/t
HCl [c]	100 €/t
Electricity	100 €/MWh
Heat	10 €/GJ
Process water	1 €/m^3
Waste water	1 €/m^3

[a] Carré and Pouzet (2014), [b] alibaba.com (2015), [c] ICIS (2006)

Cost factors (capital charge, labor, maintenance and other indirect costs) were estimated according to Turton et al. (2008) with modifications: capital charge was calculated for a 10-year investment of 6 M€ in the case of alkaline extraction and isoelectric precipitation processes, or 5 M€ in the case of water extraction processes. These investment cost estimates were based on the costs of two protein production plants: the Nutrimar fish oil and protein plant in Froya, Norway, and the BioExx canola protein plant in Saskatoon, Canada. The Nutrimar plant had an investment cost of ca. 7 M€ / 10 000 t RM/year (Nappa et al., 2013), whereas the investment cost of the BioExx plant was estimated to be 22 M€ / 40 000 t rapeseed / year based on production capacity (CNW, 2007) and equipment purchases (BioExx Specialty Proteins, 2011). Labor costs were calculated for three employ-

ees with a monthly salary of 5000 € and side costs of 37.5%. Other fixed costs consisted of maintenance and other indirect costs, which were estimated as 2.5% and 1.0% of investment costs, respectively.

4. Results

4.1 Composition and structure of raw materials

A comprehensive analysis of the chemical composition of rapeseed cold-press cakes provided guidelines for the selection of enzymes and fractionation procedures. Unprocessed, whole-seed *B. rapa* press cake contained 36% crude protein, 44% carbohydrates (incuding separately analyzed uronic acids), 14% acid-insoluble material (often termed as Klason lignin), and 12% crude oil on a dry matter basis (Table 16). Its overall monosaccharide profile consisted of mainly glucose (14.7% d.m.), galacturonic acid (8.0%), fructose (7.4%), arabinose (5.8%), galactose (3.9%) and xylose (2.3%). Glucose and fructose originated primarily from sucrose (12% of total dry matter), whereas the high galacturonic acid concentration (8.0%) indicated the presence of substantial amounts of pectin. Cellulose constituted 5.2% of the dry matter, and starch and *β*-glucan contents were negligible. In general, 44% of the carbohydrates in non-dehulled press cake and 54% in dehulled press cake were water-soluble.

SC-CO_2 extraction of the *B. rapa* press cake reduced its oil content to 2–3%, with concomitant increase of protein content to 39–40%. Further removal of most hull particles from defatted press cake by fine milling and air classification resulted in a kernel-rich material containing 46% protein. Hull removal reduced the concentration of insoluble carbohydrates and acid-insoluble material (Klason lignin) but increased the proportion of sinapic acid in the kernel-rich fraction (52% yield) when compared to unfractionated press cake (Partanen et al., 2016). Particularly, cellulose and galacturonic acid were reduced from 5.2 and 8.0% to 1.6 and 4.9%, respectively, indicating that a large proportion of cellulose and pectin was removed with the hulls. Lower concentration of cellulose and acid-insoluble material and higher concentration of protein and sinapic acid were similarly observed in the press cake from partially dehulled *B. napus* seeds than in the press cake from non-dehulled *B. rapa* seeds, in accordance with the reduced hull content.

Table 16. Chemical composition of rapeseed press cake samples used as raw materials for enzyme-assisted protein and peptide extraction.

Sample	**Non-dehulled press cake (% d.m.)**	**Partially dehulled press cake (% d.m.)**	**Kernel-rich fraction of defatted, non-dehulled press cake (% d.m.)**
Protein	35.9	40.1	45.8
Carbohydrates (including uronic acids)	43.8	40.3	34.6
Carbohydrates (excluding uronic acids)	35.4	34.5	29.3
Overall monosaccharide composition:			
Glucose	14.7	14.4	12.9
Arabinose	5.8	4.8	4.7
Galactose	3.9	3.9	3.5
Xylose	2.3	1.9	2.2
Fructose	7.4	8.6	5.3
Mannose	0.8	0.5	0.5
Rhamnose	0.5	0.4	0.2
Galacturonic acid	8.0	5.4	4.9
Glucuronic acid	0.3	0.3	0.3
Methyl-glucuronic acid	0.1	0.1	0.1
Water-soluble carbohydrates	15.7	18.7	13.5
Monosaccharides and sucrose	13.7	16.7	11.2
Polysaccharides	1.9	2.0	2.4
Insoluble carbohydrates	19.8	15.8	15.8
Acid-insoluble material (Klason lignin)	13.6	8.2	5.3
Oil	11.8	16.7	2.6
Ash	7.0	5.6	N.a.
Acid-soluble lignin	5.3	5.5	6.1
Phenolic acids	1.2	1.7	1.6
Sinapine and sinapic acid	1.18	1.69	1.6
Ferulic acid	0.01	0.02	0.1
P-coumaric acid	0.002	0.002	N.d.

N.a.) not analyzed, N.d.) not detectable. Uronic acids were separately analyzed by methanolysis. Mean results from at least two replicate analyses are presented. Overall carbohydrates (excluding uronic acids), acid-insoluble material (Klason lignin), oil, acid-soluble lignin and total phenolic acids of the kernel-rich fraction were analyzed in Partanen et al. (2016).

Fluorescence microscopy of Calcofluor- and Acid Fuchsin-stained thin sections of *B. rapa* press cake (Figure 4a) showed that disruption of protein bodies (shown as red) and partial disintegration of cell walls (shown as blue) in the embryonic tissue had occurred during cold pressing, resulting in release of some protein from the embryonic cells. Thin sections of the unprocessed *B. rapa* press cake were additionally stained with Ruthenium Red and examined under bright-field illumination in order to visualize the localization of pectins (Figure 4b). These carbohydrates were intensely stained in the seed coat and to some extent detected in cell walls of the embryo. Additionally, networks stained by Ruthenium Red were visible inside the embryonic and aleuronic cells, and particularly in cells of the embryonic axis.

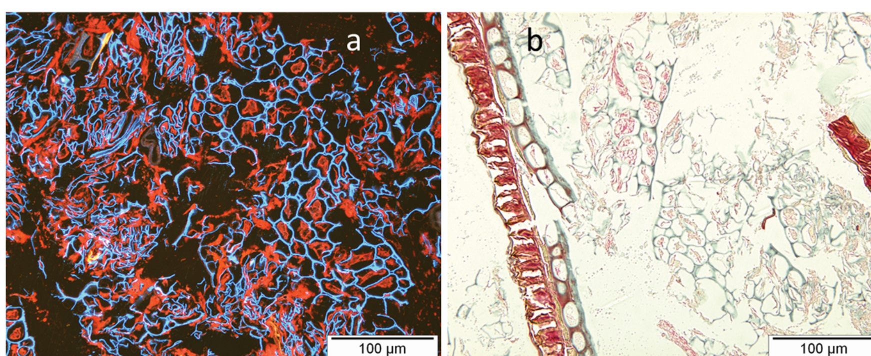

Figure 4. a) Epifluorescence micrograph of *B. rapa* cold-press cake after staining with Calcofluor White and Acid Fuchsin, showing cell wall glucans as blue and proteins as red, respectively. b) Bright-field micrograph of the corresponding press cake after staining with Ruthenium Red, showing pectins as red.

4.1.1 Influence of processing on the microstructure of rapeseed press cake

Changes occurring in the microstructure of *B. rapa* cold-press cake during mechanical or enzymatic treatments were visualized by fluorescence microscopy (Figure 5). The microstructure of defatted, ground press cake (a) largely resembled that of the unprocessed press cake, with partially disrupted embryonic tissue. Pin disc milling (b) caused further fragmentation of embryonic cell walls and seed coat particles. Treatment with a commercial pectinase preparation containing *β*-glucanase as a side activity (Pectinex Ultra SP-L, Novozymes) resulted in complete cell wall degradation in the embryo (c), but cell walls in the aleurone layer were left largely intact. Figure 5d is a micrograph of pin disc-milled, non-enzymatically treated press cake after 2-step alkaline extraction of 74% of the protein. After the extraction, protein was nearly absent from the residual solids except for some protein detected in intact aleurone cells.

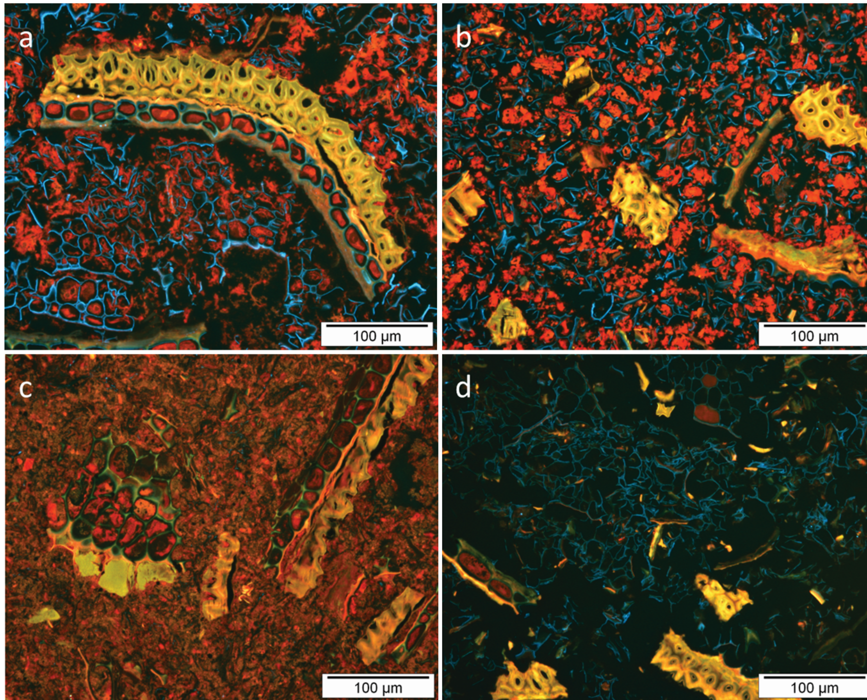

Figure 5. Epifluorescence micrographs of a) defatted, ground *B. rapa* press cake, b) defatted, pin disc-milled press cake, c) defatted, pin disc-milled press cake after enzyme treatment, and d) defatted, pin disc-milled press cake after protein extraction. The sections were stained with Calcofluor White and Acid Fuchsin, showing cell wall glucans as blue and proteins as red, respectively.

4.2 Enzyme-aided protein extraction

Approaches to enhance protein recovery from rapeseed cold-press cake were investigated by studying the extractability of protein in different extraction conditions and after varying pretreatments. Protein extraction yields from different processing schemes (Table 17), ranging from 25 to 78% of the total protein, were calculated on the basis of the extract volumes separated by centrifugation, and thus describe the practical recovery of protein in the selected centrifugation conditions. Optionally for comparison, total protein solubilization yields were calculated on the basis of the total liquid volume in each treatment (III). Higher protein recoveries were generally obtained after carbohydrate-hydrolyzing enzyme treatment of the press cake than without treatment, but the extent to which enzyme treatment enhanced protein yield depended considerably on the extraction conditions such as pH and solid content (i.e. press cake to water ratio), as specified in the following paragraphs.

Table 17. Protein recoveries from different rapeseed press cake samples during aqueous extraction in varying conditions. The samples were optionally treated with a pectinase preparation at 10% solid content (I) or 40% solid content (II, III).

Raw material	Median particle size (µm)	Protein extraction	Solid content (%)	Protein recovery without enzyme (%)	Protein recovery with pectinase (%)	Publication
B. rapa press cake, milled	54	water	10	33 ± 0.40	56 ± 0.48	I
B. napus press cake from partially dehulled seeds, milled	N.a.	water	10	47 ± 0.26	74 ± 0.44	
B. rapa press cake, defatted, milled	35	alkaline (pH 10)	10	46 ± 8.04	53 ± 3.29	II
			20	47 ± 4.70	48 ± 3.01	
		water	10	29 ± 1.41	40 ± 1.31	
			20	25 ± 0.21	32 ± 1.31	
B. rapa press cake, defatted, ground or milled	600	saline	5	61 ± 0.86	69 ± 0.27	III
		water	20	38 ± 0.56	57 ± 0.65	
	164	saline	5	61 ± 0.18	68 ± 0.48	
	21	saline	5	63 ± 0.54	67 ± 0.20	
		alkaline (pH 12)	5	74 ± 0.65	78 ± 0.09	
		water	20	37 ± 4.03	58 ± 0.29	
	7	saline	5	54 ± 0.14	59 ± 0.20	
		alkaline (pH 12)	5	65 ± 1.34	67 ± 0.82	

N.a.) not analyzed.

4.2.1 Effects of raw material pretreatments on protein extractability

In the studied fractionation schemes, particle size of *B. rapa* press cake was reduced by different grinding and dry milling procedures, with the aim to improve enzyme-substrate accessibility and protein release through disruption of the cell wall matrix. Contrary to expectations, particle size reduction of ground, defatted press cake by pin disc milling from 600 to 21 µm had no significant effect on either total protein solubilization or protein recovery during extraction (III). The effect of pin disc milling was negligible, irrespectively of the extraction procedure (saline extraction or water extraction). After particle size reduction from 600 to 7 µm by impact milling, even lower protein yields were observed than without milling. The reduced protein solubilization yields correlated with decreased protein denaturation enthalpies measured by differential scanning calorimetry.

In addition to particle size reduction, other pretreatments including dehulling and defatting were investigated (I, II). In general, protein was more effectively extracted from the press cake obtained from partially dehulled *B. napus* seeds than from the press cake obtained from non-dehulled *B. rapa* seeds (I). Removal of residual fat from *B. rapa* press cake by SC-CO_2 extraction had no clear influence on protein extractability, as similar protein yields were obtained from either non-defatted or the corresponding defatted rapeseed press cake. Only in the alkaline extraction at 20% solid content was minor improvement of protein recovery achieved by defatting.

4.2.2 Effect of carbohydrate-hydrolyzing enzyme treatment on protein extractability

Three commercial enzyme preparations, Pectinex Ultra SP-L, Depol 470L and Celluclast 1.5L, with polygalacturonase, xylanase and endo-1,4(3)-*β*-glucanase (cellulase) as their main measured activities, respectively, were compared for their ability to hydrolyze carbohydrates and enhance protein recovery from rapeseed cold-press cake (I). The pectinase preparation (Pectinex) containing high polygalacturonase activity and a *β*-glucanase side activity was the most effective preparation in terms of carbohydrate hydrolysis and protein release. The two other enzymes gave lower hydrolysis yields and enhanced protein extraction to a lesser extent. During 48-hour hydrolysis with the pectinase dosed at 10 mg total protein / g substrate, 71–75% of the total carbohydrates in non-dehulled and dehulled *B. rapa* and *B. napus* press cakes were extracted, and 63–68% were hydrolyzed into monosaccharides (based on the analyzed 35% carbohydrate content of raw materials) (I). All glucans and sucrose were converted to glucose and fructose, and most arabinose and galactose were also released as monosaccharides. In the reference treatment without added enzymes, 45–51% of the carbohydrates were extracted during 48 h (I). Complete sucrose hydrolysis observed during incubation without added enzymes at 50°C indicated the presence of endogenous invertase activity in rapeseed press cake. The pectinase preparation functioned well over a wide (10–40%) solid content range under continuous mixing (II) and was not inhibited by residual oil in the press cakes (12–17% on a dry matter basis).

Enzymatic carbohydrate hydrolysis correlated with increased protein extractability during water extraction or saline extraction at pH 6. The same trend was observed in varying solid contents of 5–20% (I, II, III). For example, only 33–47% protein yields were obtained from non-dehulled and dehulled press cakes during 48 h extraction without enzymes at 10% solid content, whereas 56–74% of the total protein was recovered after 48 h enzyme treatment (I). Carbohydrate-hydrolyzing enzyme treatment improved protein yield to a slightly lesser extent from defatted press cake when the enzyme dose and incubation time were reduced to 5 mg/g and 3 h, respectively (II).

In order to better understand the mechanism by which carbohydrate-hydrolyzing enzymes influence protein recovery, total protein solubilization and protein recovery yields from defatted rapeseed press cake in different extraction conditions were compared (III). Pectinase treatment was followed by two-step extraction into alkaline or saline buffer (0.2 M sodium phosphate, pH 12 or pH 6) at 5% solid content. The modified experimental setup including two extraction steps and higher centrifugation speeds (III) resulted in generally higher protein recoveries than in earlier experiments (I, II). Furthermore when the proteins were extracted with alkaline or saline buffer instead of water, enzyme utilization had only a minor effect or no clear influence on total protein solubilization (III). In terms of protein recovery, enzyme treatment treatment had a minor positive effect when compared to non-enzymatic alkaline or saline extraction. By contrast, when extractions were performed in water at 20% solid content, pectinase treatment clearly enhanced both the total solubilization and recovery of rapeseed protein. For example with enzyme utilization, protein solubilization from defatted, ground press cake improved from 48% to 68%, with concomitant increase of protein recovery from 38% to 57% (III).

4.2.3 Effects of extraction parameters on protein extractability

As expected, rapeseed protein extractability was higher in alkaline conditions (pH 10 or 12) than at pH 6, which is close to the isoelectric point of cruciferin. At the highest, 78% of the protein could be recovered from defatted press cake by alkaline extraction (pH 12) at 5% solid content (III). Non-enzymatic alkaline extraction at pH 12 as a reference method resulted in higher protein yields than enzyme-assisted water or saline extraction at pH 6. However, when extractions were performed at 10% solid content, similar protein yields could be obtained from defatted *B. rapa* press cake by enzyme-aided protein extraction at pH 6 (40% yield) as by non-enzymatic alkaline extraction at pH 10 (41–46% yield) (II). When the corresponding extractions were performed at higher (20%) solid content, alkaline extraction turned out to be more effective (II).

Increase of the solid content during protein extraction generally decreased the protein recovery. For example, 13–20% less protein was extracted at pH 6 when the solid content was increased from 10% to 20% (II). When at two-step extraction was used, however, almost comparable protein yields were obtained by enzyme-aided water extraction at 20% solid content as by non-enzymatic saline extraction at 5% solid content (III). Temperature increase from 30 to 50°C had no clear influence on the non-enzymatic extractability of protein or carbohydrates in water. Isoelectric precipitation enabled only partial protein recovery: when alkali-extracted proteins were subjected to isoelectric precipitation at pH 4, 35–64% of the total extracted protein precipitated (II). Proteins were even less prone to precipitation after carbohydrates had been enzymatically hydrolyzed.

4.3 Composition and solution behavior of protein fractions

Protein extracts obtained by enzyme-aided water extraction of *B. rapa* and *B. napus* press cakes at 10% solid content contained 39–54% protein and 47–49% carbohydrates on a dry matter basis (I). As anticipated, enzymatic carbohydrate hydrolysis increased the co-extraction of neutral sugars (mainly glucose, arabinose and galactose) and uronic acids (mainly galacturonic acid). Differences were also observed in the chemical composition and solution behavior of water extracts (pH 6) and isoelectric precipitates obtained from defatted rapeseed press cake at 20% total solid content (II), as presented in Table 18. The isoelectric precipitates from alkaline extraction (pH 10) had higher protein and ash contents than water extracts (pH 6), which contained relatively more carbohydrates. SDS-PAGE analysis of the samples under non-reducing and reducing conditions showed that protein extracts obtained with or without enzyme treatment at pH 10 or pH 6 did not clearly differ in their protein composition (II). Isoelectric precipitates appeared to contain relatively more cruciferin (ca. 20–50 kDa) than the water or alkaline extracts, whereas most of the extracted napins appeared to remain in the supernatants after isoelectric precipitation.

Table 18. Composition and solubility of isoelectric precipitates (from pH 10 alkaline extracts) and water extracts obtained from rapeseed press cake with or without pectinase treatment (Publication II).

Sample	P (%)	CH (%)	Ash (%)	Protein solubility (%)		ζ-potential (mV)	
				pH 4	pH 8	pH 4	pH 8
No enzyme / precipitate	65.6	10.7	10.1	6.2 ± 0.1	25.1 ± 0.2	8.7 ± 0.2	-21.9 ± 1.9
Pectinase / precipitate	57.3	13.1	10.1	6.4 ± 0.1	19.8 ± 0.1	10.2 ± 0.2	-21.3 ± 2.4
No enzyme / extract	47.3	47.6	6.0	13.4 ± 0.0	40.7 ± 0.1	-1.6 ± 0.6	-6.4 ± 0.5
Pectinase / extract	37.8	51.7	7.7	10.2 ± 0.0	39.1 ± 0.1	-5.7 ± 0.6	-11.4 ± 0.3

P) protein, CH) carbohydrates. Protein, carbohydrate and ash concentrations are expressed as % of dry matter.

When lyophilized water extracts and isoelectric precipitates were redispersed in water solutions adjusted to pH 4 and pH 8, the water extracts showed consistently better protein solubility (II) (Table 18). At pH 8, 39–41% of the protein in water extracts was soluble whereas only 20–25% protein solubility was measured in the isoelectric precipitates. Additionally, when particle size distributions of the protein dispersions were compared at pH 8, smaller particle sizes observed in the water extracts indicated better dispersion stability compared to the isoelectric precipitates. When the thermal behavior of these samples was later studied by differential scanning calorimetry at 10–150°C, both samples gave a characteristic dena-

turation peak at ca. 107°C. However, whereas denaturation enthalpy of the water extract resembled that of native rapeseed proteins (9.8 J/g protein), very low denaturation enthalpy (0.4 J/g protein) in the isoelectric precipitate indicated that the sample was already severely denatured. Differences were also observed in the ζ-potential values (describing the net charge of particles) of water extracts and isoelectric precipitates (Table 18). In contrast to the isoelectric precipitates, the water extracts exhibited consistently weak negative net charges at pH values both below (pH 4) and above (pH 8) the isoelectric point of cruciferin. At pH 4, water extracts and isoelectric precipitates possessed net charges close to zero, in accordance with their low solubility at this pH. At pH 8, the isoelectric precipitates exhibited stronger negative net charges (−21–22 mV) than the water extracts (−6–11 mV), but in this case, stronger net charge did not correlate with higher protein solubility.

4.4 Techno-economic feasibility of rapeseed protein extraction schemes

The production costs of protein-enriched fractions from defatted rapeseed cold-press cake by different fractionation schemes were estimated in order to obtain insight into the economic feasibility of enzyme-assisted process options. All data related to mass balances and consumption of enzymes and chemicals was collected from laboratory-scale experimental work in which four process schemes were compared for production of protein-enriched fractions from defatted rapeseed press cake: a) alkaline extraction and isoelectric precipitation, b) enzyme treatment, alkaline extraction and isoelectric precipitation, c) water extraction, d) enzyme treatment and water extraction (II). All enzyme treatments were performed at 40% solid content, whereas extractions were performed at both 10 and 20% solid content, generating a total of eight evaluated process schemes. Alkaline extraction and isoelectric precipitation schemes produced protein precipitates with a yield of 130–177 g dry precipitate per kg defatted press cake and a protein content of 59–77% d.m. Water extractions gave higher product yields (187–406 g dry extract/kg press cake), but the products had a lower protein content (38–52%) on a dry matter basis.

The estimated production costs for protein-enriched fractions ranged between ca. 4000 and 6000 €/t protein produced, depending on the fractionation scheme (Figure 6). When calculated on a raw material basis, the costs were 450–680 €/t press cake. Energy and raw material represented the two largest production costs. Cost of the press cake (200 €/t) was estimated on the basis of its current feed price (150–300 €/t). Energy costs were strongly associated with water consumption of the process, as heating of process water, electricity to drive machinery, and most importantly, heat needed for drying of the products and residual solids by evaporation and/or spray-drying required high inputs of energy. When the extraction was carried out at higher solid content (20% vs. 10%), energy costs were reduced by 29–41%. Enzyme utilization (included in the chemicals) resulted in a small additional cost of 14–24 €/t protein produced, depending on the product

yield from each process option. This additional cost was largely compensated by the improved product yield in the enzyme-assisted water extraction processes. A comparison of different production schemes showed that enzyme-aided water extraction and non-enzymatic alkaline extraction were the most cost-efficient options on a €/t protein basis (Figure 6). In alkaline extraction processes, enzyme utilization did not improve the product yield and was thus clearly unfeasible. The estimated production costs on a €/t protein basis were highest in the non-enzymatic water extraction due to low protein extraction yields.

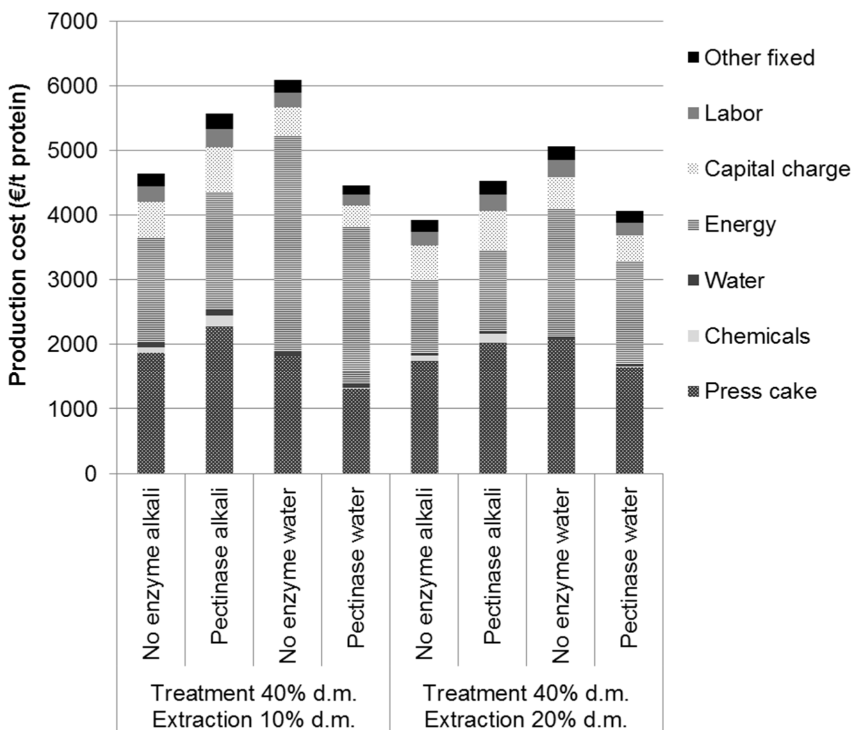

Figure 6. Estimated production costs of rapeseed protein fractions (€/t protein) obtained by different process schemes.

4.5 Production of rapeseed protein hydrolyzates

4.5.1 Composition, degree of hydrolysis and molecular weight range of rapeseed protein hydrolyzates

Four microbial endoprotease preparations (Protex, Protamex, Corolase and Alcalase) varying in substrate specificity were compared in their ability to produce bioactive protein hydrolyzates from *B. rapa* press cake and a commercial rapeseed protein isolate. Before protease treatments, the press cake was defatted by SC-CO_2 extraction, fine-milled and air classified to yield a protein-enriched material (IV). The obtained kernel-rich fraction contained 46% protein, 29% carbohydrates (ca. 50% water-soluble), 3% oil and 1.6% sinapic acid (Table 16). A commercial *B. napus* protein isolate (>90% protein) used as a reference raw material for peptide production was found to contain only 0.2% sinapic acid and traces (0.1%) of ferulic acid.

Composition and degree of hydrolysis in rapeseed protein hydrolyzates which were selected for bioactivity assessment are described in Table 19. In addition to protein and sugar concentrations, sinapic acid was determined due to its possible influence on the bioactivities of crude hydrolyzates. An unhydrolyzed protein extract obtained from the kernel-rich fraction of rapeseed press cake without enzyme treatment contained 38% protein (determined by the Lowry method) and substantial amounts of neutral sugars (31%) and sinapic acid (3.5%) on a dry matter basis. By contrast, protease treatment produced hydrolyzates with considerably higher concentrations of protein (55–69% of dry matter) and lower concentrations of non-protein compounds (16–26% neutral sugars, and 1.4–2.2% sinapic acid). At the highest, crude hydrolyzates containing over 60% protein were obtained from the kernel-rich fraction of press cake using the *B. licheniformis* serine endopeptidases, Protex and Alcalase. When compared to extracts from the dry-fractionated press cake, higher protein (69–87%) and lower sugar concentrations (1.3–7.4%) were generally measured in the corresponding extracts obtained from the commercial protein isolate. Extracts from this reference raw material were considered to be essentially devoid of the main phenolic compound, sinapic acid, due to its low initial content in the commercial protein isolate.

Table 19. Composition and degree of hydrolysis of rapeseed protein hydrolyzates obtained from the kernel-rich fraction of defatted *B. rapa* press cake (Publication IV).

Enzyme used	Protein (% d.m.)	Neutral sugars [a] (% d.m.)	Sinapic acid [b] (% d.m.)	Degree of hydrolysis (DH) (%)
No enzyme	38	31	3.5	1.5 ± 0.01
Protex	69	23	2.0	10.9 ± 0.07
Protamex	59	22	1.6	14.5 ± 0.18
Corolase	57	24	1.6	10.0 ± 0.12
Alcalase	63	26	2.2	8.5 ± 0.16
Protex-Protamex	55	18	1.4	12.6 ± 0.09
Protex-Corolase	59	22	1.5	12.6 ± 0.14
Protex-Alcalase	66	16	1.7	11.1 ± 0.07

[a] glucose, fructose, galactose, arabinose, mannose, xylose, rhamnose.
[b] free and esterified.

FAN and SDS-PAGE analyses were used to obtain an estimate of the protein and peptide size range in different rapeseed hydrolyzates. Degree of protein hydrolysis (DH) was determined on the basis of the proportion of free amino nitrogen (FAN) in the extracts out of total raw material nitrogen (Table 19). The DH of protein hydrolyzates from rapeseed press cake ranged between 8.5 and 14.5%, and expectedly increased when Protex treatment was followed by a second enzyme treatment. As an exception, Protex resulted in a slightly higher DH (14.5%) when applied individually than when Protex treatment was followed by Protamex treatment (DH 12.6%). In general, protein hydrolysis was more pronounced in the commercial protein isolate (DH 16–21%) than in the kernel-rich fraction of rapeseed press cake (DH 9–15%). SDS-PAGE analysis of the hydrolyzates and unhydrolyzed extracts under reducing conditions showed that visible peptide bands below 1.4 kDa appeared as a result of protease treatments. The molecular weight of these bands varied depending on the enzyme used. Most of the protein bands above 6.5 kDa disappeared during the treatments, indicating effective digestion of rapeseed cruciferin which contains subunits of ca. 20–30 kDa. Some bands around 5–7 kDa, corresponding to the size of napin subunits, remained visible after individual protease treatments (particularly after Alcalase treatment), but were less recognizable or no longer visible after the two-stage treatments.

4.5.2 Antioxidativity and enzyme inhibitory activity of rapeseed protein hydrolyzates

The protein hydrolyzates (Table 19) were characterized in terms of their antioxidative activity and ability to inhibit enzymes involved in skin inflammation and wrinkle formation. Antioxidativity was studied using three methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, ORAC (oxygen radical absorbance capacity) assay, and a CAA (cellular antioxidative activity) assay which was conducted in skin fibroblast cells. A cell viability assay was performed to confirm that all hydrolyzates were at least 79% compatible with the fibroblast cells at 0.2 and 2 mg/ml concentrations after 24 h of exposure. Of all the samples, only the unhydrolyzed protein from rapeseed press cake induced toxicity at 2 mg/ml.

The Protex and Alcalase hydrolyzates from rapeseed press cake were the most antioxidative hydrolyzates according to all assays. These samples showed 81–82% antioxidative activity in the CAA assay at 2 mg/ml concentration, and 85–90% and 50–60% radical scavenging activities at 0.5 mg/ml concentration in the DPPH and ORAC assays, respectively. The observed antioxidativity levels correlated with the higher protein and sinapic acid content of Protex and Alcalase hydrolyzates when compared to other hydrolyzates. Although these two hydrolyzates were identified as the most antioxidative ones, the other hydrolyzates and the unhydrolyzed extract from rapeseed press cake also showed considerable antioxidativity. For example in the DPPH assay, ca. 77–84% of the radicals were scavenged in the presence of the other hydrolyzates, and an 85% scavenging effect was observed in the presence of the unhydrolyzed extract. By contrast, the unhydrolyzed protein extract from commercial rapeseed protein isolate showed clearly lower antioxidativity in the DPPH and ORAC assays.

The ability of protein hydrolyzates to inhibit myeloperoxidase enzyme (MPO) *in vitro* was determined as an indicator of possible anti-inflammatory activity. The unhydrolyzed extract and most of the hydrolyzates from rapeseed press cake caused approximately 40% inhibition of MPO at 0.2 mg/ml concentration. The highest inhibitory level of 60% was obtained with the Protex-Alcalase hydrolyzate. Interestingly, the unhydrolyzed extract from commercial rapeseed protein isolate effectively inhibited up to 79% of the MPO activity, indicating that MPO inhibition level was not dependent on the presence of sinapic acid. The inhibitory activity of rapeseed protein hydrolyzates on human neutrophil elastase (HNE), a proteolytic enzyme involved in skin aging, was additionally assessed *in vitro*. The highest (91%) inhibition was obtained with the Alcalase hydrolyzate, followed by the Protex-Alcalase and Protamex hydrolyzates (88% inhibition). Most of the other hydrolyzates and unhydrolyzed protein extract from rapeseed press cake caused 72–84% inhibition of HNE. The unhydrolyzed extract from commercial rapeseed protein isolate was somewhat less inhibitory (65% inhibition) than the extracts obtained from rapeseed press cake.

5. Discussion

Approaches for feasible protein enrichment from rapeseed cold-press cake were investigated in the present work. These included replacement of the commonly used additives, alkali or salt, with hydrolytic enzymes, and increase of the total solid content during extraction. Data from varied experimental setups showed that enzymatic hydrolysis of pectin and cell wall glucans facilitated protein extraction from rapeseed cold-press cake. The studied pectinase (Pectinex Ultra SP-L), xylanase (Depol 740L) and cellulase preparations (Celluclast 1.5L) were mainly active on cell walls surrounding the embryo cells, where most of rapeseed protein is stored. At the highest, 68% of the total protein could be solubilized and 58% recovered from defatted, non-dehulled rapeseed press cake by enzyme-aided water extraction using the pectinase preparation. This yield was obtained during a two-step extraction at elevated (20%) solid content. A similar (56%) protein recovery was obtained from the corresponding non-defatted press cake by a single-step extraction at 10% solid content. When a non-defatted press cake from dehulled seeds was used as raw material, 74% protein recovery could be obtained by the enzyme-aided water extraction. The yields coincide well with the 56-71% yields from alkaline extraction procedures published during the past ten years (Table 5), indicating that the studied enzymatic methods provide a potentially feasible alternative to alkaline extraction of rapeseed protein.

Enzyme-aided water extraction was associated with substantial co-extraction of sugars and produced protein-rich extracts with a lower degree of denaturation than after alkaline extraction. Both factors correlated with better protein solubility and dispersion stability of water extracts when compared to isoelectric precipitates obtained after alkaline extraction. Enzymatically produced protein hydrolyzates from dry-fractionated rapeseed press cake showed high antioxidativity in correlation with their protein and sinapic acid content, and enabled moderate inhibition of two enzymes (myeloperoxidase and human neutrophil elastase) with important roles in skin condition.

5.1 Carbohydrate composition of raw materials

Comprehensive analysis of the chemical composition of rapeseed press cakes guided the selection of processing options and enzyme preparations for hydrolysis of rapeseed carbohydrates. The analyzed monosaccharide composition (14.7% glucose, 8.0% galacturonic acid, 7.4% fructose, 5.8% arabinose, 3.9% galactose, 2.3% xylose) was in accordance with previous reports of cellulose, pectin, xyloglucan, arabinogalactan and arabinan as the main rapeseed polysaccharides (Ghosh et al., 2004; Pustjens et al., 2013). This and the absence of starch and *β*-glucan, as previously reported (Bell, 1984; Eriksson et al., 1994; Schöne et al., 1996), allowed focusing of the enzyme selection on pectinolytic, cellulolytic and xylanolytic enzymes. It is notable that although press cakes from both non-dehulled and dehulled seeds had a high carbohydrate content of 35%, about half of the carbohydrates (44% in non-dehulled and 54% in dehulled press cake) were water-soluble. Similarly as reported by Pustjens et al. (2013), the water-soluble proportion consisted mainly of sucrose and other small saccharides, rather than soluble polysaccharides which accounted for only 1.9% of the press cake on a dry matter basis.

Literature values for the total carbohydrate concentration of rapeseed materials vary considerably due to different analysis methods. Eriksson et al. (1994) and Pustjens et al. (2013) subjected defatted samples to acid hydrolysis and quantified the released sugars by gas chromatography in order to obtain total sugar concentrations of 13% and 36% in whole rape seeds and rapeseed meal, respectively. Schöne et al. (1996) reported a substantially higher crude carbohydrate concentration of 44% in high-fat rapeseed press cake, calculated as the sum of crude fiber and nitrogen-free extract. In the present study, a modified acid hydrolysis procedure in comparison to Eriksson et al. (1994) and Pustjens et al. (2013) was used to achieve a better analytical yield of carbohydrates. Water-soluble carbohydrates were determined separately from insoluble carbohydrates by water extraction and a mild acid hydrolysis procedure in order to reduce degradation of soluble sugars during hydrolysis. Additionally, uronic acids were analyzed by methanolysis, which is considered more suitable for analysis of pectin-containing samples than acid hydrolysis (Willför et al., 2009). These modifications are considered to have improved the accuracy of rapeseed carbohydrate analysis, and most probably explain the relatively higher carbohydrate concentrations measured for rapeseed press cakes than those previously reported for rape seeds and rapeseed meal by Eriksson et al. (1994) and Pustjens et al. (2013).

An interesting observation was made during microscopy analysis of unprocessed rapeseed press cakes, as networks stainable with Ruthenium Red were detected inside the embryo and aleurone cells. As this stain binds selectively to the carboxyl groups of galacturonic acid residues in the polygalacturonan backbone of pectin (Hou et al., 1999), the observation suggests the presence of intracellular pectin-like material in rapeseed. Ruthenium Red has been reported to bind DNA in addition to pectin (Hanke and Northcote, 1975), but the small amounts of DNA and RNA in plant cells are not presumed to explain the clearly visible net-

works observed by microscopy. After 48 h treatment of rapeseed press cake with a pectinase preparation the networks appeared to be deformed. Non-starch polysaccharides (NSP) including cellulose, hemicelluloses and pectin are mainly insoluble structural components of plant cell walls. Intracellular NSP have been reported in some species, including mannans in palm cake (Düsterhöft and Voragen, 1991) and pectins in developing olive pistils (Suárez et al., 2013). The localization of pectins inside oilseed cells has not previously been reported, and more research is needed to determine the true composition of the detected intracellular networks as well as their role in rapeseed protein recovery.

5.2 Factors influencing rapeseed protein extractability and recovery

5.2.1 Pretreatment

In the present study, pretreatments for particle size reduction, gentle defatting and removal of hull fragments from rape seeds or press cake were investigated. Oil is generally considered as an important limiting factor for plant protein extraction (Sari et al., 2015), and particularly problematic in alkaline extraction due to formation of protein-lipid complexes, which have been suggested to hinder protein separation (Manamperi et al., 2011). In the current study, reduction of the fat content of rapeseed press cake from 12% to 2% by SC-CO_2 extraction had an unexpectedly small effect on protein recovery. Defatting resulted in minor enhancement of protein recovery by alkaline extraction at 20% solid content, but in neutral extraction conditions or at lower solid content, defatting did not appear to benefit protein extraction. These results contradict those of an earlier study in which defatting at mild temperatures improved protein solubility over a wide pH range (Sun et al., 2008). A possible reason for the observed limited effect of defatting is that the *B. rapa* press cake used in the present study had a relatively low initial fat content (12%) compared to the pressed meal (26% fat) studied in Sun et al. (2008). Deviation of the current results from previous studies may also derive from different laboratory procedures used for e.g. phase separation, and it should be taken into account that particularly in an up-scaled process, protein recovery in the presence of lipids may become a challenge.

Although the present results did not indicate positive correlation between defatting and protein recovery from rapeseed press cake, SC-CO_2 extraction may have other benefits in rapeseed protein production. Gentle defatting can improve the oxidative stability of rapeseed press cake, which has been reported to be otherwise limited (Stephens et al., 1997). SC-CO_2 extraction could serve as a gentle alternative or supplementary process to hexane extraction in defatting of rapeseed press cake. For example, mild-temperature hexane extraction followed by SC-CO_2 extraction has been found to produce defatted rapeseed meal with a lighter color and better protein solubility than hexane extraction and solvent removal by heat treatment (Sun et al., 2008). Additionally, SC-CO_2 technology offers interesting

possibilities for selective extraction of small molecular compounds from plant materials. Particularly the combination of CO_2 and ethanol as extraction solvents is a promising way for simultaneous removal of fat and phenolic components from rapeseed press cake (Sun et al., 2008; Rempel and Scanlon, 2012). Widespread industrial use of SC-CO_2 technology is currently limited by high processing and investment costs, but the above-mentioned advantages support its application in oilseed processing.

A comprehensive study of the effect of particle size reduction in protein extraction was carried out to elucidate whether protein recovery from rapeseed press cake is primarily limited by physical barriers such as intact cell walls, internal cell wall structures or covalent bonds between protein and non-protein components, or whether other factors such as protein solubility and non-covalent interactions between rapeseed components mainly determine the protein yield. Fine milling of defatted rapeseed press cake at a moderate temperature had no clear impact on protein recovery, which indicated that physical barriers in the press cake were not the main limitation for protein release. Ultrafine milling reduced protein extractability, which appeared to be linked to partial denaturation of rapeseed protein as a result of elevated temperatures during milling.

In many earlier studies, rapeseed press cakes or meals have been coarsely ground before protein extraction, but further particle reduction by dry milling has not been applied apart from a few exceptions (Yoshie-Stark et al., 2008; Helling et al., 2010). Current results of the microscopy analysis and extraction trials suggest that cell walls of the embryo were sufficiently disrupted during cold pressing of seeds to allow protein release from the cells. This conclusion is supported by the observation made using microscopy that in optimal extraction conditions, proteins appeared to be extractable from all other tissues except intact aleurone cells. Limited release of the aleuronic protein was also in accordance with lower protein extractability from non-dehulled than from dehulled press cake, in which the aleurone cells had been partially removed together with the hulls. Some of the aleurone cells in the non-dehulled press cake remained intact even after fine milling to 21 µm median particle size, and therefore, alternative approaches are needed if recovery of the aleuronic protein is desired. Possible methods to be studied include ultrafine milling at a mild temperature and enzyme treatments optimized to target the aleuronic cell walls.

Dry fractionation methods used in the present study include dehulling of seeds and air classification of milled press cake, which influence the composition of rapeseed press cake in similar ways. By partially removing the fiber- and polyphenolic-rich hull particles, these pretreatments increase the protein content of rapeseed materials (Mustafa et al., 1996). In the present study, a press cake from partially dehulled rape seeds contained more protein and oil and less acid-insoluble material (termed as Klason lignin for wood biomass) and water-insoluble carbohydrates than non-dehulled press cake. In addition, a larger fraction of the protein was extractable from the dehulled than non-dehulled press cake, as was also reported by (Ghodsvali et al., 2005).

The most probable reason for the improved protein extractability after dehulling is that proteins associated with the hull fraction are mainly entrapped inside aleurone cells and are thus difficult to recover. Hence, hull removal increased the relative proportion of recoverable protein from the press cake. Additionally, microscopy of dehulled and non-dehulled press cakes revealed differences in the packing density of proteins, suggesting that pressing of partially dehulled vs. non-dehulled seeds may have had different impacts on the state of the protein. Another advantage of hull removal is that fewer protein-polyphenolic interactions are formed during processing, which makes it easier to produce mild tasting, light-colored protein concentrates (Naczk and Amarowicz, 1998; Von Der Haar et al., 2014). An obvious drawback of dehulling is that a large fraction of the kernel particles is lost in the hull fraction. Therefore, value-added applications for the hull fraction need to be developed in order for the hull removal to be feasible. The hulls are a good source of fiber and antioxidative polyphenolics, which could have potential uses in e.g. health foods or packaging applications.
On the basis of the obtained results and literature data, projections on the influence of the applied pretreatments on rapeseed protein enrichment were made (Table 20). SC-CO_2 extraction offers an interesting approach for mild-temperature defatting of rapeseed press cake, with potential to aid protein recovery and improve oxidative stability of the resulting protein concentrates. However, the feasibility of this technology is hindered by its high investment and operation costs. Dehulling of seeds before oil pressing is generally considered to aid production of light-colored rapeseed protein concentrates low in polyphenolics. Since the applicability of seed dehulling in industrial scale is limited by varying seed sizes and reduced oil yields, milling and air classification is a promising alternative for partial removal of hulls from defatted press cake or meal before protein extraction. A clear drawback of both dehulling approaches is the partial loss of protein in the hull fraction. Particle size reduction, which often helps to disrupt plant matrices for improved release of compounds, did not improve protein recovery from rapeseed press cake in the present study. On the contrary, ultrafine milling exposed the protein to denaturation, resulting in reduced extraction efficiency.

Table 20. Influence of pretreatments and enzymatic treatments carried out in this work on protein extractability from rapeseed cold-press cake.

Treatment	Aim	Influence
Dehulling of seeds	partial removal of fiber-rich hulls	+ relative increase of extractable protein + prevention of protein-polyphenolic interactions – partial loss of protein in the hull fraction – reduction of oil pressing efficiency
SC-CO_2 extraction	partial removal of residual oil	+ prevention of protein-lipid interactions + improvement of oxidative stability – major increase in processing costs
Pin disc milling	reduction of particle size	– no clear influence on protein extractability
Impact milling	reduction of particle size	– reduction of protein extractability due to denaturation
Air classification	partial removal of fiber-rich hull particles	+ relative increase of extractable protein + prevention of protein-polyphenolic interactions – partial loss of protein in the hull fraction

5.2.2 Carbohydrate-hydrolyzing enzyme treatment

The use of carbohydrate-hydrolyzing enzymes to facilitate protein recovery from rapeseed press cake was a major focus area in this study. Three commercial enzyme preparations containing polygalacturonase (Pectinex Ultra SP-L), cellulase (Celluclast 1.5L) and xylanase (Depol 740L) as their main measured activities were used to enhance protein recovery from rapeseed press cake through hydrolysis of cell wall polysaccharides. In addition to Celluclast, the other products also contained *β*-glucanase as a side activity. Whereas the xylanase (Depol) and cellulase preparations (Celluclast) lacking polygalacturonase activity caused only partial disruption of embryonic cell walls, the preparation containing high polygalacturonase activity (Pectinex) enabled complete embryonic cell wall disintegration already within 4 h of hydrolysis as detected by microscopy. Commercial carbohydrate hydrolase preparations containing pectinase activity have generally been successful in hydrolyzing rapeseed carbohydrates (Zhang et al., 2007; Chen et al., 2011; Pustjens et al., 2012), supporting the conclusion that pectin hydrolysis is needed for effective rapeseed cell wall disintegration.

All enzyme treatments in the present study were associated with enhanced protein recovery in the extracts, the greatest improvement being obtained using the pectinase preparation. At the highest, 58% protein recovery was achieved from defatted *B. rapa* press cake by water extraction at 20% solid content after pectinase treatment, compared to 37% recovery by a corresponding extraction without enzyme treatment. The results clearly indicate that pectin hydrolysis enhances protein extraction from rapeseed press cake. Enzymatic carbohydrate hydrolysis with a pectinolytic enzyme preparation was also previously associated with im-

proved protein extraction from rape seeds (Zhang et al., 2007; Latif et al., 2008). By contrast, Niu et al. (2012) found another enzyme product containing cellulase, *β*-glucanase, xylanase, arabinase and other hemicellulase activities (Viscozyme L) to be more effective than a pectinase (Pectinex Ultra SP-L) in enhancing protein extraction from rapeseed cold-press cake.

A set of experiments was designed to understand the mechanism behind improved rapeseed protein extractability after cell wall hydrolysis. The effects of mechanical and enzymatic tissue disruption on protein solubilization and recovery were compared in an extraction environment in which overall rapeseed protein solubility was expected to be close to an optimal level, i.e. at pH 12 and 5% solid content. During a two-step extraction in these conditions, max. 79% of the total protein was solubilized and a similar percentage (78%) was recovered in the extracts. Similarly to particle size reduction, enzyme treatment appeared to have no major impact on total protein solubilization in favorable extraction conditions such as in alkaline or saline buffer at 5% solid content. Carbohydrate hydrolysis was only associated with minor improvement of extract recovery in these conditions. By contrast, enzyme utilization appeared to be particularly beneficial when protein extraction was subsequently performed at increased (20%) solid content and in water without pH adjustment.

The results from particle size reduction and enzyme treatments suggest that protein extraction from rapeseed cold-press cake was in fact not physically hindered by remaining cell walls or internal cell structures. Entrapment of proteins by intact cell walls was only observed in the aleuronic layer, which accounts for a minor proportion of rape seeds. Interestingly, enzyme treatment influenced protein extraction in different ways depending on the extraction scheme: in conditions supporting high protein solubility, enzyme utilization mainly increased the volume of recovered protein extracts. On the other hand in conditions in which protein solubility was expected to be limited (in water at 20% solid content), enzyme treatment also increased the protein solubility in addition to significant improvement of extract recovery. The main limiting factors for protein recovery appear to be inefficient solid-liquid separation due to high water holding capacity of the press cake, limited solubility of the main rapeseed proteins, and entrapment of protein in the aleurone cells.

On the basis of the data obtained, it is concluded that treatment of rapeseed press cake with a pectinase preparation affects protein extractability in two major ways. First, reduction of water holding capacity of the press cake through enzymatic hydrolysis of carbohydrates facilitated solid-liquid separation and recovery of extracts by centrifugation. Pectinase treatment has previously been reported to reduce the water holding capacity of rapeseed meal (Pustjens et al., 2012), and in the present study, liquefaction of the press cake during hydrolysis was also observed.

The second suggested mechanism by which pectinolytic enzymes influence rapeseed protein extraction is related to soluble hydrolysis products which were expected to improve protein solubility during water extraction. Particularly the release of negatively charged uronic acids increased ionic strength, with positive

effects on cruciferin solubility. In addition, solubilization of pectic oligosaccharides after limited pectin hydrolysis may have had a role in improving protein solubility through electrostatic and steric stabilization. Certain anionic carbohydrates are known to enhance rapeseed protein solubility via the formation of soluble complexes stabilized by electrostatic forces (Klassen et al., 2011; Stone et al., 2014). The solubility of protein-polysaccharide complexes depends on the pH and mixing ratio of compounds (Klassen et al., 2011), and in the case of pectin, complexation with proteins is also influenced by the molecular weight and degree of esterification of pectin (Zhao et al., 2013). The stabilizing effect of soluble pectin hydrolysis products is similarly expected to be the reason for hindered isoelectric precipitation of proteins in alkaline extracts which were obtained after enzyme treatment in the current study. Controlled hydrolysis of pectin into soluble oligosaccharides of desired size and degree of esterification, using enzymes with selected pectinolytic activities, has a strong potential to improve rapeseed protein recovery and at the same time produce protein-carbohydrate extracts with enhanced techno-functional properties due to electrostatic stabilization.

Other factors which may have influenced protein extractability during enzymatic treatment include partial hydrolysis of protein into soluble polypeptides. As the aim of the current study was to extract intact proteins, enzyme preparations with low protease side activity were selected. Particularly the negligible protease activities of the pectinase (Pectinex) and cellulase products (Celluclast) were expected to have no influence on protein solubilization. This was supported by SDS-PAGE analysis, which did not reveal substantial protein hydrolysis. By contrast, higher protease side activity in the xylanase product (Depol) caused protein hydrolysis and changes in the staining intensity of protein as visualized by SDS-PAGE and microscopy. The observed partial proteolysis may have improved the solubility of rapeseed protein, but without pectin hydrolysis the extract recovery remained lower than after pectinase treatment. In previous studies, combinations of enzymes hydrolyzing carbohydrates and protein have proven effective for extraction of rapeseed protein hydrolyzates (Latif et al., 2008; Niu et al., 2012), suggesting that supplementation of the pectinase product used here with a low dosage of protease could boost protein solubilization. Carefully controlled partial proteolysis also has the potential to improve the techno-functional properties of the protein (Vioque et al., 2000) or release bioactive peptides, as additionally investigated in the present study.

5.2.3 Extraction conditions

Protein solubility is a key aspect in wet fractionation of protein-rich biomass. Several research groups have investigated how extraction pH and various additives influence the yield and selectivity of rapeseed protein extraction. Based on literature data summarized in Table 5 and Table 6, and a recent optimization study by Das Purkayastha and Mahanta (2015), optimal overall solubility of rapeseed protein is achieved in highly alkaline conditions (pH 12–12.5) and at a low meal to solvent ratio (1:27). Although such extraction conditions give high protein yields, a

drawback is that several neutralization and washing steps may be needed for the extract and residual solids. In alkaline conditions, the interactions between protein and phenolic compounds of rapeseed are also increased, and oxidation of phenolics results in dark-colored products (Xu and Diosady, 2000). Since processing in 'optimal conditions' requires extensive amounts of water, carefully designed concepts are clearly needed for sustainable and economically feasible rapeseed protein production.

In the current study, higher protein yields from rapeseed press cake were expectedly obtained at elevated pH (10–12) or in saline conditions (0.2 M sodium phosphate) when compared to extraction in water. This was considered to result from the increased solubility of rapeseed cruciferin in saline solutions or at alkaline pH values far from its isoelectric range (pH 4–7), as demonstrated by a number of earlier studies. As expected, rapeseed protein extraction was also less efficient at 20% than 10% total solid content, as was reported by Das Purkayastha and Mahanta (2015), who observed a decrease in protein yield when the solid content during alkaline extraction was increased. At higher solid content the difference between total protein solubilization and protein recovery values was clearly larger, indicating that solid-liquid separation becomes a major hindrance in slurries containing over 10% solids. One option to improve extract recovery in such conditions is to increase the number of extractions: Experimental data of the present study showed that protein recovery at 20% solid content could be enhanced by performing a second extraction step which functioned as a washing step. As this procedure requires more water than a single step extraction, its techno-economic impacts should be assessed. In addition to modification of the number of extraction steps, more efficient and scalable phase separation practices should be investigated to facilitate high-solids wet processing.

The present study focused on simple and robust, but non-selective recovery of protein-enriched fractions containing both of the major rapeseed proteins. Although a low-cost production method may be achieved, the applicability of impure protein fractions is expected to be limited to fewer applications than that of protein isolates. Purity is likewise expected to influence the product price: For example, the price of milk protein concentrates (34–42% protein) has been estimated to range at approximately 30% of the price of corresponding protein isolates (Frost & Sullivan, 2013; Burcon NutraScience, 2015). If higher product quality is desired, optimization of the extraction process or downstream processing of the extracts is needed. Recently, the different properties of cruciferin and napin have attracted more interest in the production of individual protein fractions for distinct high-value applications. Carefully designed extraction procedures can allow selective recovery of napins and cruciferins: For example, acidic conditions have been utilized to first extract napin, which remains soluble over a wide pH range (Wanasundara and McIntosh, 2008). Alternatively, isoelectric precipitation or "salting out" of cruciferins from protein extracts can partially separate the two main proteins (Blaicher et al., 1983; Murray, 1999).

5.2.4 Techno-economic feasibility of enzyme utilization in rapeseed protein extraction

The costs and benefits of enzyme utilization in industrial scale will determine whether enzyme-aided rapeseed protein extraction is a feasible technology with potential for commercialization. An early-stage light techno-economic assessment of process options was performed by estimation of variable, fixed and capital costs of a theoretical production plant with 10 000 t/a raw material capacity. Techno-economic calculations regarding rapeseed protein production have not previously been reported, and hence the results provided valuable guidelines for further research work. A major observation was that the estimated overall production costs of protein-enriched fractions by enzyme-aided water extraction (ca. 4100–4500 € per ton protein produced) were similar to those of alkaline extraction and isoelectric precipitation (ca. 3900–4600 € per ton protein produced). The enzyme-aided extraction schemes produced less pure protein streams but with a higher protein yield.

Energy and raw material together represented the largest share of rapeseed protein production costs. Therefore, regional and seasonal fluctuations in energy prices and the feed price of rapeseed press cake can have strong effects on the production costs of rapeseed protein. Increase of total solid content during protein extraction clearly reduced the costs of both concepts due to lower energy consumption, supporting further investigation of water-saving enrichment technologies. Reduction of water and energy use is a key task for improving the economic feasibility and sustainability of plant protein production.

According to the evaluation based on the data from laboratory-scale experiments, enzyme utilization generated a relatively small additional cost to the enrichment of rapeseed protein by wet fractionation. This was despite the fact that enzyme consumption was not optimized – instead a high enzyme dose of 10 mg/g was used to ensure that the dose was not a limiting factor for carbohydrate hydrolysis. The enzyme treatment was also found to improve protein yield when a reduced enzyme dose (5 mg/g) and shorter hydrolysis time (2 h) was used, suggesting that lower enzyme dosages and hydrolysis times can be sufficient. Carbohydrate-hydrolyzing enzyme treatments have been mentioned in the patents of BioExx (Tang, 2010) and Wanasundara and McIntosh (2008) in relation to rapeseed protein extraction, and the growing availability of food-grade industrial enzymes is expected to further increase interest in enzyme-aided processes for production of plant protein ingredients.

5.3 Factors influencing the functional properties of rapeseed protein fractions

5.3.1 Technical functionality

In today's food products, proteins not only serve as nutrients but increasingly as technical ingredients providing desired structure and mouthfeel. Technical functionality of rapeseed proteins is an extensively studied research topic, with particular attention given to the emulsion and gelling properties and their improvement using protein modification or additives. In the current study, solution behavior of dried rapeseed water extracts and isoelectric precipitates was investigated as a means to compare different production methods in their ability to produce functional protein fractions.

The major observation was that crude protein-enriched fractions from enzyme-assisted water extraction (i.e. water extracts) were more soluble at pH 4 and 8, and appeared to form more stable dispersions than isoelectric precipitates from alkaline extraction. At pH 8 the isoelectric precipitates showed lower protein solubility than water extracts despite stronger ζ-potential, which is typically associated with improved solubility. Similar results were previously reported by Karaca et al. (2011) and Yoshie-Stark et al. (2008), who compared the solubility of rapeseed isoelectric precipitates with that of rapeseed protein isolates produced by saline extraction or alkaline extraction and ultrafiltration, respectively. In the present study, protein solubility of the water extracts and isoelectric precipitates (analyzed by a Lowry-based method) was pH-dependent and in accordance with the solution behaviour of cruciferin, with lower solubility values observed at pH 4 than at pH 8. In addition to better protein solubility of water extracts at pH 8, the smaller particle size range detected by dynamic light scattering indicated higher dispersion stability when compared to the isoelectric precipitates.

Solubility and dispersion stability differences between rapeseed water extracts and isoelectric precipitates are suggested to have resulted from differences in protein composition, protein state, and concentration of different non-protein compounds. SDS-PAGE analysis indicated that the isoelectric precipitates contained a higher proportion of cruciferins, which are generally characterized by lower water solubility in comparison to the small, readily soluble napins (Wu and Muir, 2008). Protein solubility in the isoelectric precipitates was additionally hindered by partial protein denaturation: low denaturation enthalpy in DSC analysis and smeared bands observed by non-reducing SDS-PAGE indicated that the protein in isoelectric precipitates had become almost completely denatured during either alkaline extraction or isoelectric precipitation. Alkaline pH is known to expose proteins to denaturation (Ma et al., 1990). Krause and Schwenke (2001) and Manamperi et al. (2011) also linked the low solubility of rapeseed isoelectric precipitates to partial unfolding of proteins during their production.

In addition to a higher proportion of napins and better preservation of the native state of proteins in rapeseed water extracts, a composition analysis of the samples showed that the water extracts contained at least as much carbohydrates as protein. Negatively charged carbohydrates such as pectin are known to form soluble complexes with rapeseed proteins, particularly with napins which are positively charged at pH values below their alkaline isoelectric point (Klassen et al., 2011; Stone et al., 2014). It is probable that anionic carbohydrates which were solubilized during enzyme treatment electrostatically stabilized proteins in the water extracts, resulting in more stable dispersions. Other non-protein compounds such as phenolics and fat were not analyzed in the present study, but these compounds are likewise expected to have influenced the techno-functional properties of rapeseed water extracts and isoelectric precipitates. Particularly phenolic compounds and phytates have been reported to hinder rapeseed protein solubility and surface activity by formation of insoluble complexes (Blaicher et al., 1983; Xu and Diosady, 2000). These interactions have been associated with detrimental effects on the gelation and emulsification properties of rapeseed protein products (Rubino et al., 1996; Krause and Schwenke, 2001).

Phenolic compounds and their oxidation products are considered to be the main cause of undesirable color and taste in rapeseed protein products. Even small amounts of phenolic acids (e.g. 1–2% relative to protein) in protein isolates may cause undesirable color (Xu and Diosady, 2002) and impaired gelation properties (Rubino et al., 1996). In the present study, rapeseed protein fractions from enzyme-aided water extraction had a light yellow color, in contrast to the brown shade of isoelectric precipitates, suggesting lower concentration or less severe oxidation of phenolic compounds in the water extracts. This is in accordance with a previous study in which the dark color of alkali-extracted rapeseed protein products was associated with non-enzymatic oxidation of phenolics in alkaline conditions (Xu and Diosady, 2000). Substantial efforts have been made for the removal of phenolics from rapeseed and mustard meal and their protein fractions using membrane filtration, solvent extraction and additives that reduce protein-phenolic interactions (Xu and Diosady, 2002; Xu et al., 2003; Das et al., 2009; Das Purkayastha et al., 2013). In general, modification of extraction and precipitation conditions has been suggested to result in substantial improvement of the techno-functional properties of rapeseed protein isolates, without major reduction of protein yield (Manamperi et al., 2011).

5.3.2 Bioactivity

Partial hydrolysis of proteins into peptides is another well studied approach to improve the extractability and functional properties of rapeseed protein. In the present work, protease treatment was used to produce soluble, bioactive peptides which could be water-extracted from dry-fractionated rapeseed press cake as crude protein hydrolyzates without pH adjustment. The crude hydrolyzates were characterized in terms of their ability to prevent radical-induced oxidation and to inhibit enzymes involved in inflammation and skin aging. All hydrolyzates showed

antioxidative activity *in vitro* and in a skin fibroblast cell model, the most antioxidative hydrolyzates being obtained by *B. licheniformis* serine endopeptidase (Alcalase or Protex) treatment. The results from *in vitro* assays are in accordance with a number of reports on the radical scavenging activity of rapeseed protein hydrolyzates (Cumby et al., 2008; Mäkinen et al., 2012; He et al., 2013).

Radical scavenging activity and cellular antioxidativity of the hydrolyzates obtained from rapeseed press cake showed correlation with their protein and sinapic acid concentration, suggesting that one or both of these compounds were involved in antioxidativity of the samples. All hydrolyzates contained a much higher concentration of protein and peptides (55–69%) than sinapic acid (1.4–2.2%), whereas the unhydrolyzed protein extract from rapeseed press cake had a somewhat lower protein (38%) and higher sinapic acid content (3.5%) than the hydrolyzates. The unhydrolyzed extract was also an effective antioxidant, indicating that sinapic acid had a role in the antioxidativity of this sample. The influence of rapeseed phenolics on the antioxidative properties of rapeseed protein hydrolyzates was recently studied by Hernández-Jabalera et al. (2015), who found that peptide-phenolic interactions diminished the radical scavenging activity and increased the cellular antioxidativity of rapeseed hydrolyzates. The results are not fully consistent with those of the present study, and therefore, more research is needed to elucidate the antioxidative role of phenolics in rapeseed protein hydrolyzates. In practical terms, the co-extraction of peptides and phenolics during protease treatment can advance the antioxidativity of hydrolyzates, but cause challenges in terms of the color and taste of the products.

Radical scavenging activity of proteins and peptides depends largely on the proportion of exposed amino acids with active side groups (Huang et al., 2005). Proteolysis has potential to enhance the exposure of active amino acids and could therefore enhance the antioxidativity of proteins. Varying reports exist on the influence of hydrolysis on the DPPH radical scavenging activity of plant proteins. Radical scavenging activity of peanut peptides has been reported to increase with the degree of hydrolysis (11–21%), suggesting that active amino acid residues are exposed as a result of more extensive proteolysis (Zhang et al., 2011). On the other hand, Yoshie-Stark et al. (2006) reported no effect of hydrolysis on the DPPH radical scavenging activity of rapeseed protein concentrates. In the current study, antioxidativity of rapeseed protein hydrolyzates did not correlate with the degree of hydrolysis. In addition to the studied hydrolyzates, unhydrolyzed rapeseed protein extracts also exhibited antioxidative activity, which could be related to the antioxidativity of sinapic acid in these samples. It is also possible that after the heat treatments carried out during extract production in the current study to inactivate endogenous enzymes, the unhydrolyzed rapeseed protein was in an unfolded conformation, with antioxidative amino acid side groups sufficiently exposed to suppress radical-induced oxidation *in vitro* or in fibroblast cells.

The observed cellular antioxidative activity of rapeseed hydrolyzates and unhydrolyzed protein extracts opens up interesting possibilities for the use of these fractions as active ingredients in anti-aging skin products. For this purpose, the biological activities still need to be tested *in vivo* on skin using animal models

and/or clinical trials. The hydrolyzates could additionally serve as natural preservatives, since their radical scavenging activity could prevent rancidity formation in e.g. skin creams.

Most of the studied samples mediated high inhibition of human neutrophil elastase and moderate inhibition of myeloperoxidase enzymes, which have not previously been reported in enzymatically produced rapeseed protein hydrolyzates. The most promising hydrolyzate in terms of these two bioactivities was obtained by sequential Protex and Alcalase treatment. Moderate inhibition of cellular MPO activity is considered favorable for the balanced control of skin inflammation. The hydrolyzates and unhydrolyzed protein extracts from rapeseed press cake caused partial inhibition (25–60%) of MPO activity *in vitro*, indicating potential applicability in skin inflammation control. Furthermore, the moderately high HNE inhibitory activity (ca. 80%) of nearly all samples suggests that rapeseed hydrolyzates could serve as anti-wrinkle agents in skin care products. Final hydrolyzate concentrations in the antioxidativity and enzyme inhibition assays (0.2–2 mg/ml) were considered to be relevant for special ingredients in skin care products or foods.

Bioactive peptides such as those with enzyme inhibitory activity are generally incorporated in the primary structure of proteins and become activated only after cleavage of the proteins into fragments of specific size and sequence. The enzyme inhibitory activities analyzed in the present study did not correlate with DH, most probably because proteolysis resulted in a mixture of active and inactive peptides and free amino acids, all of which were present in the crude hydrolyzates. In the literature, bioactivities such as ACE inhibitory activity have been similarly detected in rapeseed protein hydrolyzates of varying DH (Yoshie-Stark et al., 2006; Cumby et al., 2008; Wu and Muir, 2008). Better understanding of the active and inactive components present in the hydrolyzates would require purification and assessment of individual proteins and peptides.

A probable factor which influenced the bioactivity of protein hydrolyzates is the cleavage site specificity of protease enzymes, which affects both the efficiency of hydrolysis and the amino acid sequence of the peptides produced. All of the four protease preparations studied originated from *Bacillus* species and contained serine endopeptidase activity, but differed in their declared minor protease activities, pH optima, and the overall level of neutral protease activity per gram of protein. MPO and HNE inhibitory activity is expected to have resulted from small peptides which can act as competitive substrates for these enzymes, but the exact size and sequence of these peptides is yet to be determined.

5.4 Limitations of the study

5.4.1 Design of experiments and data analysis

The present study aimed to obtain experimental data with relevance for industrial applications, which required some compromises in the design of experiments. For example, commercial enzyme preparations containing a mixture of activities were used due to their industrial-scale availability, and mechanistic studies with specific pure enzymes were not attempted. Because of the presence of various enzyme activities, preparations were dosed on the basis of total protein content, not main activity. The use of purified enzymes would be interesting from a scientific point of view and also useful for process development, as it could allow detection of the most crucial individual activities, optimization of enzyme mixtures, and controlled hydrolysis of carbohydrates and proteins at specific sites.

Some improvements to the extraction protocols were made during the work, and the influence of these changes should be noted when comparing results from different experimental setups. For example, a two-step extraction procedure and tubes allowing higher centrifugation speeds than in earlier experiments were later used when the influence of particle size on protein extractability was studied (III). These modifications are expected to have improved the phase separation efficiency and recovery of protein extracts, resulting in increased protein yields particularly at 20% solid content. A factor limiting the applicability of the presented results is the lack of experimental data from downstream processes such as membrane filtration and removal of salt and phenolics, as isoelectric precipitation and freeze-drying were the only investigated concentration methods. Downstream processes are expected to have remarkable effects on production costs, and should be additionally included in the techno-economic analysis for cost estimation of the full production process.

The method of calculation used in determination of extraction yields should be noted for accurate interpretation of the data and comparison against literature values. In aqueous extraction of components from biomass, the yield calculation method can have a remarkable influence on results, particularly when fractionation is performed at increased solid content or with materials possessing high water holding capacity. In such environments, components such as protein may exist in the soluble phase, but are not recovered in extracts because a large fraction of the solubles remains associated with residual solids. In the present study, different calculation methods were applied to distinguish between solubilized and recovered protein as described in the Materials and methods section. Due to practical relevance, the extraction yields were primarily described as recovered protein. In addition, total solubilization yields were presented when specified (III). When small differences between protein recovery yields from enzyme-aided extractions are compared, it should additionally be noted that the yields were not corrected for protein originating from the added enzyme preparation. However, with the enzyme dosages applied (5–10 mg/g), enzyme protein corresponded to only 1.3–2.8% of

the raw material protein and thus had only a minor contribution to the observed protein yield.

5.4.2 Analytics

In the present study, the protein concentration of raw materials and fractionation of protein into extracts and residual solids was determined as ‘crude protein’, based on the total nitrogen (N) content of fractions which was measured using the Kjeldahl combustion method (Kane, 1986). This analysis was selected as sufficiently accurate, repeatable and feasible to monitor rapeseed protein fractionation during aqueous extraction. Kjeldahl was considered more reliable than colorimetric protein assays such as the Lowry method which suffers from interference by rapeseed phenolics (Lindeboom and Wanasundara, 2007), and the Bicinchoninic acid (BCA) method which is affected by other small molecules such as glucose (Brown et al., 1989). In Kjeldahl analysis, protein is quantified on the basis of total N content of the sample using a conversion factor for N to protein. Although protein constitutes most of rapeseed nitrogen, varying amounts of nitrogenous non-protein compounds such as glucosinolates, nucleic acids and phospholipids in rapeseed fractions may cause some inaccuracy of the protein analysis results. Non-protein N has been calculated to constitute 4.2–7.2% of total N in rapeseed meal (Rogulski, 1989), although in another study, up to 24% of crude rapeseed protein was suggested to be non-protein N (Mustafa et al., 1996).

The N to protein conversion factor of 6.25, which has been used in most previous studies for conversion of rapeseed nitrogen into protein, was also used in the present study in order to produce data comparable with the literature values. This factor is based on the assumption that protein contains approximately 16% nitrogen. The 6.25 factor is widely applied for cereals and oilseeds today for generic estimation of ‘crude protein’, but has confronted justified criticism due to the varying N to protein ratios in different raw materials (Mariotti et al., 2008). After the amino acid-based protein analysis of various cereals and vegetables was found to give 12–14% lower results than total N multiplied by a factor of 6.25, alternative conversion factors for foods were suggested (Salo-väänänen and Koivistoinen, 1996). For rapeseed meal, 5.53 or 5.83 have been proposed as more accurate conversion factors which take rapeseed protein composition and non-protein N better into account (Tkachuk, 1969; Simbaya et al., 1996).

To avoid comparison of divergent results from different protein analysis methods, consistent use of the same protein quantification method and conversion factors is crucial. In the present study, this principle was used on all except two occasions, when small sample size prevented the use of the Kjeldahl method. First, protein solubility of dried rapeseed water extracts and isoelectric precipitates was determined by comparing results from a Lowry-based protein analysis against the total protein content determined by Kjeldahl. Due to this discrepancy, the solubility values are only indicative, although considered useful for comparing the solution behavior of similar protein fractions. The total peptide and protein concentration of rapeseed protein hydrolyzates was similarly determined using a Lowry-

based method. The data is considered feasible for comparison of different hydrolyzates, but should otherwise be treated as indicative.

Another biomass characterization method which should be treated with caution is the Klason lignin analysis, which is commonly used for crude estimation of acid-insoluble lignin in biomass. Although considered as suitable for wood and other lignocellulosic feedstocks, reliable estimation of the lignin content by this method has been suggested to be unfeasible with protein-rich raw materials such as rapeseed (Theander et al., 1977), brewer's spent grain (Rencoret et al., 2015) and wheat bran (Bunzel et al., 2011). Klason lignin is determined on the basis of the total dry mass of insoluble material after acid hydrolysis of a defatted sample, and in addition to lignin has been reported to contain non-lignin substances such as protein, ash and cutin in various plant materials (Bunzel et al., 2011). For this reason, Klason lignin was primarily described as 'acid-insoluble material' in the present study, and the ash content of this material (ca. 0.7%) was analyzed and subtracted from the presented results. The compounds other than ash are expected to be lignin, protein and possibly non-lignin polyphenolics complexed with the protein. Overlapping of Klason lignin with protein was the most probable reason why the sum of components exceeded 100% in the composition analysis of press cakes. Acid-soluble lignin, which was determined in the present study on the basis of the absorption of acid-soluble material at 215 and 280 nm, may likewise encompass protein due to the absorption of aromatic and sulfur amino acids at 280 nm (Pace et al., 1995). Oxidative characterization offers more accurate estimation of rapeseed lignin and other polyphenolics than the Klason lignin method (Theander et al., 1977).

The level of rapeseed protein denaturation was evaluated using differential scanning calorimetry (DSC). This method allows detection of the excess heat capacity associated with conformational changes of biomolecules such as protein and starch at a certain temperature, as reviewed by Johnson (2013). DSC has been used to assess the denaturation level of various plant proteins, including rapeseed protein isolates (Wu and Muir, 2008). In the present study, DSC was applied directly to water suspensions of differently milled rapeseed press cake samples to obtain insight into how milling influenced the level of protein denaturation. When corrected against the protein content of the press cakes, the obtained denaturation enthalpies were similar to those reported by Yang et al. (2011) for isolated rapeseed cruciferin and napin, but substantially lower than the values reported for native rapeseed proteins by Wu and Muir (2008) and Li et al. (2012). Meanwhile, the measured denaturation enthalpy of a water extract obtained from the corresponding press cake was much higher, approaching the enthalpies reported by Wu and Muir (2008).

Results of the present study suggest that direct comparison of denaturation enthalpies between complex (press cakes) and relatively pure samples (protein isolates) may be impaired by differences in their composition, microstructure and water binding capacity. Hitherto it has not been investigated whether these factors influence the performance of different rapeseed samples in the DSC analysis, but it is possible that the ratio and interactions of non-protein and protein components

affect the size of endothermic peaks in DSC. In the present study, DSC was used to compare crude rapeseed press cake samples which differed only in particle size, not in composition. Although the data was regarded as comparable between different samples, the measured values may not give a reliable estimation of the denaturation enthalpy of proteins in the studied rapeseed press cakes.

5.5 Future prospects

A substantial amount of research work is still needed to improve the feasibility of rapeseed protein production, but with the current pace of development, food and cosmetic products containing rapeseed protein or peptides could well reach the market within a couple of years. One of the key targets is the reduction of energy costs, which are strongly connected with water use in the fractionation process. For example, extraction at increased solid content appears to substantially reduce the energy costs of rapeseed protein production, but the feasibility of high-solids processing and solid-liquid separation should still be demonstrated in pilot scale. As shown in the present study, enzymes provide useful tools to improve the sustainability of rapeseed protein production, particularly when food ingredients with multiple valuable components are targeted instead of pure protein isolates. The feasibility of enzyme-aided extraction methods depends on the costs of possible downstream processing, and therefore, low-cost concentration technologies suitable for enzymatically produced, high-solids protein extracts should be developed.

Improvement of product quality in terms of color, taste, technical functionality and bioavailability is another key aspect which needs to be addressed. Taste, emulsion foaming and gelation properties of rapeseed protein concentrates were not assessed in the present study, and suitability of the fractions in food models was not tested. These characteristics are expected to be influenced by the processing history as well as protein-interacting compounds such as carbohydrates, phenolics and phytates. Sustainable, solvent-free and water-saving approaches to remove, modify or prevent co-extraction of these compounds with protein are required in order to yield rapeseed protein products with desirable sensory and functional properties.

The nutritional and health effects of rapeseed protein in humans are still relatively unknown. Hence, more clinical trials are needed to assess the bioavailability of protein in differently processed rapeseed protein products. With bioactive rapeseed protein hydrolyzates, *in vivo* bioactivity studies are necessary to verify their biological effects. Finally, assessment of the toxicity, allergenicity and microbiological safety of novel rapeseed-derived protein and peptide ingredients will be required in order to obtain permission for marketing. Another important aspect which has been studied only to a limited extent is the oxidative and technological stability of rapeseed protein concentrates. Oxidation of lipids and phenolics may reduce the shelf life of these products unless suitable processing and storage conditions are determined.

Emerging technologies including supercritical extraction, enzymatic modification of biopolymers, and dry fractionation open up new possibilities in the valorization of rapeseed press cake. Recent studies on SC-CO_2 extraction of rapeseed press cake with ethanol as a co-solvent have shown promising results on the suitability of this method for simultaneous removal of residual oil and phenolics. The resulting defatted, low-phenol press cake could potentially be applied as a fiber- and protein-rich ingredient in food and feed, or it could be gently processed to improve its digestibility and functional properties. In particular, controlled enzymatic hydrolysis of carbohydrates at low water content is a potential new way to enhance the technical functionality of rapeseed meal or protein concentrates. Finally, dry fractionation offers sustainable solutions for valorization of both rapeseed press cake and industrial heat-treated rapeseed meal. Several recent studies have focused on the use of gently desolventized meal or cold-press cake for protein extraction. However, currently the largest volumes of rapeseed meal are generated in industrial solvent extraction processes in which high-temperature desolventizing procedures are in general use. The higher level of protein denaturation in such meals limits their suitability for aqueous protein extraction, whereas the low oil content makes them potentially favorable raw materials for air classification. Removal of hulls by dry fractionation could allow the production of value-added, protein-enriched feed or food components also from heat-treated rapeseed meal.

6. Conclusions

Rapeseed press cake is a promising raw material for sustainable production of food protein and bioactive peptides. Although intensive research in the past decade has yielded a number of technologies for extraction and modification of rapeseed proteins, commercialization of rapeseed protein production has been hindered in part due to high costs of the current extraction processes. In the present study, enzyme-aided methods were developed to facilitate protein extraction from rapeseed cold-press cake at increased total solid content and without chemicals such as alkali or salt. Effects of mechanical pretreatment and extraction conditions on protein extractability and the solution behavior of the resulting protein fractions were studied.

Enzyme treatment with a preparation containing high polygalacturonase activity and a *β*-glucanase side activity effectively hydrolyzed carbohydrates, disrupted embryonic cell walls and enhanced protein recovery from rapeseed press cake by water extraction. The main mechanisms by which enzymes increased protein extractability are suggested to be reduction of water holding capacity of the press cake, and release of hydrolysis products which increased protein solubility. Although enzymatic cell wall disintegration appeared to be associated with enhanced protein release, inability to evoke similar effects by mechanical cell wall fragmentation indicated that protein release from SC-CO_2-defatted press cake was not markedly hindered by microstructural barriers. As an exception, a fraction of the protein appeared to remain entrapped in the intact aleurone cells of rapeseed press cake. Limited release of the aleuronic protein was in accordance with the observed relatively lower protein extractability from non-dehulled than from dehulled press cake. Enzyme-aided water extraction produced protein-enriched fractions with a similar yield and estimated production costs as alkaline extraction and isoelectric precipitation, suggesting that enzyme-aided methods could successfully replace chemical rapeseed protein extraction technologies.

Carbohydrate-hydrolyzing enzymes have strong potential in improving rapeseed protein extraction at reduced water content, at which extract recovery is limited by the high water holding capacity of rapeseed press cake. The present study revealed that almost comparable protein yields could be obtained from defatted press cake by enzyme-aided water extraction at 20% solid content as by water-intensive saline extraction at 5% solid content. According to a techno-

economic evaluation of selected processing schemes, enzymes represented only a minor share of overall production costs. The major costs originated from energy consumption, which was associated with the volume of water that needed to be processed and finally removed from the product. When protein extraction was carried out at higher solid content (20% vs. 10%), substantial energy cost savings could be achieved according to the evaluation.

Co-extraction of sugars and soluble polysaccharides released during carbohydrate hydrolysis resulted in protein extracts with lower protein concentration when compared to extracts from non-enzymatic procedures. Hence, purification steps are needed if protein concentrates (>65% protein) are targeted. On the other hand, carbohydrates are suggested to be one reason for the better solubility and dispersion stability observed in water extracts than in isoelectric precipitates. These favorable properties support the applicability of crude protein extracts in liquid or semi-solid products, in which high dispersion stability is desired.

Production of bioactive protein hydrolyzates from rapeseed press cake by dry fractionation and protease treatment was additionally studied in the present work. Seven hydrolyzates and two unhydrolyzed extracts were characterized in terms of chemical composition, degree of hydrolysis, antioxidativity and ability to inhibit enzymes involved in skin aging and inflammation. Protein hydrolyzates showed high antioxidative activity *in vitro* and in a skin cell model, in correlation with their protein and sinapic acid concentrations. Sinapic acid was suggested to particularly influence the antioxidativity of unhydrolyzed crude protein extracts from dry-fractionated press cake. Hydrolyzates and unhydrolyzed extracts also exhibited moderate to high MPO and HNE inhibitory activity *in vitro* at concentrations which are considered relevant for application in skin care products. Overall, the most bioactive hydrolyzates were produced using two serine endopeptidase preparations from *Bacillus licheniformis*. On the basis of the presented results, crude rapeseed protein hydrolyzates containing bioactive peptides and phenolics have potential uses in skin care products as functional ingredients protecting from oxidation, inflammation or wrinkle formation, or as natural preservatives to prevent rancidity formation in cosmetic formulations. Clinical trials are still needed to confirm the bioactivity and safety of the hydrolyzates *in vivo*.

The present work provided increased understanding of the microstructure and composition of rapeseed cold-press cake, the main of barriers to protein recovery from this material, and the solution behavior of rapeseed proteins during and after wet processing. Enzyme-assisted methods for rapeseed protein extraction at increased solid content or without chemicals such as alkali or salt were developed, and an early-stage evaluation of the techno-economic feasibility of rapeseed protein extraction using alkali or enzymes was presented for the first time. In addition, new bioactivities of rapeseed protein hydrolyzates with potential value in skin care applications were identified. The results encourage further development of enzyme-aided processes for water-saving rapeseed protein enrichment. As shown in the present study, carbohydrate- and protein-hydrolyzing enzymes represent valuable tools to improve the techno-economic feasibility and sustainability of rapeseed protein and protein hydrolyzate production.

References

Aachary, A.A., Thiyam, U., 2012. A pursuit of the functional nutritional and bioactive properties of canola proteins and peptides. Critical Reviews in Food Science and Nutrition 52, 965–979. doi:10.1080/10408398.2010.516033

Aider, M., Barbana, C., 2011. Canola proteins: composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity – A practical and critical review. Trends in Food Science & Technology 22, 21–39. doi:10.1016/j.tifs.2010.11.002

Akbari, A., Wu, J., 2015. An integrated method of isolating napin and cruciferin from defatted canola meal. LWT - Food Science and Technology 64, 308–315. doi:10.1016/j.lwt.2015.05.046

Alashi, A.M., Blanchard, C.L., Mailer, R.J., Agboola, S.O., Mawson, A.J., He, R., Girgih, A., Aluko, R.E., 2014. Antioxidant properties of Australian canola meal protein hydrolysates. Food Chemistry 146, 500–506. doi:10.1016/j.foodchem.2013.09.081

alibaba.com, 2015. Manufacturers, suppliers, exporters & importers from the world's largest online B2B marketplace [WWW Document]. URL http://www.alibaba.com (accessed 12.29.15).

Aluko, R.E., McIntosh, T., 2001. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. Journal of the Science of Food and Agriculture 81, 391–396. doi:10.1002/1097-0010(200103)81:4<391::AID-JSFA823>3.0.CO;2-S

Analytica EBC, 1997. 4.10 Free Amino Nitrogen of Malt by Spectrophotometry, in: Analytica-EBC. Fachverlag Hans Carl, Nürnberg.

Aspinall, G.O., Kuo-Shii, J., 1974. Rapeseed hull pectin. Carbohydrate Research 38, 247–255. doi:10.1016/S0008-6215(00)82355-1

Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23, 257–270. doi:10.1016/0168-1656(92)90074-J

Bailey, M.J., Nevalainen, K.M., 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enzyme and Microbial Technology 3, 153–157. doi:10.1016/0141-0229(81)90076-4

Bailey, M.J., Pessa, E., 1990. Strain and process for production of polygalacturonase. Enzyme and Microbial Technology 12, 266–271. doi:10.1016/0141-0229(90)90098-B

Barakat, A., Jérôme, F., Rouau, X., 2015. A dry platform for separation of proteins from biomass- containing polysaccharides, lignin, and polyphenols. ChemSusChem 8, 1161–1166. doi:10.1002/cssc.201403473

Barker, L.D., Martens, R.W., Murray, E.D., 2002. Production of oil seed protein isolate. WO 2002/089597 A1.

Bate-Smith, E.C., 1973. Haemanalysis of tannins: The concept of relative astringency. Phytochemistry 12, 907–912. doi:10.1016/0031-9422(73)80701-0

Bell, J.M., 1984. Nutrients and toxicants in rapeseed meal : a review. Journal of Animal Science 58, 996–1010. doi:10.2134/jas1984.584996x

Bell, J.M., 1993. Factors affecting the nutritional value of canola meal: a review. Canadian Journal of Animal Science 73, 689–697. doi:10.4141/cjas93-075

Bell, J.M., Keith, M.O., 1991. A survey of variation in the chemical composition of commercial canola meal produced in Western Canadian crushing plants. Canadian Journal of Animal Science 71, 469–480. doi:10.4141/cjas91-056

Berghout, J.A.M., Pelgrom, P.J.M., Schutyser, M.A.I., Boom, R.M., van der Goot, A.J., 2015. Sustainability assessment of oilseed fractionation processes: A case study on lupin seeds. Journal of Food Engineering 150, 117–124. doi:10.1016/j.jfoodeng.2014.11.005

Bérot, S., Compoint, J.P., Larré, C., Malabat, C., Guéguen, J., 2005. Large scale purification of rapeseed proteins (*Brassica napus* L.). Journal of chromatography B 818, 35–42. doi:10.1016/j.jchromb.2004.08.001

BioExx Specialty Proteins, 2011. BioExx Specialty Proteins Inc. Annual Report [WWW Document]. URL http://quote.morningstar.com/stock-filing/Annual-Report/2011/12/31/t.aspx?t=PINX:BIXZF&ft=&d=82b88515f911b65f26f418260adaeb7e (accessed 12.29.15).

Blaicher, F.M., Elstner, F., Stein, W., Mukherjee, K.D., 1983. Rapeseed protein isolates : effect of processing on yield and composition of protein. Journal of Agricultural and Food Chemistry 31, 358–362. doi:10.1021/jf00116a044

Blair, R., Reichert, R.D., 1984. Carbohydrate and phenolic constituents in a comprehensive range of rapeseed and canola fractions: Nutritional significance for animals. Journal of the Science of Food and Agriculture 35, 29–35. doi:10.1002/jsfa.2740350106

Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P.M., Fischer, A.R.H., van Boekel, M.A.J.S., Rutherfurd, S.M., Gruppen, H., Moughan, P.J., Hendriks, W.H., 2013. The future supply of animal-derived protein for human consumption. Trends in Food Science & Technology 29, 62–73. doi:10.1016/j.tifs.2012.07.002

Bos, C., Airinei, G., Mariotti, F., Benamouzig, R., Bérot, S., Evrard, J., Fénart, E., Tomé, D., Gaudichon, C., 2007. The poor digestibility of rapeseed protein is balanced by its very high metabolic utilization in humans. The Journal of Nutrition 137, 594–600.

Brown, R.E., Jarvis, K.L., Hyland, K.J., 1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. Analytical biochemistry 180, 136–139. doi:10.1016/0003-2697(89)90101-2

Bunzel, M., Schüßler, A., Tchetseubu Saha, G., 2011. Chemical characterization of Klason lignin preparations from plant-based foods. Journal of Agricultural and Food Chemistry 59, 12506–12513. doi:10.1021/jf2031378

Burcon NutraScience, 2015. Corporate presentation [WWW Document]. URL http://www.burcon.ca/investors/corporate_factsheet.php (accessed 12.29.15).

Carré, P., Pouzet, A., 2014. Rapeseed market, worldwide and in Europe. OCL 21, D102. doi:10.1051/ocl/2013054

Cartea, M.E., Velasco, P., 2008. Glucosinolates in *Brassica* foods: Bioavailability in food and significance for human health. Phytochemistry Reviews 7, 213–229. doi:10.1007/s11101-007-9072-2

Chang, C., Nickerson, M.T., 2014. Effect of plasticizer-type and genipin on the mechanical, optical, and water vapor barrier properties of canola protein isolate-based edible films. European Food Research and Technology 238, 35–46. doi:10.1007/s00217-013-2075-x

Chen, K., Zhang, H., Miao, Y., Wei, P., Chen, J., 2011. Simultaneous saccharification and fermentation of acid-pretreated rapeseed meal for succinic acid production using Actinobacillus succinogenes. Enzyme and Microbial Technology 48, 339–344. doi:10.1016/j.enzmictec.2010.12.009

Cheung, L., Wanasundara, J., Nickerson, M.T., 2014. The effect of pH and NaCl levels on the physicochemical and emulsifying properties of a cruciferin protein isolate. Food Biophysics 9, 105–113. doi:10.1007/s11483-013-9323-2

CNW, 2007. BioExx announces 40,000,000 kg extraction facility. Canada NewsWire.

Cumby, N., Zhong, Y., Naczk, M., Shahidi, F., 2008. Antioxidant activity and water-holding capacity of canola protein hydrolysates. Food Chemistry 109, 144–148. doi:10.1016/j.foodchem.2007.12.039

Das Purkayastha, M., Das, S., Manhar, A.K., Deka, D., Mandal, M., Mahanta, C.L., 2013. Removing antinutrients from rapeseed press-cake and their benevolent role in waste cooking oil-derived biodiesel: conjoining the valorization of two disparate industrial wastes. Journal of Agricultural and Food Chemistry 61, 10746–10756. doi:10.1021/jf403657c

Das Purkayastha, M., Mahanta, C.L., 2015. Statistically designed optimal process conditions for recuperation of protein from rapeseed meal. Journal of Food Science and Technology 52, 3203–3218. doi:10.1007/s13197-014-1299-5

Das, R., Bhattacherjee, C., Ghosh, S., 2009. Preparation of mustard (*Brassica juncea* L.) protein isolate and recovery of phenolic compounds by ultrafiltration. Industrial & Engineering Chemistry Research 48, 4939–4947. doi:10.1021/ie801474q

Day, L., 2013. Proteins from land plants - potential resources for human nutrition and food security. Trends in Food Science and Technology 32, 25–42. doi:10.1016/j.tifs.2013.05.005

de Vries, S., Pustjens, A.M., Kabel, M.A., Kwakkel, R.P., Gerrits, W.J.J., 2014. Effects of processing technologies and pectolytic enzymes on degradability of nonstarch polysaccharides from rapeseed meal in broilers. Poultry Science 93, 589–598. doi:10.3382/ps.2013-03476

Deng, Q.Y., Barefoot, R.R., Diosady, L.L., Rubin, L.J., Tzeng, Y.-M., 1990. Lysinoalanine concentrations in rapeseed protein meals and isolates. Canadian Institute of Food Science and Technology Journal 23, 140–142. doi:10.1016/S0315-5463(90)70218-0

Diosady, L.L., Rubin, L.J., Tar, C.G., 1989. Extraction of particulate materials. 4,859,371.

Dong, X.Y., Guo, L.L., Wei, F., Li, J.F., Jiang, M.L., Li, G.M., Zhao, Y. Di, Chen, H., 2011. Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. Journal of the Science of Food and Agriculture 91, 1488–1498. doi:10.1002/jsfa.4339

Downey, R.K., 1983. The origin and description of the *Brassica* oilseed crops, in: Kramer, J.K.G., Sauer, F.D., Pigden, W.J. (Eds.), High and Low Erucic Acid Rapeseed Oils. Academic Press, Toronto, pp. 1–17.

Düsterhöft, E.-M., Voragen, A.G.J., 1991. Non-starch polysaccharides from sunflower (Helianthus annuus) meal and palm kernel (Elaeis guineenis) meal - preparation of cell wall material and extraction of polysaccharide fractions. Journal of the Science of Food and Agriculture 55, 411–422. doi:10.1002/jsfa.2740550309

Eriksson, I., Andersson, R., Westerlund, E., Andersson, R., Åman, P., 1996. Structural features of an arabinan fragment isolated from the water-soluble fraction of dehulled rapeseed. Carbohydrate Research 281, 161–172. doi:10.1016/0008-6215(95)00337-1

Eriksson, I., Westerlund, E., Åman, P., 1994. Chemical composition in varieties of rapeseed and turnip rapeseed, including several samples of hull and dehulled seed. Journal of the Science of Food and Agriculture 66, 233–240. doi:10.1002/jsfa.2740660219

European Commission, 2006. Integrated Pollution Prevention and Control. Reference document on best available techniques in the food, drink and milk industries [WWW Document]. URL http://eippcb.jrc.ec.europa.eu/reference/BREF/fdm_bref_0806.pdf (accessed 12.29.15).

European Commission, 2014. Commission implementing decision, of 1 July 2014, authorising the placing on the market of rapeseed protein as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. Official Journal of the European Union L196, 27–29.

Fang, J., Reichelt, M., Hidalgo, W., Agnolet, S., Schneider, B., 2012. Tissue-specific distribution of secondary metabolites in rapeseed (*Brassica napus* L.). PLoS ONE 7, e48006. doi:10.1371/journal.pone.0048006

FAO, 2015. FAOSTAT Database [WWW Document]. URL http://faostat3.fao.org (accessed 6.26.15).

Fleddermann, M., Fechner, A., Rössler, A., Bähr, M., Pastor, A., Liebert, F., Jahreis, G., 2013. Nutritional evaluation of rapeseed protein compared to soy protein for quality, plasma amino acids, and nitrogen balance - A randomized cross-over intervention study in humans. Clinical Nutrition 32, 519–526. doi:10.1016/j.clnu.2012.11.005

Frost & Sullivan, 2013. Analysis of the global protein ingredients market. Rapid Market Insight Series 9A75-88, 1–18.

Ghodsvali, A., Khodaparast, M.H.H., Vosoughi, M., Diosady, L.L., 2005. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. Food Research International 38, 223–231. doi:10.1016/j.foodres.2004.10.007

Ghosh, P., Ghosal, P., Thakur, S., Lerouge, P., Loutelier-Bourhis, C., Driouich, A., Ray, B., 2004. Cell wall polysaccharides of *Brassica campestris* seed cake: isolation and structural features. Carbohydrate Polymers 57, 7–13. doi:10.1016/j.carbpol.2004.01.012

Gillberg, L., Tornell, B., 1976. Preparation of rapeseed protein isolates. Precipitation of rapeseed proteins in the presence of polyacids. Journal of Food Science 41, 1070–1075. doi:10.1111/j.1365-2621.1976.tb14390.x

Goldschmid, O., 1971. Ultraviolet spectra, in: Sarkanen, K. V, Ludwig, C.H. (Eds.), Lignins: Occurrence, Formation, Structure and Reactions. John Wiley & Sons, New York, NJ, pp. 241–198.

Guo, X., Tian, S., Small, D.M., 2010. Generation of meat-like flavourings from enzymatic hydrolysates of proteins from *Brassica* sp. Food Chemistry 119, 167–172. doi:10.1016/j.foodchem.2009.05.089

Hanke, D.E., Northcote, D.H., 1975. Molecular visualization of pectin and DNA by ruthenium red. Biopolymers 14, 1–17. doi:10.1002/bip.1975.360140102

He, R., Girgih, A.T., Malomo, S.A., Ju, X., Aluko, R.E., 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. Journal of Functional Foods 5, 219–227. doi:10.1016/j.jff.2012.10.008

Heinonen, M., 2001. Rypsiöljy vähintään oliiviöljyn veroista. Kehittyvä elintarvike 3.

Helling, R.K., Patterson, T.G., Campbell, S.J., 2010. Aqueous processing of oilseed press cake. US 2010/0234569 A1.

Hernández-Jabalera, A., Cortés-Giraldo, I., Dávila-Ortíz, G., Vioque, J., Alaiz, M., Girón-Calle, J., Megías, C., Jiménez-Martínez, C., 2015. Influence of peptides–phenolics interaction on the antioxidant profile of protein hydrolysates from *Brassica napus*. Food Chemistry 178, 346–357. doi:10.1016/j.foodchem.2014.12.063

Holopainen-Mantila, U., Marjamaa, K., Merali, Z., Käsper, A., de Bot, P., Jääskeläinen, A.-S., Waldron, K., Kruus, K., Tamminen, T., 2013. Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. Bioresource Technology 138, 156–162. doi:10.1016/j.biortech.2013.03.152

Hou, W., Chang, W., Jiang, C., 1999. Qualitative distinction of carboxyl group distributions in pectins with ruthenium red. Botanical Bulletin of Academia Sinica 40, 115–119.

Hu, Z.-Y., Hua, W., Zhang, L., Deng, L.-B., Wang, X.-F., Liu, G.-H., Hao, W.-J., Wang, H.-Z., 2013. Seed structure characteristics to form ultrahigh oil content in rapeseed. PLoS ONE 8, 1–10. doi:10.1371/journal.pone.0062099

Huang, D., Boxin, O.U., Prior, R.L., 2005. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry 53, 1841–1856. doi:10.1021/jf030723c

Höglund, A., Rödin, J., Larsson, E., Rask, L., 1992. Distribution of napin and cruciferin in developing rape seed embryos. Plant physiology 98, 509–515. doi:10.1104/pp.98.2.509

ICIS, 2006. Indicative Chemical Prices [WWW Document]. URL http://www.icis.com/chemicals/channel-info-chemicals-a-z/ (accessed 12.29.15).

Jang, S.-A., Lim, G.-O., Song, K. Bin, 2011. Preparation and mechanical properties of edible rapeseed protein films. Journal of Food Science 76, C218–223. doi:10.1111/j.1750-3841.2010.02026.x

Johnson, C.M., 2013. Differential scanning calorimetry as a tool for protein folding and stability. Archives of Biochemistry and Biophysics 531, 100–109. doi:10.1016/j.abb.2012.09.008

Jolivet, P., Boulard, C., Bellamy, A., Valot, B., d'Andréa, S., Zivy, M., Nesi, N., Chardot, T., 2011. Oil body proteins sequentially accumulate throughout seed development in *Brassica napus*. Journal of Plant Physiology 168, 2015–2020. doi:10.1016/j.jplph.2011.06.007

Jones, J.D., Holme, J., 1979. Oilseed processing. 4,158,656.

Kane, P.F., 1986. $CuSO_4$-TiO_2 as Kjeldahl digestion catalyst in manual determination of crude protein in animal feeds. Journal - Association of Official Analytical Chemists 69, 664–666.

Karaca, A.C., Low, N., Nickerson, M., 2011. Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. Food Research International 44, 2991–2998. doi:10.1016/j.foodres.2011.07.009

Keshavarz, E., Cheung, R.K.M., Lui, R.C.M., Nakai, S., 1977. Adaptation of the three stage extraction process to rapeseed meal for preparation of colourless protein extracts. Canadian Institute of Food Science and Technology Journal 10, 73–77. doi:10.1016/S0315-5463(77)73460-1

Khattab, R.Y., Arntfield, S.D., 2009. Functional properties of raw and processed canola meal. LWT - Food Science and Technology 42, 1119–1124. doi:10.1016/j.lwt.2009.02.009

Kiarie, E.G., Slominski, B.A., Krause, D.O., Nyachoti, C.M., 2008. Nonstarch polysaccharide hydrolysis products of soybean and canola meal protect against enterotoxigenic Escherichia coli in piglets. The Journal of Nutrition 138, 502–508.

Kim, H.O., Li-Chan, E.C.Y., 2006. Quantitative structure-activity relationship study of bitter peptides. Journal of Agricultural and Food Chemistry 54, 10102–10111. doi:10.1021/jf062422j

King, R.D., Dietz, H.M., 1987. Air classification of rapeseed meal. Cereal Chemistry 64, 411–413.

Klassen, D.R., Elmer, C.M., Nickerson, M.T., 2011. Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. Food Chemistry 126, 1094–1101. doi:10.1016/j.foodchem.2010.11.138

Klockeman, D.M., Toledo, R., Sims, K.A., 1997. Isolation and characterization of defatted canola meal protein. Journal of Agricultural and Food Chemistry 45, 3867–3870. doi:10.1021/jf970026i

Krause, J.-P., Schultz, M., Dudek, S., 2002. Effect of extraction conditions on composition, surface activity and rheological properties of protein isolates from flaxseed (*Linum usitativissimum* L). Journal of the Science of Food and Agriculture 82, 970–976. doi:10.1002/jsfa.1140

Krause, J.-P., Schwenke, K.D., 2001. Behaviour of a protein isolate from rapeseed (*Brassica napus*) and its main protein components - globulin and albumin - at air/solution and solid interfaces, and in emulsions. Colloids and surfaces. B, Biointerfaces 21, 29–36. doi:10.1016/S0927-7765(01)00181-3

Krygier, K., Sosulski, F., Hogge, L., 1982. Free, esterified, and insoluble-bound phenolic acids. 2. Composition of phenolic acids in rapeseed flour and hulls. Journal of Agricultural and Food Chemistry 30, 334–336. doi:10.1021/jf00110a029

Kvist, S.U., Carlsson, T.I., Lawther, J.M., DeCastro, F.B., 2005. Process for the fractionation of oilseed presscakes and meals. US 2005/0136162 A1.

Lacki, K., Duvnjak, Z., 1998. Decrease of phenolic content in canola meal using a polyphenol oxidase preparation from Trametes versicolor: Effect of meal saccharification. Biotechnology Techniques 12, 31–34. doi:10.1023/A:1008847324408

Larré, C., Mulder, W., Sánchez-Vioque, R., Lazko, J., Bérot, S., Guéguen, J., Popineau, Y., 2006. Characterisation and foaming properties of hydrolysates derived from rapeseed isolate. Colloids and Surfaces B: Biointerfaces 49, 40–48. doi:10.1016/j.colsurfb.2006.02.009

Latif, S., Diosady, L.L., Anwar, F., 2008. Enzyme-assisted aqueous extraction of oil and protein from canola (*Brassica napus* L.) seeds. European Journal of Lipid Science and Technology 110, 887–892. doi:10.1002/ejlt.200700319

Li, N., Qi, G., Sun, X.S., Stamm, M.J., Wang, D., 2012. Effects of sodium bisulfite on the physicochemical and adhesion properties of canola protein fractions. Journal of Polymers and the Environment 20, 905–915. doi:10.1007/s11746-011-1977-7

Lindeboom, N., Wanasundara, P.K.J.P.D., 2007. Interference of phenolic compounds in *Brassica napus*, *Brassica rapa* and *Sinapis alba* seed extracts with the Lowry protein assay. Food Chemistry 104, 30–38. doi:10.1016/j.foodchem.2006.10.067

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.-C., Fine, F., 2012. Rapeseed and sunflower meal: a review on biotechnology status and challenges. Applied Microbiology and Biotechnology 95, 1105–1114. doi:10.1007/s00253-012-4250-6

Lowry, O.H., Rosebrough, N., Farr, A., Randall, R., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Ma, C.-Y., Harwalkar, V.R., Paquet, A., 1990. Physicochemical properties of alkali-treated oat globulin. Journal of Agricultural and Food Chemistry 38, 1707–1711. doi:10.1021/jf00098a017

Mahdi-Pour, B., Jothy, S.L., Latha, L.Y., Chen, Y., Sasidharan, S., 2012. Antioxidant activity of methanol extracts of different parts of *Lantana camara*. Asian Pacific Journal of Tropical Biomedicine 2, 960–965. doi:10.1016/S2221-1691(13)60007-6

Manamperi, W.A.R., Chang, S.K.C., Ulven, C.A., Pryor, S.W., 2010. Plastics from an improved canola protein isolate: Preparation and properties. Journal of the American Oil Chemists' Society 87, 909–915. doi:10.1007/s11746-010-1616-8

Manamperi, W.A.R., Wiesenborn, D.P., Chang, S.K.C., Pryor, S.W., 2011. Effects of protein separation conditions on the functional and thermal properties of canola protein isolates. Journal of Food Science 76, 266–273. doi:10.1111/j.1750-3841.2011.02087.x

Mansour, E.H., Dworschák, E., Pollhamer, Z., Gergely, Á., Hóvári, J., 1999. Pumpkin and canola seed proteins and bread quality. Acta Alimentaria 28, 59–70.

Mariotti, F., Tomé, D., Mirand, P.P., 2008. Converting nitrogen into protein—beyond 6.25 and Jones' factors. Critical Reviews in Food Science and Nutrition 48, 177–184. doi:10.1080/10408390701279749

McCleary, B. V, Gibson, T.S., Mugford, D.C., 1997. Measurement of total starch in cereal products by amyloglucosidase-alpha-amylase method: Collaborative study. Journal of AOAC International 80, 571–579.

Mejia, L.A., Korgaonkar, C.K., Schweizer, M., Chengelis, C., Marit, G., Ziemer, E., Grabiel, R., Empie, M., 2009a. A 13-week dietary toxicity study in rats of a napin-rich canola protein isolate. Regulatory Toxicology and Pharmacology 55, 394–402. doi:10.1016/j.fct.2009.07.029

Mejia, L.A., Korgaonkar, C.K., Schweizer, M., Chengelis, C., Marit, G., Ziemer, E., Grabiel, R., Empie, M., 2009b. A 13-week sub-chronic dietary toxicity study of a cruciferin-rich canola protein isolate in rats. Food and Chemical Toxicology 47, 2645–2654. doi:10.1016/j.fct.2009.07.029

Meng, X., Slominski, B.A., Nyachoti, C.M., Campbell, L.D., Guenter, W., 2005. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. Poultry Science 84, 37–47. doi:10.1093/ps/84.1.37

Monsalve, R.I., Rodriguez, R., 1990. Purification and characterization of proteins from the 2S fraction from seeds of the *Brassicaceae* family. Journal of Experimental Botany 41, 89–94. doi:10.1093/jxb/41.1.89

Moure, A., Sineiro, J., Domínguez, H., Parajó, J.C., 2006. Functionality of oilseed protein products: A review. Food Research International 39, 945–963. doi:10.1016/j.foodres.2006.07.002

Munck, L., Jorgensen, K.G., Ruud-Hansen, J., Hansen, K.T., 1989. The EBC methods for determination of high molecular weight *β*-glucan in barley, malt, wort and beer. Journal of the Institute of Brewing 95, 79–82. doi:10.1002/j.2050-0416.1989.tb04612.x

Murray, E.D., 1999. Oilseed protein extraction. 6,005,076.

Mustafa, A.F., Christensen, D.A., McKinnon, J.J., 1996. Chemical characterization and nutrient availability of high and low fiber canola meal. Canadian Journal of Animal Science 76, 579–586. doi:10.4141/cjas96-086

Mustafa, A.F., Christensen, D.A., McKinnon, J.J., Newkirk, R., 2000. Effects of stage of processing of canola seed on chemical composition and in vitro protein degradability of canola meal and intermediate products. Canadian Journal of Animal Science 80, 211–214. doi:10.4141/A99-079

Mäkinen, S., Johannson, T., Vegarud Gerd, E., Pihlava, J.M., Pihlanto, A., 2012. Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. Journal of Functional Foods 4, 575–583. doi:10.1016/j.jff.2012.03.003

Naczk, M., Amarowicz, R., 1998. Role of phenolics in flavor of rapeseed protein products. Developments in Food Science, Developments in Food Science 40, 597–613. doi:10.1016/S0167-4501(98)80080-0

Nappa, M., Kangas, P., Slizyte, R., Navikas, K., Vensulauskas, K., Nekrosius, A., 2013. Concepts and profitability for utilisation of fish industry side-stream, in: Rural Development. pp. 95–101.

NATECO2, 2014. Personal communication on November 11, 2014.

Neumuller, W., 2000. Preparation of vegetable protein concentrates, especially soya protein concentrate, with improved protein contents and with production of animal feeds as by-products. DE 2000/19907723 A1.

Newkirk, R.W., Classen, H.L., Edney, M.J., 2003. Effects of prepress-solvent extraction on the nutritional value of canola meal for broiler chickens. Animal Feed Science and Technology 104, 111–119. doi:10.1016/S0377-8401(02)00331-0

Niu, Y.X., Li, W., Zhu, J., Huang, Q., Jiang, M., Huang, F., 2012. Aqueous enzymatic extraction of rapeseed oil and protein from dehulled cold-pressed double-low rapeseed cake. International Journal of Food Engineering 8, 1–14. doi:10.1515/1556-3758.2530

Ostergaard, J., Hojrup, P., Knudsen, J., 1995. Amino acid sequences of three acyl-binding / lipid-transfer proteins from rape seedlings. Biochimica et Biophysica Acta 1254, 169–179. doi:10.1016/0005-2760(94)00180-7

Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., Gray, T., 1995. How to measure and predict the molar absorption coefficient of a protein. Protein Science 4, 2411–2423. doi:10.1002/pro.5560041120

Palomäki, A., Pohjantähti-Maaroos, H., Wallenius, M., Kankkunen, P., Aro, H., Husgafvel, S., Pihlava, J.-M., Oksanen, K., 2010. Effects of dietary cold-pressed turnip rapeseed oil and butter on serum lipids, oxidized LDL and arterial elasticity in men with metabolic syndrome. Lipids in Health and Disease 9, 137. doi:10.1186/1476-511X-9-137

Pantoja-Uceda, D., Palomares, O., Bruix, M., Villalba, M., Rodríguez, R., Rico, M., Santoro, J., 2004. Solution structure and stability against digestion of rproBnIb, a recombinant 2S albumin from rapeseed: Relationship to its allergenic properties. Biochemistry 43, 16036–16045. doi:10.1021/bi048069x

Partanen, R., Sibakov, J., Rommi, K., Hakala, T., Holopainen-Mantila, U., Lahtinen, P., Ercili-Cura, D., Lantto, R., 2016. Dispersion stability of non-refined turnip rapeseed (*Brassica rapa*) protein concentrate; impact of thermal, mechanical and enzymatic treatments. Food and Bioproducts Processing 99, 29–37. doi:10.1016/j.fbp.2016.03.006

Pekkarinen, S., Hopia, A., Heinonen, M., 1998. Effect of processing on the oxidative stability of low erucic acid turnip rapeseed (*Brassica rapa*) oil. Fett / Lipid 100, 69–74. doi:10.1002/(SICI)1521-4133(199803)100:3<69::AID-LIPI69>3.0.CO;2-H

Pinterits, A., Arntfield, S.D., 2007. The effect of limited proteolysis on canola protein gelation. Food Chemistry 102, 1337–1343. doi:10.1016/j.foodchem.2006.07.025

Pudel, F., 2012. Rapeseed proteins, in: Thiyam-Holländer, U., Eskin, M.N.A., Matthäus, B. (Eds.), Canola and Rapeseed. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp. 187–202. doi:doi:10.1201/b13023-11

Pustjens, A.M., de Vries, S., Gerrits, W.J.J., Kabel, M.A., Schols, H.A., Gruppen, H., 2012. Residual carbohydrates from in vitro digested processed rapeseed (*Brassica napus*) meal. Journal of Agricultural and Food Chemistry 60, 8257–8263. doi:10.1021/jf301160m

Pustjens, A.M., de Vries, S., Schols, H.A., Gruppen, H., Gerrits, W.J.J., Kabel, M.A., 2014. Understanding carbohydrate structures fermented or resistant to fermentation in broilers fed rapeseed (*Brassica napus*) meal to evaluate the effect of acid treatment and enzyme addition. Poultry Science 93, 926–934. doi:10.3382/ps.2013-03519

Pustjens, A.M., Schols, H.A., Kabel, M.A., Gruppen, H., 2013. Characterisation of cell wall polysaccharides from rapeseed (*Brassica napus*) meal. Carbohydrate Polymers 98, 1650–1656. doi:10.1016/j.carbpol.2013.07.059

Puumalainen, T.J., Poikonen, S., Kotovuori, A., Vaali, K., Kalkkinen, N., Reunala, T., Turjanmaa, K., Palosuo, T., 2006. Napins, 2S albumins, are major allergens in oilseed rape and turnip rape. Journal of Allergy and Clinical Immunology 117, 426–432. doi:10.1016/j.jaci.2005.10.004

Rass, M., Schein, C., 2013. Utilization of hulled rape seed. US 2013/0001333 A1.

Rempel, C., Scanlon, M.G., 2012. The potential for ultrasound and supercritical fluid extraction for value-added processing of canola, in: Thiyam-Holländer, U., Eskin, M.N.A., Matthäus, B. (Eds.), Canola and Rapeseed. CRC Press, Boca Raton, FL, pp. 145–170. doi:doi:10.1201/b13023-9

Rencoret, J., Prinsen, P., Gutiérrez, A., Martínez, Á.T., del Río, J.C., 2015. Isolation and structural characterization of the milled wood lignin, dioxane lignin, and cellulolytic lignin preparations from brewer's spent grain. Journal of Agricultural and Food Chemistry 63, 603–613. doi:10.1021/jf505808c

Rodrigues, I.M., Carvalho, M.G.V.S., Rocha, J.M.S., 2014. Increasing the protein content of rapeseed meal by enzymatic hydrolysis of carbohydrates. BioResources 9, 2010–2025.

Rogulski, W., 1989. Non-protein nitrogen from the rapeseed meal. Roczniki Nauk Rolniczych. Seria B - Zootechniczna (Poland) 105, 7–37.

Rozenszain, L., Beye, G., 2012. Aqueous process for preparing protein isolate and hydrolyzed protein from an oilseed. US 2012/0252065 A1.

Rubino, M.I., Amfield, S.D., Nadon, C.A., Bernatsky, A., 1996. Phenolic protein interactions in relation to the gelation properties of canola protein. Food Research International 29, 653–659. doi:10.1016/S0963-9969(97)89643-3

Russin, T.A., Arcand, Y., Boye, J.I., 2007. Particle size effect on soy protein isolate extraction. Journal of Food Processing and Preservation 31, 308–319. doi:10.1111/j.1745-4549.2007.00127.x

Salleh, M.R.B.M., Maruyama, N., Adachi, M., Hontani, N., Saka, S., Kato, N., Ohkawa, Y., Utsumi, S., 2002. Comparison of protein chemical and physicochemical properties of rapeseed cruciferin with those of soybean glycinin. Journal of Agricultural and Food Chemistry 50, 7380–7385. doi:10.1021/jf0202537

Salo-väänänen, P.P., Koivistoinen, P.E., 1996. Determination of protein in foods: comparison of net protein and crude protein (N × 6.25) values. Food Chemistry 57, 27–31. doi:10.1016/0308-8146(96)00157-4

Sánchez-Vioque, R., Bagger, C.L., Larré, C., Guéguen, J., 2004. Emulsifying properties of acylated rapeseed (*Brassica napus* L.) peptides. Journal of Colloid and Interface Science 271, 220–226. doi:10.1016/j.jcis.2003.10.028

Sari, Y.W., Bruins, M.E., Sanders, J.P.M., 2013. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Industrial Crops and Products 43, 78–83. doi:10.1016/j.indcrop.2012.07.014

Sari, Y.W., Syafitri, U., Sanders, J.P.M., Bruins, M.E., 2015. How biomass composition determines protein extractability. Industrial Crops and Products 70, 125–133. doi:10.1016/j.indcrop.2015.03.020

Schmidt, I., Novales, B., Boué, F., Axelos, M.A. V, 2010. Foaming properties of protein/pectin electrostatic complexes and foam structure at nanoscale. Journal of Colloid and Interface Science 345, 316–324. doi:10.1016/j.jcis.2010.01.016

Schutyser, M.A.I., Pelgrom, P.J.M., van der Goot, A.J., Boom, R.M., 2015. Dry fractionation for sustainable production of functional legume protein concentrates. Trends in Food Science & Technology 45, 327–335. doi:10.1016/j.tifs.2015.04.013

Schutyser, M.A.I., van der Goot, A.J., 2011. The potential of dry fractionation processes for sustainable plant protein production. Trends in Food Science & Technology 22, 154–164. doi:10.1016/j.tifs.2010.11.006

Schwenke, K.D., Mothes, R., Marzilger, K., Borowska, J., Kozlowska, H., 1987. Rapeseed protein polyanion interactions —Turbidimetric studies in systems with phosphate-containing polyanions: phytic acid and octametaphosphate. Food / Nahrung 31, 1001–1013. doi:10.1002/food.19870311014

Schwenke, K.D., Raab, B., Plietz, P., Damaschun, G., 1983. The structure of the 12 S globulin from rapeseed (*Brassica napus* L.). Food / Nahrung 27, 165–175. doi:10.1002/food.19830270208

Schöne, F., Kirchheim, U., Schumann, W., Lüdke, H., 1996. Apparent digestibility of high-fat rapeseed press cake in growing pigs and effects on feed intake, growth and weight of thyroid and liver. Animal Feed Science and Technology 62, 97–110. doi:10.1016/S0377-8401(96)00993-5

Segall, K.I., Green, B.E., Schweizer, M., 2010. Production of canola protein isolate without heat treatment ("C200Ca"). WO 2010/020038 A1.

Shahidi, F., Naczk, M., 1989. Effect of processing on the content of condensed tannins in rapeseed meals. Journal of Food Science 54, 1082–1083. doi:10.1111/j.1365-2621.1989.tb07951.x

Siddiqui, I.R., Wood, P.J., 1972. Structural investigation of water-soluble rapeseed (*Brassica campestris*) polysaccharides. Carbohydrate Research 24, 1–9. doi:10.1016/S0008-6215(00)82254-5

Siddiqui, I.R., Wood, P.J., 1977. Carbohydrates of rapeseed: a review. Journal of the Science of Food and Agriculture 28, 530–538.

Simbaya, J., Slominski, B.A., Guenter, W., Morgan, A., Campbell, L.D., 1996. The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: In vitro and in vivo studies. Animal Feed Science and Technology 61, 219–234. doi:10.1016/0377-8401(95)00939-6

Simopoulos, A.P., 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine and Pharmacotherapy 56, 365–379. doi:10.1016/S0753-3322(02)00253-6

Siy, R.D., Talbot, F.D.F., 1981. Preparation of low-phytate rapeseed protein by ultrafiltration: II. Membrane development and testing. Journal of the American Oil Chemists' Society 58, 1021–1023. doi:10.1007/BF02679317

Slominski, B.A., Campbell, L.D., 1990. Non-starch polysaccharides of canola meal: quantification, digestibility in poultry and potential benefit of dietary enzyme supplementation. Journal of the Science of Food and Agriculture 53, 175–184. doi:10.1002/jsfa.2740530205

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignins in biomass, in: Laboratory Analytical Procedure (LAP). National Renewable Energy Laboratory, Golden, CO, p. 18 pp.

Solanic, 2013. Native potato proteins - Unique nutrition & functionality for“ free from” solutions, in: Bridge2Food 8th Food Proteins Course. 26-28 November 2013, Amsterdam, p. 35.

Spragg, J., Mailer, R., 2007. Canola meal value chain quality improvement. Berwick.

Stephens, S.D., Watkins, B.A., Nielsen, S.S., 1997. Storage stability of screwpress-extracted oils and residual meals from CELSS candidate oilseed crops. Advances in Space Research 20, 1879–1889. doi:10.1016/S0273-1177(97)00855-7

Stone, A.K., Teymurova, A., Dang, Q., Abeysekara, S., Karalash, A., Nickerson, M.T., 2014. Formation and functional attributes of electrostatic complexes involving napin protein isolate and anionic polysaccharides. European Food Research and Technology 238, 773–780. doi:10.1007/s00217-014-2159-2

Suárez, C., Zienkiewicz, A., Castro, A.J., Zienkiewicz, K., Majewska-Sawka, A., Rodríguez-García, M.I., 2013. Cellular localization and levels of pectins and arabinogalactan proteins in olive (*Olea europaea* L.) pistil tissues during development: implications for pollen-pistil interaction. Planta 237, 305–319. doi:10.1007/s00425-012-1774-z

Sun, M., Xu, L., Saldaña, M.D.A., Temelli, F., 2008. Comparison of canola meals obtained with conventional methods and supercritical CO_2 with and without ethanol. Journal of the American Oil Chemists’ Society 85, 667–675. doi:10.1007/s11746-008-1239-5

Tan, S.H., Mailer, R.J., Blanchard, C.L., Agboola, S.O., 2011. Canola proteins for human consumption: extraction, profile, and functional properties. Journal of Food Science 76, R16–28. doi:10.1111/j.1750-3841.2010.01930.x

Tang, Q.N., 2010. Protein concentrates and isolates, and processes for the production thereof. US 2010/0136173 A1.

Tenkanen, M., Siika-Aho, M., 2000. An *α*-glucuronidase of Schizophyllum commune acting on polymeric xylan. Journal of Biotechnology 78, 149–161. doi:10.1016/S0168-1656(99)00240-0

Terras, F.R., Torrekens, S., Van Leuven, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P., Broekaert, W.F., 1993. A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. FEBS Letters 316, 233–240. doi:10.1016/0014-5793(93)81299-F

Tessier, B., Harscoat-Schiavo, C., Marc, I., 2006. Selective separation of peptides contained in a rapeseed (*Brassica campestris* L.) protein hydrolysate using UF/NF membranes. Journal of Agricultural and Food Chemistry 54, 3578–3584. doi:10.1021/jf0527605

Theander, O., Aman, P., Miksche, G.E., Yasuda, S., 1977. Carbohydrates, polyphenols, and lignin in seed hulls of different colors from turnip rapeseed. Journal of Agricultural and Food Chemistry 25, 270–273. doi:10.1021/jf60210a042

Thompson, L.U., 1990. Phytates in canola/rapeseed, in: Shahidi, F. (Ed.), Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology. Van Norstrand Reinhold, New York, NJ, pp. 173–192.

Thompson, L.U., Allum-Poon, P., Procope, C., 1976. Isolation of rapeseed protein using sodium hexametaphosphate. Canadian Institute of Food Science and Technology Journal 9, 15–19. doi:10.1016/S0315-5463(76)73586-7

Tkachuk, R., 1969. Nitrogen-to-protein conversion factors for cereals and oilseed meals. Cereal Chemistry 46, 419–424.

Turton, R., Bailie, R.C., Whiting, W.B., Shaeiwitz, J.A., 2008. Analysis, Synthesis and Design of Chemical Processes, 3rd ed. Pearson Education, New York, NJ.

Udenigwe, C.C., Aluko, R.E., 2012. Food protein-derived bioactive peptides: Production, processing, and potential health benefits. Journal of Food Science 77, R11–R24. doi:10.1111/j.1750-3841.2011.02455.x

Uruakpa, F.O., Arntfield, S.D., 2005. The physico-chemical properties of commercial canola protein isolate-guar gum gels. International Journal of Food Science and Technology 40, 643–653. doi:10.1111/j.1365-2621.2005.00975.x

Uruakpa, F.O., Arntfield, S.D., 2006. Network formation of canola protein-κ-carrageenan mixtures as affected by salts, urea and dithiothreitol. LWT - Food Science and Technology 39, 939–946. doi:10.1016/j.lwt.2005.06.007

van der Spiegel, M., Noordam, M.Y., van der Fels-Klerx, H.J., 2013. Safety of novel protein sources (insects, microalgae, seaweed, duckweed, and rapeseed) and legislative aspects for their application in food and feed production. Comprehensive Reviews in Food Science and Food Safety 12, 662–678. doi:10.1111/1541-4337.12032

Wanasundara, J.P.D., 2011. Proteins of *Brassicaceae* oilseeds and their potential as a plant protein source. Critical Reviews in Food Science and Nutrition 51, 635–677. doi:10.1080/10408391003749942

Wanasundara, J.P.D., McIntosh, T.C., 2008. A process for aqueous protein extraction from *Brassicaceae* oilseeds. WO 2008/144939 A1.

Vasconcelos, A., Azoia, N.G., Carvalho, A.C., Gomes, A.C., Güebitz, G., Cavaco-Paulo, A., 2011. Tailoring elastase inhibition with synthetic peptides. European Journal of Pharmacology 666, 53–60. doi:10.1016/j.ejphar.2011.05.056

Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., Saake, B., Uotila, K., Simolin, H., Hemming, J., Holmbom, B., 2009. Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides–A comparison between different hydrolysis and subsequent chromatographic analytical techniques. Industrial Crops and Products 29, 571–580. doi:10.1016/j.indcrop.2008.11.003

Vioque, J., Sanchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., Millan, F., 1999. Production and characterization of an extensive rapeseed protein hydrolysate. Journal of the American Oil Chemists' Society 76, 819–823. doi:10.1007/s11746-999-0071-x

Vioque, J., Sánchez-vioque, R., Clemente, A., Pedroche, J., Millán, F., 2000. Partially hydrolyzed rapeseed protein isolates with improved functional properties. Journal of the American Oil Chemists' Society 77, 447–450. doi:10.1007/s11746-000-0072-y

Wolfe, K.L., Liu, R.H., 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. Journal of Agricultural and Food Chemistry 55, 8896–8907. doi:10.1021/jf0715166

Von Der Haar, D., Müller, K., Bader-Mittermaier, S., Eisner, P., 2014. Rapeseed proteins - Production methods and possible application ranges. OCL 21, 1–8. doi:10.1051/ocl/2013038

Woyewoda, A.D., Nakai, S., Watson, E.L., 1978. Detoxification of rapeseed protein products by an activated carbon treatment. Canadian Institute of Food Science and Technology Journal 11, 107–112. doi:10.1016/S0315-5463(78)73222-0

Wu, J., Aluko, R.E., Muir, A.D., 2008. Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. Food Chemistry 111, 942–950. doi:10.1016/j.foodchem.2008.05.009

Wu, J., Muir, A.D., 2008. Comparative structural, emulsifying, and biological properties of 2 major canola proteins, cruciferin and napin. Journal of Food Science 73, 210–216. doi:10.1111/j.1750-3841.2008.00675.x

Vuorela, S., Meyer, A.S., Heinonen, M., 2003. Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. European Food Research and Technology 217, 517–523. doi:10.1007/s00217-003-0811-3

Xu, L., Diosady, L., 2012. Processing of canola proteins, in: Thiyam-Holländer, U., Eskin, M.N.A., Matthäus, B. (Eds.), Canola and Rapeseed. CRC Press, Boca Raton, FL, pp. 59–78. doi:doi:10.1201/b13023-5

Xu, L., Diosady, L.L., 2000. Interactions between canola proteins and phenolic compounds in aqueous media. Food Research International 33, 725–731. doi:10.1016/S0963-9969(00)00062-4

Xu, L., Diosady, L.L., 2002. Removal of phenolic compounds in the production of high-quality canola protein isolates. Food Research International 35, 23–30. doi:10.1016/S0963-9969(00)00159-9

Xu, L., Lui, F., Luo, H., Diosady, L.., 2003. Production of protein isolates from yellow mustard meals by membrane processes. Food Research International 36, 849–856. doi:10.1016/S0963-9969(03)00082-6

Yang, M., Liu, C., Huang, F., Zheng, C., Zhou, Q., 2011. Effect of dehulling treatment on the oxidative stability of cold-pressed low erucic acid rapeseed oil. Journal of the American Oil Chemists' Society 88, 1633–1639. doi:10.1007/s11746-011-1822-z

Yoshie-Stark, Y., Wada, Y., Schott, M., Wäsche, A., 2006. Functional and bioactive properties of rapeseed protein concentrates and sensory analysis of food application with rapeseed protein concentrates. LWT - Food Science and Technology 39, 503–512. doi:10.1016/j.lwt.2005.03.006

Yoshie-Stark, Y., Wada, Y., Wäsche, A., 2008. Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. Food Chemistry 107, 32–39. doi:10.1016/j.foodchem.2007.07.061

Zhang, L., Falla, T.J., 2009. Cosmeceuticals and peptides. Clinics in Dermatology 27, 485–494. doi:10.1016/j.clindermatol.2009.05.013

Zhang, S.B., Wang, Z., Xu, S.Y., 2007. Optimization of the aqueous enzymatic extraction of rapeseed oil and protein hydrolysates. Journal of the American Oil Chemists' Society 84, 97–105. doi:10.1007/s11746-006-1004-6

Zhang, S.B., Wang, Z., Xu, S.Y., 2008. Antioxidant and antithrombotic activities of rapeseed peptides. Journal of the American Oil Chemists' Society 85, 521–527. doi:10.1007/s11746-008-1217-y

Zhang, Y., Zhang, H., Wang, L., Guo, X., Qi, X., Qian, H., 2011. Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal. European Food Research and Technology 232, 941–950. doi:10.1007/s00217-011-1466-0

Zhao, G.-Y., Diao, H.-J., Zong, W., 2013. Nature of pectin-protein-catechin interactions in model systems: pectin-protein-catechin interactions. Food science and technology international 19, 153–165. doi:10.1177/1082013212442191

Zhou, B., He, Z., Yu, H., Mukherjee, K.D., 1990. Proteins from double-zero rapeseed. Journal of Agricultural and Food Chemistry 38, 690–694. doi:10.1021/jf00093a023

PUBLICATION I

Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (*Brassica rapa* L. and *Brassica napus* L.) press cakes

Journal of Agricultural and Food Chemistry 62: 7989−7997.
Copyright 2014 American Chemical Society.
Reprinted with permission from the publisher.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Effect of Enzyme-Aided Cell Wall Disintegration on Protein Extractability from Intact and Dehulled Rapeseed (*Brassica rapa* L. and *Brassica napus* L.) Press Cakes

Katariina Rommi,* Terhi K. Hakala, Ulla Holopainen, Emilia Nordlund, Kaisa Poutanen, and Raija Lantto

VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Finland

ABSTRACT: Cell-wall- and pectin-degrading enzyme preparations were used to enhance extractability of proteins from rapeseed press cake. Rapeseed press cakes from cold pressing of intact *Brassica rapa* and partially dehulled *Brassica napus* seeds, containing 36−40% protein and 35% carbohydrates, were treated with pectinolytic (Pectinex Ultra SP-L), xylanolytic (Depol 740L), and cellulolytic (Celluclast 1.5L) enzyme preparations. Pectinex caused effective disintegration of embryonic cell walls through hydrolysis of pectic polysaccharides and glucans and increased protein extraction by up to 1.7-fold in comparison to treatment without enzyme addition. Accordingly, 56% and 74% of the total protein in the intact and dehulled press cakes was extracted. Light microscopy of the press cakes suggested the presence of pectins colocalized with proteins inside the embryo cells. Hydrolysis of these intracellular pectins and deconstruction of embryonic cell walls during Pectinex treatment were concluded to relate with enhanced protein release.

KEYWORDS: *enzymatic treatment, protein extraction, microstructure, pectinase*

INTRODUCTION

Rapeseed press cake is a promising source of food-quality protein. It is currently used as high-value feed, but interest for its human consumption is growing due to increasing demand for vegetable-based protein sources. Rapeseed is the second most abundant oilseed crop after soybean, with a worldwide cultivation of 65 million tons in 2012 (FAOSTAT 2012). The main cultivated species are oilseed rape (*Brassica napus*) and the closely related turnip rape (*Brassica rapa*). Rapeseeds contain 40% oil, which is separated mainly for food or biodiesel, while around 50−60% of the seed dry matter is obtained as a byproduct and used as feed.

Rapeseed press cake contains proteins, carbohydrates, lignin, oil, and ash as its main chemical components.[1] Its nutrient composition and proteins are affected by processes used in rapeseed oil production.[2] Most of the industrially produced rapeseed oil is obtained using hexane extraction combined with heat pressing. In a gentler alternative, cold pressing, the oil is pressed from rapeseeds at 50−60 °C. Rapeseed hulls, which are rich in fiber, lignin, and other polyphenolics,[3] can be partially removed before oil pressing. Dehulling increases the proportion of protein-rich kernels and reduces the proportion of fiber-rich hulls in the press cake.[4] Although both dehulling and oil pressing are expected to have an impact on the level of protein denaturation in a press cake, the effects of upstream processing on rapeseed protein extractability have not received major attention.

Depending on the type of pressing, a rapeseed press cake contains approximately 30−40% of protein on a dry matter basis. The proteins consist of mainly two types of storage proteins, globulins and albumins,[1,3,5] which have shown good technological functionality such as solubility and foaming capacity.[6] In addition, rapeseed proteins contain adequate amounts of lysine and sulfur-containing amino acids[3,7] to meet the recommendations for daily amino acid intake. On the other hand, rapeseed press cake is also rich in phytates and phenolic compounds which can be antinutritional or otherwise undesirable in food applications, and the high carbohydrate content[8] (36% on dry matter basis) influences the technological functionality and digestibility of the press cake. Rapeseed press cake polysaccharides consist of cellulose, xyloglucan, xylan, arabinan, arabinogalactan, and pectins.[8,9] Arabinogalactans consist of a galactan backbone substituted by galactose chains which often terminate in an arabinose residue.[10] They can be covalently bonded to proteins via hydroxyproline residues to form proteoglycans. Although some nondigestible polysaccharides (i.e., dietary fiber) have widely recognized health benefits for humans,[11] others such as galactooligosaccharides may limit the usability of rapeseed press cake as such by causing flatulence.[3,12,13]

The sufficient nutritional value and the technological functionality of rapeseed proteins makes rapeseed press cake a potential vegetable-based protein source for human use. However, due to the described undesirable components in press cake, the proteins need to be enriched in order to enhance their technical functionality, bioavailability, and sensory properties in food and cosmetic applications. Industrially used protein extraction methods include aqueous extraction in alkaline or saline conditions.[6,14] In addition, protease treatment alone or in combination with cell wall degrading enzymes has proven successful for the extraction of protein hydrolysates from rapeseed press cake.[15,16] By using a combination of proteases, hemicellulases, pectinases, and cellulases, Niu et al.[16] extracted up to 82% of the total protein in dehulled, cold-pressed *B. napus* press cake, and a respective

Received: April 15, 2014
Revised: July 4, 2014
Accepted: July 21, 2014
Published: July 21, 2014

ACS Publications © 2014 American Chemical Society

approach using cellulase and protease treatment has been commercialized by Tang et al.[17] Enzymatic hydrolysis of carbohydrates has been commonly used to improve the feed quality, such as digestibility and bioavailability, of rapeseed press cake,[18,19] but it is also a potential technology to facilitate rapeseed protein extraction. A patent by Kvist et al.[20] reports 83% total protein extraction yield into four fractions from wet-milled rapeseed press cake after treatment with pectinase, β-glucanase, and hemicellulase enzymes, without the addition of proteases.

Although rapeseed protein extraction has been studied extensively, deeper understanding of the protein−carbohydrate interactions and the impact of processing on press cake cell wall structure could open up new possibilities for efficient protein release. Rapeseed cell walls are composed of a strongly interconnected network of polysaccharides, where xyloglucan, xylan, and pectic polysaccharides cross-link with cellulose and each other through noncovalent and sometimes covalent interactions.[8,21] Meanwhile, the majority of rapeseed protein is stored in protein bodies inside cells that are surrounded by these cell walls.[5,22] Transmission electron micrographs have shown partial disruption of rapeseed cell structure during cold pressing,[16] and Srivastava et al.[23] reported a correlation between microstructural changes and oil extraction yield after enzyme treatment of rapeseeds. However, a respective correlation for structural changes and protein extraction is yet to be established.

The aim of this study was to determine the effects of polysaccharide hydrolases on cell wall disintegration and protein extractability from two rapeseed press cakes obtained by cold pressing of intact *B. rapa* and partially dehulled *B. napus* seeds.

MATERIALS AND METHODS

Rapeseed press cakes. Two rapeseed press cakes were used as raw materials for mechanical and enzymatic processing. The intact press cake (6.8% moisture) was obtained from Kankaisten Öljykasvit Oy (Turenki, Finland) after cold pressing of oil from *B. rapa* L. seeds at 50−60 °C, pelletizing, and air-drying. The dehulled press cake (6.6% moisture) was received from Kroppenstedter Ölmühle Walter Doepelheuer GmbH (Kroppenstedt, Germany) after cold pressing of partially dehulled *B. napus* L. seeds and drying.

Enzymes. Three enzyme products were used: Pectinex Ultra SP-L and Celluclast 1.5L from Novozymes A/S (Bagsvaerd, Denmark) and Depol 740L from Biocatalysts Ltd. (Cardiff, United Kingdom). Their protein concentration was quantified using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Activity profiles of the enzyme preparations, including β-glucanase,[24] β-glucosidase,[25] *endo*-1,3(4)-β-glucanase, polygalacturonase,[26] xylanase,[27] and protease[28] activity, were determined (Table 1). The polysaccharide hydrolase assays were performed at 40 °C, pH 6 and the protease assay at 30 °C, pH 5.5.

Mechanical Pretreatment. Rapeseed press cakes were dry-milled at 17 800 rpm using a 100 UPZ-II fine impact mill (Hosokawa Alpine Ag, Ausburg, Germany). Prior to dry milling, the pelletized, intact press cake was ground at 1000 rpm at room temperature using an SM 300 cutting mill (Retsch, Düsseldorf, Germany).

Enzymatic Treatments. Dry-milled intact and dehulled rapeseed press cakes were treated with Pectinex, Depol, and Celluclast for 0, 4, and 48 h at an enzyme dosage of 10 mg protein/g dry substrate. On the basis of the temperature optima obtained from the manufacturer of the enzymes, the incubation temperature was 50 °C for Depol and Celluclast and 30 °C for Pectinex. The enzymatic treatments were performed in duplicate in 25 mL volume at 10% (w/v) consistency in distilled water containing 0.02% (w/v) sodium azide as an antimicrobial agent. After the treatment, liquid fractions were separated from the residual solids by centrifugation for 15 min at 3220*g*. The liquid fractions were stored in aliquots at −20 °C prior to analysis. The residual solid fractions obtained from 48 h hydrolysis were freeze-dried and weighed.

Table 1. Protein Concentration and Activities of Pectinex Ultra SP-L, Depol 740L, and Celluclast 1.5L[a]

	Pectinex Ultra SP-L	Depol 740 L	Celluclast 1.5L
protein concn (mg/mL)	61	48	170
activity (nkat/mg protein)			
endo-1,3(4)-β-glucanase (EC 3.2.1.6)	135	223	158
β-glucosidase (EC 3.2.1.21)	0.07	10	1.3
cellulase (EC 3.2.1.4)	22	23	112
polygalacturonase (EC 3.2.1.15)	2876	0.2	1.6
endo-1,4-β-xylanase (EC 3.2.1.8)	7.0	288	52
protease	0.3	0.6	nd

[a]Polysaccharide hydrolase activities were determined at pH 6 and protease activity at pH 5.5; nd = not detected.

Analysis of the Liquid Fractions from Enzymatic Treatments. Protein hydrolysis was monitored by reducing SDS−PAGE on a Criterion TGX, stain-free precast 18% gel (Bio-Rad, Hercules, CA). Molecular weights of the protein bands were determined on the basis of the migration of recombinant 10−250 kDa Precision Plus Protein standards (Bio-Rad). The protein bands were identified by correspondence of the band molecular weights with known molecular weights of the major rapeseed proteins. Monosaccharides (glucose, fructose, galactose, rhamnose, arabinose, xylose, and mannose), uronic acids, and sucrose in the 48-h enzymatic hydrolysates were identified directly by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) using a ICS-3000 ion chromatography system equipped with a CarboPac PA1 column (Dionex, Sunnyvale, CA).[29] Likewise, monosaccharide profiles of extracted polysaccharides were analyzed by HPAEC-PAD after secondary hydrolysis of the 48-h enzymatic hydrolysates.[29,30] The secondary hydrolyses were performed in duplicate in 4% sulfuric acid for 1 h at 120 °C.

Compositional Analysis of Rapeseed Press Cakes and Solid Fractions after Enzymatic Treatment. Protein concentration was determined in duplicate by Kjeldahl total nitrogen analysis (N × 6.25), according to the method by Kane.[31] Ash content of the press cakes was quantified gravimetrically after combustion for 23 h at 550 °C in an N 11 muffle furnace in triplicate (Nabertherm GmbH, Lilienthal, Germany). For total lipid content, the dry-milled press cakes were defatted by heptane extraction for 5 h in a Soxhlet apparatus. Total lipid content was determined by gravimetrical analysis of the heptane-extracted dry mass. To extract water-soluble carbohydrates, the heptane-defatted press cakes were mixed with water at 10% (w/v) consistency, 60 °C for 2 h in duplicate. Water-soluble mono- and disaccharides were analyzed from the water extracts by HPAEC-PAD. Monosaccharide profiles of water-soluble polysaccharides were determined by HPAEC-PAD after acid hydrolysis of the water extracts in 4% sulfuric acid.[29,30]

The water-extracted and enzyme-treated press cakes were analyzed for insoluble polysaccharides and lignin. The press cakes were remilled and hydrolyzed in triplicate with 70% sulfuric acid for 1 h at 30 °C followed by hydrolysis with 4% sulfuric acid for 50 min at 120 °C. The released neutral monosaccharides were analyzed by HPAEC-PAD.[29,30] Acid-soluble lignin was measured spectrophotometrically at 215 and 280 nm[32] from the acid hydrolysates, and acid-insoluble lignin was determined as the weight of the acid hydrolysis residues. Ash content of the acid hydrolysis residue was quantified gravimetrically after combustion and subtracted from the acid-insoluble lignin. Additionally, insoluble noncellulosic polysaccharides were determined after dilute acid hydrolysis of the water-extracted and enzyme-treated press cakes in triplicate with 4% sulfuric acid for 1 h at 120 °C. Cellulose content was calculated as the difference of glucose in the total and noncellulosic polysaccharides. Digestible starch content of the press cakes was determined enzymatically according to the method of

McCleary et al.[33] β-Glucan content was determined enzymatically according to the work of Munck et al.[34]

Microstructure of the Rapeseed Press Cakes before and after Enzymatic Hydrolysis. The press cakes were prepared for microscopy according to the method of Holopainen-Mantila et al.[35] As an exception, the press cakes before enzyme treatment were not embedded in 2% agar prior to fixation. The sections were stained with 0.1% (w/v) aqueous Acid Fuchsin (BDH Chemicals Ltd., Poole, United Kingdom) in 1.0% acetic acid for 1 min and with 0.01% (w/v) aqueous Calcofluor White (Fluorescent brightener 28, Aldrich, Germany) for 1 min. When the stained sections were examined with a fluorescence microscope (excitation λ 400−410 nm, emission λ >455 nm), glucans in intact cell walls appear blue (Calcofluor) and proteins appear red (Acid Fuchsin).[36,37] Additionally, the sections were stained with 0.2% (w/v) Ruthenium Red (Fluka Chemie AG, Buchs, Switzerland) in water for 2 h. Ruthenium Red associates with the carboxyl groups of galacturonic acid residues and thus shows pectins as red in bright-field illumination.[38,39] The sections were imaged with an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan). Micrographs were obtained using a PCO SensiCam CCD color camera (PCO AG, Kelheim, Germany) and the cell^P imaging software (Olympus). Images taken from replicate sample blocks were examined and representative images were selected for publication.

Protein extraction yield was expressed as the proportion of total nitrogen that was lost from the solid fraction during 48-h enzymatic and reference treatments. The yield represents the average of two Kjeldahl total nitrogen analysis results from two parallel reactions. Carbohydrate extraction yield was expressed as the proportion of total press cake carbohydrates (monosaccharides, sucrose, or polysaccharides) that were recovered in the liquid fraction after 48-h enzymatic and reference treatments. Extracted polysaccharides were calculated as the difference of the monosaccharide concentration before and after secondary acid hydrolysis. Two replicate secondary acid hydrolyses and HPLC analyses were performed for each of the two parallel reactions; thus, the carbohydrate yields represent the average of four parallel analysis results.

RESULTS AND DISCUSSION

Chemical Composition of Intact and Dehulled Rapeseed Press Cakes. Differences in the chemical composition of the intact and dehulled rapeseed press cakes were identified. The intact *B. rapa* press cake consisted of 36% crude protein, 19% acid-soluble and insoluble lignin, 12% crude oil, and 7% ash, on a dry matter basis (Table 2). In the dehulled *B. napus* press cake, the protein and oil concentrations were higher, whereas the proportion of lignin and ash was expectedly lower. The values were consistent with earlier reports for cold-pressed *B. rapa*[9] and *B. napus*[1] press cakes. Compositional differences between the intact *B. rapa* and dehulled *B. napus* press cake were expected to result rather from the removal of lignin-rich hulls than from species-related variation.[40] Dehulling reduces the carbohydrate and lignin content and enriches protein and ash in rapeseed press cake,[4] whereas equally processed, solvent-extracted *B. rapa* and *B. napus* meals have been reported to possess similar chemical composition despite the species variation.[8,12]

Both press cakes contained 35% of carbohydrates as quantified from the total neutral monosaccharides recovered after acid hydrolysis. A substantial portion of the carbohydrates consisted of water-soluble mono- and disaccharides (Table 2). Glucose originated mainly from sucrose and cellulose, while starch and β-glucan accounted for less than 0.3% of the dry matter (Table 3). The *B. napus* press cake contained more sucrose and other water-soluble sugars and less cellulose than the *B. rapa* press cake (Tables 2 and 3). Monosaccharide profiles of the press cakes (Table 2) were consistent with the rapeseed polysaccharide composition comprising cellulose, xyloglucan, xylan, and pectic polysaccharides, including homogalacturonan, rhamnogalacturonan, arabinan, and arabinogalactan.[8,9] Pectin-derived uronic acids could not be quantified from the press cakes before enzyme treatment due to their degradation during secondary acid hydrolysis.

Table 2. Chemical Composition of the Intact *B. rapa* and Dehulled *B. napus* Rapeseed Press Cakes (PC) and Solid Fractions Obtained from Treatment at 30 °C without Enzyme Addition (no 30) or with Pectinex (P)[a]

	concentration (% dm)					
	intact *B. rapa* press cake			dehulled *B. napus* press cake		
	PC	no 30	P	PC	no 30	P
protein	35.9	30.7	32.3	40.1	29.6	31.4
carbohydrates	35.4	21.0	9.3	34.5	20.7	8.2
monosaccharide composition						
glucose	14.7	9.4	5.0	14.4	9.2	4.2
arabinose	5.8	4.8	1.5	4.8	5.1	1.4
galactose	3.9	2.1	0.9	3.9	2.1	0.8
xylose	2.3	2.5	0.6	1.9	2.6	0.8
fructose	7.4	0.6	0.4	8.6	0.7	0.5
mannose	0.8	1.0	0.7	0.5	0.5	0.3
rhamnose	0.5	0.5	0.2	0.4	0.5	0.2
water-soluble carbohydrates	15.7	na	na	18.7	na	na
monosaccharides and sucrose	13.7			16.7		
polysaccharides	1.9			2.0		
insoluble carbohydrates	19.8	21.0	9.3	15.8	20.7	8.2
lignin	18.8	17.3	23.5	13.7	11.8	19.8
acid-insoluble lignin	13.5	12.7	19.4	8.2	8.3	15.8
acid-soluble lignin	5.3	4.6	4.0	5.5	3.6	3.9
oil	11.8	13.9	20.5	16.7	20.5	28.2
ash	7.0	na	na	5.6	na	na

[a]na = not analyzed. Standard deviations were <12% of the measured values from at least two parallel analyses.

Table 3. Composition of Glucose Polysaccharides in Intact *B. rapa* and Dehulled *B. napus* Rapeseed Press Cakes[a]

	content (% dm)	
	intact *B. rapa* press cake	dehulled *B. napus* press cake
sucrose	11.7	13.8
cellulose	5.2	1.6
starch	0.2	0.2
β-glucan	0.1	0.1

[a]Standard deviations <15% of the measured values (N = 2).

Microstructure of Intact and Dehulled Rapeseed Press Cakes. Rapeseed press cakes and solid fractions obtained after enzymatic treatment of the intact press cake were analyzed by microscopy to visualize the cell structure as well as integrity and localization of polysaccharides and proteins and to understand the effects of enzymatic treatment. In thin sections of the press cakes, structures originating from seed coat (hull) and embryo including cotyledons of the rapeseed were

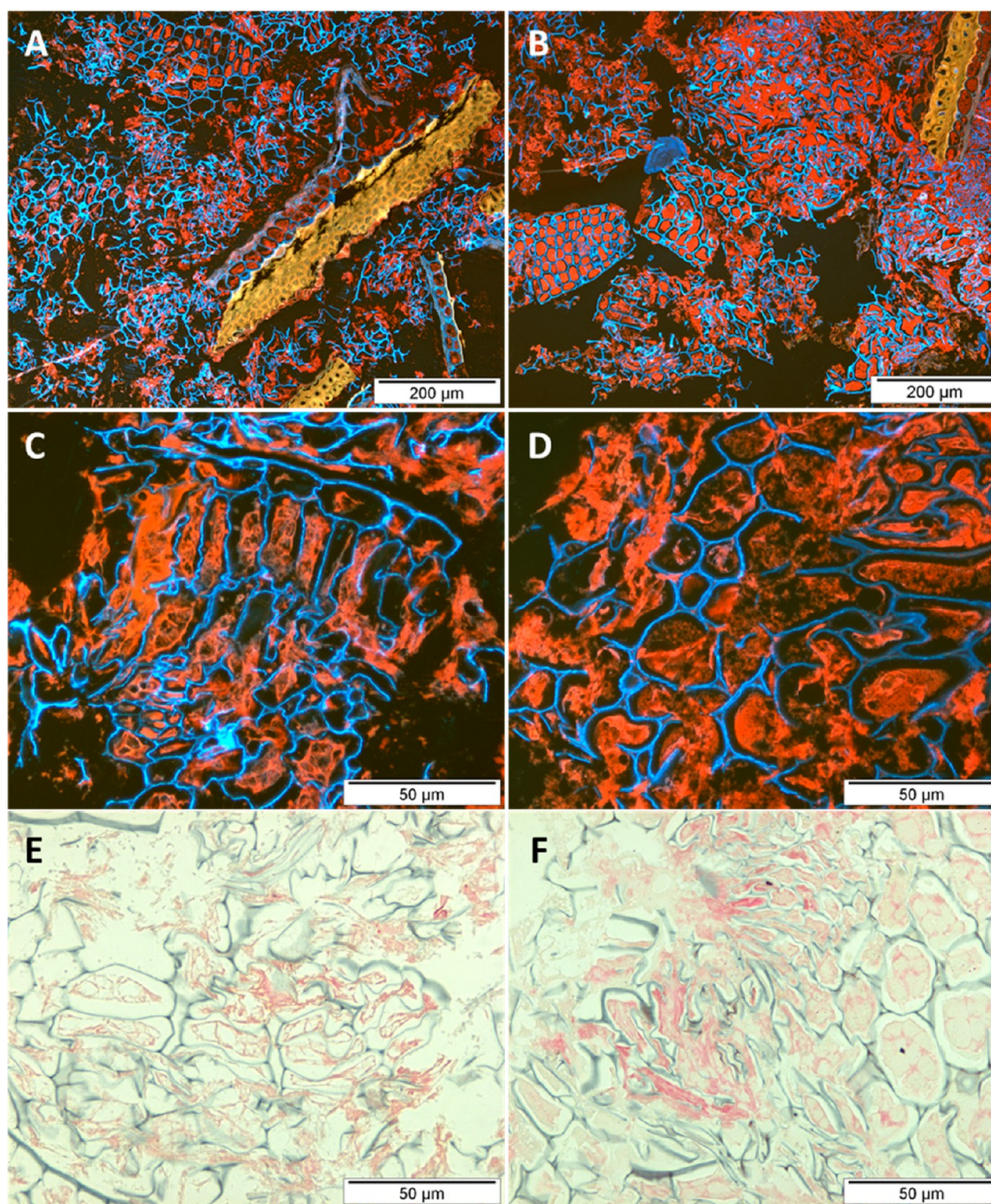

Figure 1. Micrographs of press cakes obtained from cold pressing of intact *B. rapa* seeds (A, C, E) and partially dehulled *B. napus* seeds (B, D, F). The sections were stained with Calcofluor White and Acid Fuchsin (A–D), showing cell wall glucans as blue and proteins as red, and with Ruthenium Red (E, F), showing pectins as red.

recognized (Figure 1A,B). Seed coat fragments were detected in all samples both before and after the enzymatic treatment and appear in some of the selected micrographs. Staining of the press cakes with Calcofluor White visualized cellulose and other β-linked glucans[41] in the cell walls of embryo and seed coat, whereas staining with Acid Fuchsin showed the proteins inside the embryonic cells. Cold pressing had partially disrupted the structure of embryonic cell walls (shown as blue) and protein bodies (shown as red) (Figure 1A–D). Dry milling caused further fragmentation of the embryonic cell walls without noticeable effect on proteins (Figure 3A). No clear difference was observed in the cell wall fluorescence of the two press cakes. However, proteins seemed to form more compact structures in the dehulled press cake than in the intact press cake (Figure 1A,B). Such variation in the packing density of protein bodies was not observed in intact *B. rapa* and dehulled *B. napus* seeds obtained before oil pressing (data not shown).

Pectins were visualized by staining with Ruthenium Red, which binds to the pectin polygalacturonan backbone.[39] Pectins were abundant in the seed coat (Figure 2C) and detected in smaller amounts in the embryonic cell walls (data not shown). Pectins were more intensely stained by Ruthenium Red in the seed coat than in the embryo, which may be related to differences in their composition: pectins in rapeseed hulls have been reported to contain substantially more galacturonic acid than pectins in the embryo.[3] In addition to the cell wall pectins, networks stained by Ruthenium Red were observed inside the embryo cells (Figure 1E,F), suggesting the presence of

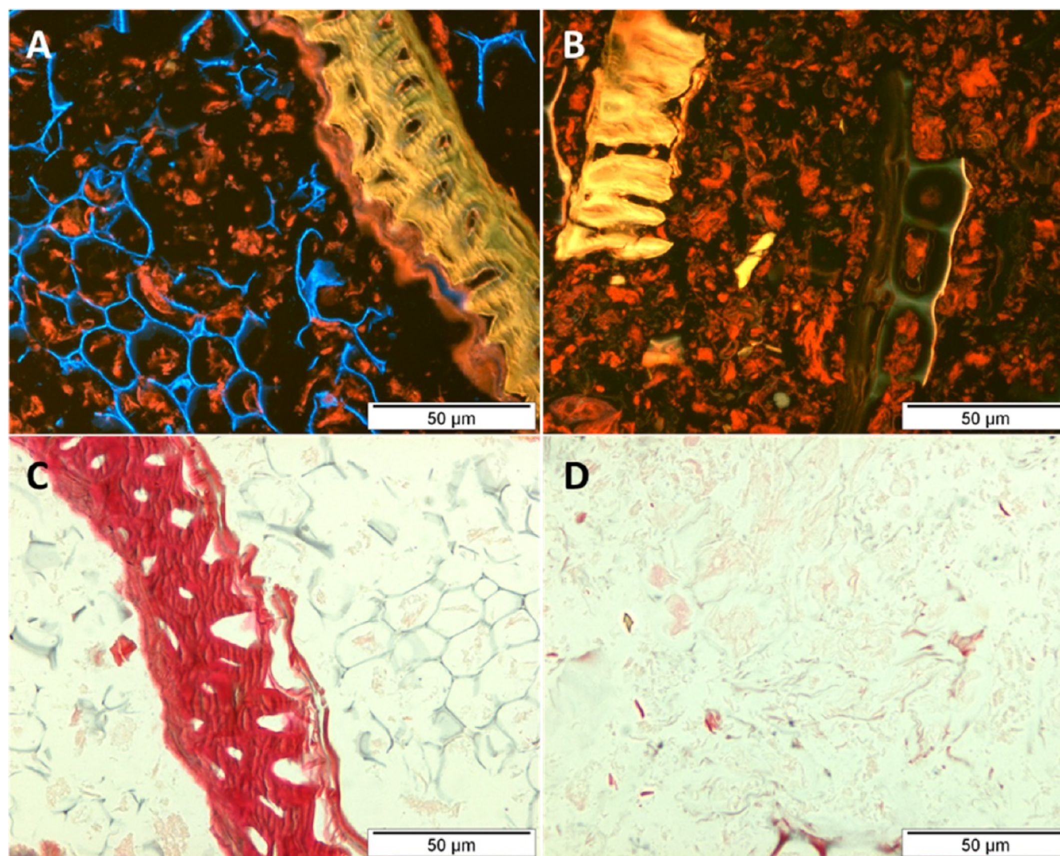

Figure 2. Micrographs of the solid fractions obtained from treatment of intact *B. rapa* press cake at 30 °C without enzyme addition (A, C) or with Pectinex (B, D). The sections were stained with Calcofluor White and Acid Fuchsin (A, B), showing cell wall glucans as blue and proteins as red, and with Ruthenium Red (C, D), showing pectins as red. Panels A–C include fragments of the seed coat.

intracellular pectin-type material. These networks were colocalized with proteins in the cells (shown by Acid Fuchsin staining in Figure 1C,D). Intracellular pectic polysaccharides have not been reported in rapeseed, but their presence was recently detected in the olive pistil.[42]

Enzymatic Hydrolysis of Rapeseed Carbohydrates. The commercial enzyme product Pectinex Ultra SP-L hydrolyzed the rapeseed carbohydrates most effectively, solubilizing up to 75% of the carbohydrates (Figure 4) and causing complete degradation of the embryonic cell walls, as shown by microscopy (Figure 2). The total carbohydrate hydrolysis yield was higher than in the study reported by Pustjens et al.,[18] who extracted 64% of the total carbohydrates in solvent-extracted *B. napus* meal during 4.75 h hydrolysis with amyloglucosidase and proteases. The higher yield is most probably explained by longer hydrolysis time and a better targeted enzyme product used in our study.

According to the sugar profiles of the 48-h enzymatic hydrolysates, carbohydrates were released almost exclusively as monosaccharides into the liquid fractions (Table 4). Release of galacturonic acid by Pectinex indicated hydrolysis of pectin (homogalacturonan, rhamnogalacturonan), whereas the release of galactose and arabinose by both Pectinex and Depol suggested hydrolysis of other pectic polysaccharides (arabinan and arabinogalactan). Substantial amounts of fructose and glucose were released as a result of sucrose hydrolysis during all enzymatic and reference treatments at 50 °C (Table 4). Respectively, the sucrose content in these hydrolysates was remarkably lower or nondetectable in comparison to the 30 °C reference treatments. Sucrose hydrolysis at 50 °C suggests that rapeseed contains an endogenous fructosyltransferase (i.e., invertase) with enhanced activity at above 30 °C. On the other hand, the high fructosyltransferase activity of Pectinex[43] was obviously responsible for the hydrolysis of sucrose into glucose and fructose at 30 °C.

The three commercial enzyme preparations were selected on the basis of the correspondence of their activity profiles with rapeseed carbohydrate composition. The Pectinex Ultra SP-L product contains a variety of activities that target the rapeseed cell walls. According to enzyme activity assays, it is rich in polygalacturonase, which is responsible for the release of galacturonic acid from pectins. The preparation also contains *endo*-β-1,4-galactanase, active on arabinogalactan,[44] and β-glucanase, which hydrolyzes glucans (cellulose and xyloglucan in rapeseed). High β-glucanase and endoxylanase activities in the Depol 740L product hydrolyzed the cellulose, xyloglucans, and xylan. However, due to the lack of polygalacturonase activity in Depol, no galacturonic acid was released from pectin. Celluclast 1.5L, which contained *endo*-1,3(4)-β-glucanase and β-glucanase (EC 3.2.1.4) as its main activities, was expected to hydrolyze cellulose and xyloglucan.

Effects of Enzymatic Treatment on the Microstructure of Rapeseed Press Cakes. Light microscopy of the solid fractions obtained after enzyme treatment of the intact press cake revealed differences in cell wall and pectin stainability after each treatment. Enzyme action was mainly detected in the cell walls of the embryo, which forms the major part of the seed and where most of rapeseed protein is stored.[5,22] By comparison,

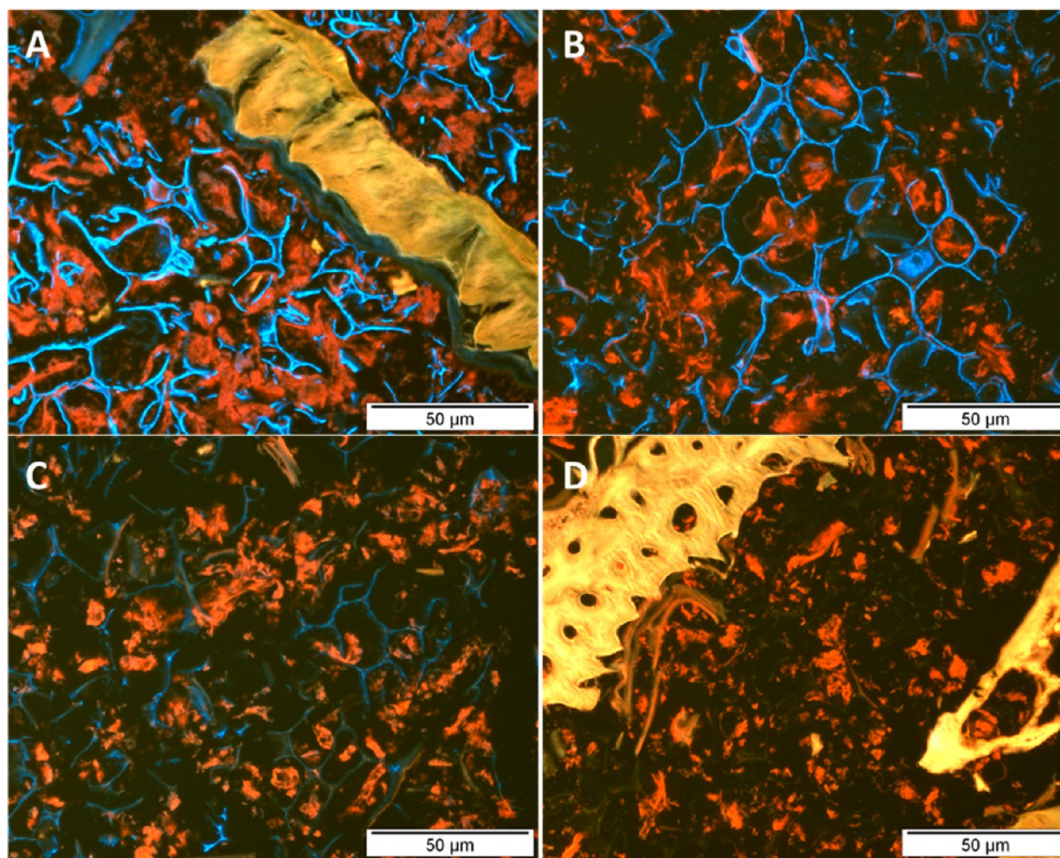

Figure 3. Micrographs of the intact *B. rapa* press cake after dry-milling (A) and of the solid fractions obtained from treatment of the press cake at 50 °C without enzyme addition (B), with Celluclast (C) or with Depol (D). The sections were stained with Calcofluor White and Acid Fuchsin, showing cell wall glucans as blue and proteins as red. Panels A and D include fragments of the seed coat.

the seed coat fragments were mostly unaffected by the enzymes. The embryonic cell walls were stainable with Calcofluor after the reference treatment but not after the Pectinex treatment (Figure 2A,B), suggesting complete cell wall glucan hydrolysis at least on the surface of the cell walls. The cell walls were likewise detected in the bright-field image of the reference sample but not in the Pectinex-treated sample (Figure 2C,D). According to Ruthenium Red staining, the seed coat pectins were still present after the Pectinex treatment (data not shown). The Ruthenium Red-stained networks inside the embryo cells were also detectable in both the Pectinex-treated and the reference sample, but their staining intensity had been reduced, suggesting partial hydrolysis of this pectin-type material. The substantial effect of Pectinex on the press cake microstructure was obviously caused by the hydrolysis of pectins and pectic polysaccharides, which are among the main rapeseed carbohydrates[3,8,45]

Depol had a strong disruptive effect on the cell wall structure, despite the lower carbohydrate extraction yield in comparison to that of Pectinex. Similarly, as after Pectinex treatment, the embryonic cell walls were no longer stainable with Calcofluor after Depol treatment (Figure 3D). However, while Pectinex degraded the embryonic cell walls completely, skeletons of the cell walls were still detectable after Depol treatment. This suggests that complete cell wall degradation by Depol may have been hindered by its inability to hydrolyze pectin. Proteins in the Depol-treated solids seemed more degraded and scattered than after other treatments (Figure 3D), indicating partial protein hydrolysis by the minor protease activity in Depol (Table 1).

In the Celluclast-treated solids (Figure 3C), cell wall stainability with Calcofluor was only slightly reduced in comparison to the reference sample (Figure 3B). Since cellulose and xyloglucan are among the major components of rapeseed cotyledon cell walls, the weak impact of Celluclast suggests that cellulase accessibility may be limited by the interconnections of cellulose with other polysaccharides.[8] The data indicates that efficient disintegration of rapeseed embryonic cell walls requires targeting of both glucans and pectic polysaccharides.

Protein Extractability from Rapeseed Press Cakes. Protein extractability was generally higher from the dehulled *B. napus* press cake than from the intact *B. rapa* press cake. Even without enzyme addition, 48% of the protein in the dehulled press cake was extractable in 50 °C water, whereas the corresponding yield was 34% from the intact press cake (Figure 4). Proteins have been previously reported to be more soluble in solvent-extracted *B. napus* meals than in the respective *B. rapa* meals,[46] but the effects of genetic variation on protein extractability are not yet well understood. Upstream processes such as seed drying and dehulling are presumably the main cause of variation in rapeseed protein extractability. Tighter protein compaction observed in the cold-pressed, dehulled *B. napus* press cake than in intact *B. rapa* press cake suggests that protein solubility had been better retained during cold pressing of dehulled seeds. It is well-known that excess heat used to facilitate seed drying or oil pressing may cause denaturation and

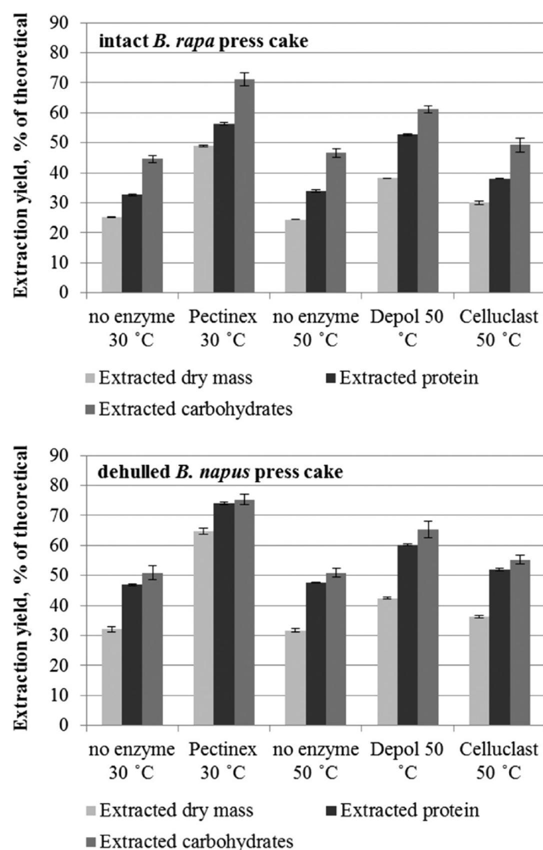

Figure 4. Effect of enzymatic treatment on extraction of proteins and carbohydrates from intact *B. rapa* and dehulled *B. napus* press cakes, in comparison to reference treatment without enzyme addition (no enzyme) at 30 or 50 °C. The press cakes were treated with Pectinex, Depol, and Celluclast for 48 h at 30 or 50 °C. Error bars represent standard deviations of at least two parallel reactions.

alteration of the proteins, reducing their solubility,[5] but denaturation may also occur due to mechanical force during cold pressing. For example, protein solubility has been previously reported to decrease during screw pressing of oil from flaked, cooked rapeseeds.[2] Cold pressing of intact seeds may require harsher conditions than pressing of dehulled seeds, which could have had a negative impact on protein solubility.

All enzyme treatments enhanced protein release from the press cakes. Pectinex increased protein extraction most, by 73% from the intact press cake and by 58% from the dehulled press cake in comparison to reference treatments without enzyme addition (Figure 4). In total, 56 and 74% of the protein in the intact and dehulled press cakes were extracted during the 48-h Pectinex treatment. The resulting liquid fraction from Pectinex treatment contained 39−54% of protein (as calculated from dry mass and protein concentration of the solid fraction) and 47−49% of carbohydrates per dry weight. SDS−PAGE analysis of the liquid fractions showed strong protein bands in the area of 20−30 kDa, corresponding to the subunits of rapeseed cruciferins (Figure 5). The small protein bands of 5−7 kDa corresponded to the subunits of napins, whereas 35−50 kDa bands observed after enzyme treatments originated from the enzymes. According to SDS−PAGE, the proteins remained mostly unhydrolyzed during Celluclast and Pectinex treatments. However, some of the bands corresponding to 20−30 kDa cruciferin subunits were hydrolyzed into 12−15 kDa fragments when the press cakes were treated with Depol due to the minor protease activity.

In the present work, combined structural and chemical characterization of rapeseed press cake and its enzymatically treated soluble and insoluble fractions provided understanding on how polysaccharide hydrolysis facilitates rapeseed protein extraction. Enzymatic hydrolysis of pectic polysaccharides (i.e., homogalacturonan, rhamnogalacturonan, arabinan and arabinogalactan) enhanced disintegration of the rapeseed embryonic cell walls, which facilitated protein extraction from cold-pressed rapeseed press cake. The presence of intracellular pectin-type networks colocalized with protein in the cotyledons and embryo was revealed, and such networks are proposed to affect protein release. However, more research on their presence and interactions with rapeseed storage proteins is needed. Our data supports the utilization of pectin-degrading enzymes for rapeseed press cake disassembly and subsequent fractionation and suggests that, in addition to the cell walls, the intracellular

Table 4. Concentration of Released Monosaccharides, Uronic Acids, Surcose, and Polysaccharides (glucose, fructose, galactose, rhamnose, arabinose, xylose and mannose) in the Liquid Fractions Obtained from Treatment of Intact *B. rapa* and Dehulled *B. napus* Rapeseed Press Cakes with Pectinex (P), Depol (D), and Celluclast (C) and without Enzyme Addition at 30°C (no 30) and 50°C (no 50)[a]

	concentration (g/L)									
	intact *B. rapa* press cake					dehulled *B. napus* press cake				
	no 30	P	no 50	D	C	no 30	P	no 50	D	C
glucose	5.4	9.1	6.2	7.1	6.8	4.2	9.9	6.3	6.7	6.9
fructose	6.4	6.7	7.1	6.3	6.5	4.3	6.6	6.6	5.5	6.3
galactose	0.8	2.0	1.2	1.5	1.3	1.4	2.5	1.7	2.2	1.9
rhamnose	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
arabinose	0.1	3.9	0.1	3.7	0.2	0.1	3.7	0.1	3.1	0.2
xylose	0.0	0.5	0.0	0.1	0.1	0.0	0.4	0.0	0.2	0.2
mannose	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1
galacturonic acid	0.1	0.6	0.0	0.1	0.0	0.0	0.5	0.0	0.0	0.0
methyl glucuronic acid	0.4	0.3	0.1	0.1	0.0	0.1	0.2	0.0	0.1	0.0
glucuronic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
sucrose	0.3	0.0	0.0	0.0	0.0	5.5	0.0	1.5	2.3	1.7
polysaccharides	2.7	2.9	2.0	2.9	2.6	1.9	2.6	1.4	2.6	1.8

[a]Standard deviations were <12% of the measured values from at least two parallel reactions.

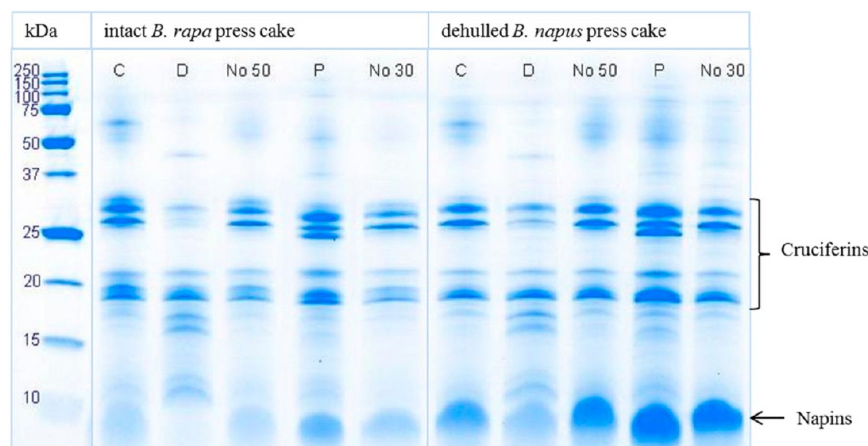

Figure 5. SDS−PAGE patterns of proteins released during enzymatic treatment of intact and dehulled rapeseed press cakes. The materials were hydrolyzed for 48 h with Celluclast (C), Depol (D), and Pectinex (P) at an enzyme dosage of 10 mg protein/g dry substrate. No enzyme was added in the reference treatments carried out at 50 °C (No 50) and 30 °C (No 30).

matrix should be targeted for enhanced protein release. Once qualified for technological functionality, rapeseed protein concentrates produced by enzyme-aided extraction may find use in food, feed, and cosmetic applications.

AUTHOR INFORMATION

Corresponding Author

*Phone: +358 20 722 4514. Fax: +358 20 722 7071. E-mail: katariina.rommi@vtt.fi.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 289170−APROPOS. Funding from Academy of Finland to K.P. is likewise gratefully acknowledged.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to thank Kankaisten Öljykasvit Oy, Kroppenstedter Ölmühle Walter Doepelheuer GmbH, and IGV Institut für Getreideverarbeitung GmbH for rapeseed press cakes. The authors thank Mari Raulio, Juhani Sibakov, and Atte Mikkelson at the VTT Technical Research Centre of Finland for cooperation in the rapeseed microscopy mechanical pretreatment, and carbohydrate analytics and VTT's experienced technical staff for their valuable experimental work.

REFERENCES

(1) Schöne, F.; Kirchheim, U.; Schumann, W.; Lüdke, H. Apparent digestibility of high-fat rapeseed press cake in growing pigs and effects on feed intake, growth and weight of thyroid and liver. *Anim. Feed Sci. Technol.* **1996**, *62*, 97−110.

(2) Mustafa, A. F.; Christensen, D.; McKinnon, J.; Newkirk, R. Effects of stage of processing of canola seed on chemical composition and in vitro protein degradability of canola meal and intermediate products. *Can. J. Anim. Sci.* **2000**, *80*, 211−214.

(3) Shahidi, F. *Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology*; Van Nostrand Reinhold: New York, 1990; 355 pp.

(4) Mustafa, A. F.; Christensen, D.; McKinnon, J. Chemical characterization and nutrient availability of high and low fiber canola meal. *Can. J. Anim. Sci.* **1996**, *76*, 579−586.

(5) Wanasundara, J. Proteins of *Brassicaceae* oilseeds and their potential as a plant protein source. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 635−677.

(6) Ghodsvali, A.; Khodaparast, M. H.; Vosoughi, M.; Diosady, L. L. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Res. Int.* **2005**, *38*, 223−231.

(7) Bell, J.; Keith, M. A. Survey of variation in the chemical composition of commercial canola meal produced in Western Canadian crushing plants. *Can. J. Anim. Sci.* **1991**, *71*, 469−480.

(8) Pustjens, A. M.; Schols, H. A.; Kabel, M. A.; Gruppen, H. Characterisation of cell wall polysaccharides from rapeseed (*Brassica napus*) meal. *Carbohydr. Polym.* **2013**, *98*, 1650−1656.

(9) Ghosh, P.; Ghosal, P.; Thakur, S.; Lerouge, P.; Loutelier-Bourhis, C.; Driouich, A.; Ray, B. Cell wall polysaccharides of *Brassica campestris* seed cake: isolation and structural features. *Carbohydr. Polym.* **2004**, *57*, 7−13.

(10) Gaspar, Y.; Johnson, K. L.; McKenna, J. A.; Bacic, A.; Schultz, C. J. The complex structures of arabinogalactan−proteins and the journey towards understanding function. *Plant Mol. Biol.* **2001**, *47*, 161−176.

(11) Anderson, J. W.; Baird, P.; Davis, R. H., Jr; Ferreri, S.; Knudtson, M.; Koraym, A.; Waters, V.; Williams, C. L. Health benefits of dietary fiber. *Nutr. Rev.* **2009**, *67*, 188−205.

(12) Simbaya, J.; Slominski, B.; Rakow, G.; Campbell, L.; Downey, R.; Bell, J. Quality characteristics of yellow-seeded *Brassica* seed meals: Protein, carbohydrates and dietary fiber components. *J. Agric. Food Chem.* **1995**, *43*, 2062−2066.

(13) Slominski, B. A.; Campbell, L. D. Non-starch polysaccharides of canola meal: quantification, digestibility in poultry and potential benefit of dietary enzyme supplementation. *J. Sci. Food Agric.* **1990**, *53*, 175−184.

(14) Bérot, S.; Compoint, J. P.; Larré, C.; Malabat, C.; Guéguen, J. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2005**, *818*, 35−42.

(15) Sari, Y. W.; Bruins, M. E.; Sanders, J. P. Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Ind. Crops Prod.* **2013**, *43*, 78−83.

(16) Niu, Y. X.; Li, W.; Zhu, J.; Huang, Q.; Jiang, M.; Huang, F. Aqueous enzymatic extraction of rapeseed oil and protein from dehulled cold-pressed double-low rapeseed cake. *Int. J. Food Eng.* **2012**, *8*, 1−14.

(17) Tang, Q. N. *Protein concentrates and isolates, and processes for the production thereof.* 2010, US 2010/0136173 A1.

(18) Pustjens, A. M.; de Vries, S.; Gerrits, W.; Kabel, M.; Schols, H.; Gruppen, H. Residual carbohydrates from in vitro digested processed rapeseed (*Brassica napus*) meal. *J. Agric. Food Chem.* **2012**, *60*, 8257−8263.

(19) Meng, X.; Slominski, B. A.; Nyachoti, C. M.; Campbell, L. D.; Guenter, W. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. *Poult. Sci.* **2005**, *84*, 37−47.
(20) Kvist, S.; Carlsson, T.; Lawther, J. M.; Basile, F. Process for the fractionation of oilseed press cakes and meals. 2005, US2005/0136162 A1.
(21) Keegstra, K.; Talmadge, K. W.; Bauer, W. D.; Albersheim, P. The structure of plant cell walls: III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiol.* **1973**, *51*, 188−197.
(22) Hu, Z.; Hua, W.; Zhang, L.; Deng, L.; Wang, X.; Liu, G.; Hao, W.; Wang, H. Seed structure characteristics to form ultrahigh oil content in rapeseed. *PLoS One* **2013**, *8*, 1−10.
(23) Srivastava, B.; Agrawal, Y. C.; Sarker, B. C.; Kushwaha, Y. P.; Singh, B. P. Effect of enzyme extract on rapeseed microstructure and oil recovery. *J. Food Sci. Technol.* **2004**, *41*, 88−91.
(24) Zurbriggen, B.; Bailey, M.; Penttilä, M.; Poutanen, K.; Linko, M. Pilot scale production of a heterologous *Trichoderma reesei* cellulase in *Saccharomyces cerevisiae*. *J. Biotechnol.* **1990**, *13*, 267−278.
(25) Bailey, M. J.; Nevalainen, K. M. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microb. Technol.* **1981**, *3*, 153−157.
(26) Bailey, M. J.; Pessa, E. Strain and process for production of polygalacturonase. *Enzyme Microb. Technol.* **1990**, *12*, 266−271.
(27) Bailey, M. J.; Biely, P.; Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **1992**, *23*, 257−270.
(28) Lowry, O. H.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(29) Hausalo, T. Analysis of wood and pulp carbohydrates by anion exchange chromatography with pulsed amperometric detection. *Proceedings of the 8th International Symposium on Wood and Pulping Chemistry. June 6−9, 1995, Helsinki, Finland*; 1995; pp 131−136.
(30) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of structural carbohydrates and lignins in biomass. In *Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, 2008; 18 pp.
(31) Kane, P. F. $CuSO_4$−TiO_2 as Kjeldahl digestion catalyst in manual determination of crude protein in animal feeds. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 664−666.
(32) Goldschmid, O. Ultraviolet spectra. In *Lignins: Occurrence, Formation, Structure and Reactions*; Sarkanen, K. V., Ludwig, C. H., Eds.; John Wiley & Sons: New York, 1971; Chapter 6, pp 241−298.
(33) McCleary, B. V.; Gibson, T. S.; Mugford, D. C. Measurement of total starch in cereal products by amyloglucosidase-alpha-amylase method: Collaborative study. *J. AOAC Int.* **1997**, *80*, 571−579.
(34) Munck, L.; Jorgensen, K. G.; Ruud-Hansen, J.; Hansen, K. T. The EBC methods for determination of high molecular weight β-glucan in barley, malt, wort and beer. *J. Inst. Brewing* **1989**, *95*, 79−82.
(35) Holopainen-Mantila, U.; Marjamaa, K.; Merali, Z.; Käsper, A.; de Bot, P.; Jääskeläinen, A.; Waldron, K.; Kruus, K.; Tamminen, T. Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. *Bioresour. Technol.* **2013**, *138*, 156−162.
(36) Fulcher, R. G.; Wong, S. J. Inside cereals—A fluorescence microchemical view. In *Cereals for Food and Beverages*; Inglett, G. E., Munck, L., Eds.; Academic Press: New York, 1980; pp 1−26.
(37) Maeda, H.; Ishida, N. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. *J. Biochem.* **1967**, *62*, 276−278.
(38) Hanke, D. E.; Northcote, D. H. Molecular visualization of pectin and DNA by ruthenium red. *Biopolymers* **1975**, *14*, 1−17.
(39) Hou, W. C.; Chang, W. H.; Jiang, C. M. Qualitative distinction of carboxyl group distributions in pectins with ruthenium red. *Bot. Bull. Acad. Sin.* **1999**, *40*, 115−119.
(40) Eriksson, I.; Westerlund, E.; Åman, P. Chemical composition in varieties of rapeseed and turnip rapeseed, including several samples of hull and dehulled seed. *J. Sci. Food Agric.* **1994**, *66*, 233−240.
(41) Herth, W.; Schnepf, E. The fluorochrome, calcofluor white, binds oriented to structural polysaccharide fibrils. *Protoplasma* **1980**, *105*, 129−133.
(42) Suárez, C.; Zienkiewicz, A.; Castro, A.; Zienkiewicz, K.; Majewska-Sawka, A.; Rodríguez-García, M. Cellular localization and levels of pectins and arabinogalactan proteins in olive (*Olea europaea* L.) pistil tissues during development: Implications for pollen−pistil interaction. *Planta* **2013**, *237*, 305−319.
(43) Ghazi, I.; Fernandez-Arrojo, L.; Garcia-Arellano, H.; Ferrer, M.; Ballesteros, A.; Plou, F. Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. *J. Biotechnol.* **2007**, *128*, 204−211.
(44) Luonteri, E.; Laine, C.; Uusitalo, S.; Teleman, A.; Siika-Aho, M.; Tenkanen, M. Purification and characterization of *Aspergillus* β-d-galactanases acting on β-1,4- and β-1,3/6-linked arabinogalactans. *Carbohydr. Polym.* **2003**, *53*, 155−168.
(45) Siddiqui, I. R.; Wood, P. J. Carbohydrates of rapeseed: A review. *J. Sci. Food Agric.* **1977**, *28*, 530−538.
(46) Aluko, R. E.; Mcintosh, T. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *J. Sci. Food Agric.* **2001**, *81*, 391−396.

PUBLICATION II

Impact of total solid content and extraction pH on enzyme-aided recovery of protein from defatted rapeseed (*Brassica rapa* L.) press cake and physicochemical properties of the protein fractions

Journal of Agricultural and Food Chemistry 63: 2997–3003.
Copyright 2015 American Chemical Society.
Reprinted with permission from the publisher.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Impact of Total Solid Content and Extraction pH on Enzyme-Aided Recovery of Protein from Defatted Rapeseed (*Brassica rapa* L.) Press Cake and Physicochemical Properties of the Protein Fractions

Katariina Rommi,* Dilek Ercili-Cura, Terhi K. Hakala, Emilia Nordlund, Kaisa Poutanen, and Raija Lantto

VTT Technical Research Centre of Finland Ltd., P.O. Box 1000, FI-02044 VTT, Finland

ABSTRACT: Pectinase treatment was used to facilitate protein recovery from defatted rapeseed (*Brassica rapa*) cold-pressing residue in water-lean conditions and without pH adjustment. Effect of extraction pH on protein yield and physiochemical properties of the protein concentrates was assessed. Enzymatic hydrolysis of carbohydrates was feasible at high (40%) solid content and improved protein recovery at pH 6. Comparable protein yields (40−41% of total protein) from enzyme-aided water extraction (pH 6) and nonenzymatic alkaline extraction (pH10) at 10% solid content suggested that after enzymatic treatment, rapeseed protein could be extracted without exposure to alkali. However, water extraction required dilute conditions, whereas alkaline extraction was feasible also at 20% solid content. The water extracts possessed better protein solubility, higher ζ-potential, and smaller particle size than isoelectric precipitates from alkaline extraction, indicating higher dispersion stability. This is suggested to be mediated by electrostatic interactions between proteins and pectic carbohydrates in the water extracts.

KEYWORDS: *pectinase treatment, protein extraction, isoelectric precipitation, protein solubility, anionic carbohydrates*

INTRODUCTION

The use of plant proteins and protein-rich co-streams in foods and cosmetics is gaining growing importance due to the global necessity for sustainable use of raw materials and production of novel protein-rich ingredients. Rapeseed press cake, a coproduct of oil pressing, represents a potential source of protein having a favorable amino acid composition for human nutrition.[1,2] The press cake obtained by cold pressing the rapeseed oil contains approximately 35−40% of protein on a dry matter basis. The main storage proteins, cruciferin and napin, constitute 60 and 20% of the total protein, respectively.[3] Cruciferin (12S globulin) is soluble in saline conditions and has an isoelectric point (p*I*) within the pH range of 4−7.[4] It is a ca. 300 kDa hexamer of ca. 50 kDa subunits, each consisting of approximately 20 and 30 kDa polypeptides linked with a single disulfide bridge.[5] Napin (2S albumin), on the other hand, is a water-soluble, basic protein with p*I* > 10. This low-molecular-weight protein is composed of two disulfide-linked polypeptide chains of ca. 4.5 and 10 kDa.[5]

For successful application in food and skin care products, plant proteins must be concentrated and have adequate technological functionality, often including solubility, emulsifying, gelling, or foaming properties. When the aim is to utilize the protein in rapeseed press cake, the influence of oil extraction conditions on protein solubility also needs to be considered. Although the commonly used hexane extraction effectively removes oil from rapeseeds, the process requires high-temperature treatments, which may promote protein denaturation.[6,7] On the other hand, press cakes from cold pressing generally contain higher amounts (10−20% dry matter (dm)) of residual oil in comparison to solvent-extracted press cakes.[8,9] The increased oil content may enhance complex formation between lipids and proteins and thus hinder their fractionation.[10,11] Defatting of the cold-pressing residue using supercritical carbon dioxide (SC-CO_2) represents a gentle technology that prevents protein denaturation and does not require subsequent heat treatments. SC-CO_2 extraction has been shown to increase protein recovery from rapeseed[12,13] as well as oats.[14,15]

Proteins from rapeseed press cake are most commonly extracted in aqueous alkaline or saline conditions in which the solubility of napin and cruciferin is increased.[4,16] The extracted proteins may be concentrated using membrane technology or precipitated by adjusting the pH or ionic strength to produce protein isolates with >90% protein content.[17,18] Extraction and precipitation pH affects not only the yield but also the functional properties of the recovered proteins. Highly alkaline conditions (pH 11−12) are commonly used to optimize extraction of both cruciferin and napin. Although these conditions have been reported to enhance water and fat absorption capacities and the emulsifying activity of the protein isolates,[10] protein solubility may be reduced due to partial denaturation. At pH extremes also degradation, cross-linking, or racemization of amino acids may occur.[10,19]

Production of rapeseed protein isolates by aqueous extraction has been commercially established,[20] but faced problems due to high costs. One drawback of the current extraction and concentration processes is their water-intensiveness. Extraction is commonly carried out at 5−15% solid content, and the use of alkali or salt introduces ions that usually are removed by additional membrane filtration or washing steps. As a more sustainable and simple alternative to alkaline extraction and isoelectric precipitation, protein concentrates could be produced at higher solid content by combining enzymatic treatment and neutral or mild alkaline conditions.

Received: November 25, 2014
Revised: March 2, 2015
Accepted: March 5, 2015
Published: March 5, 2015

© 2015 American Chemical Society

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

Proteolytic and carbohydrate-degrading enzymes have been shown to enhance protein extractability from rapeseed press cake.[21] We have also previously shown that hydrolysis of the embryonic cell walls (consisting of pectin, arabinogalactan, arabinan, xyloglucan, xylan, and cellulose) with a commercial pectinase preparation can increase protein recovery from cold-pressed rapeseed press cake by 73%.[8] However, the effect of solid content on protein recovery during combined enzymatic treatment and aqueous extraction has not been previously reported.

In the current study, we aimed to increase the total solid content during pectinase treatment of cold-pressed, SC-CO_2-defatted rapeseed press cake to limit water consumption in the production of a rapeseed protein concentrate. Another aim was to investigate the impact of extraction pH and isoelectric precipitation on protein recovery as well as on the solution behavior of the protein concentrates.

■ MATERIALS AND METHODS

Materials and Enzymes. Rapeseed press cake (6.8% moisture) was obtained from Kankaisten Öljykasvit Oy (Turenki, Finland). The press cake had been produced by cold pressing of oil from *Brassica rapa* L. seeds at 50−60 °C, pelletizing, and air-drying. A commercial pectinase preparation, Pectinex Ultra SP-L, was obtained from Novozymes A/S (Bagsvaerd, Denmark). It had a total protein content of 61 mg/mL and contained 2876 nkat of polygalacturonase (pectinase, assayed using polygalacturonic acid as a substrate), 135 nkat of endo-β-glucanase (assayed using β-glucan as a substrate), 22 nkat of endoglucanase (cellulase, assayed using hydroxyethyl cellulose as a substrate), and 7 nkat of endo-1,4-β-xylanase activity (assayed using glucuronoxylan as a substrate) per total protein, as measured at 30 °C and pH 5.5.[8]

Pretreatment. Rapeseed press cake was ground at 1000 rpm using an SM 300 cutting mill (Retsch GmbH, Düsseldorf, Germany) and defatted by SC-CO_2 extraction in a Nova Swiss extraction vessel (Nova Werke Ag, Effretikon, Switzerland) equipped with a Chematur Ecoplanning compressor (Chematur Engineering Ltd., Pori, Finland). The vessel was operated for approximately 5 h under 300 bar of pressure at 40 °C. The defatted press cake was dry-milled twice at 17 800 rpm using a 100 UPZ-II Fine Impact mill (Hosokawa Alpine Ag, Ausburg, Germany). The defatted press cake contained 39% crude protein, 2% crude fat, 24% carbohydrates, and 8% ash on dry matter basis, as measured by using the methods described in Rommi et al.[8]

Enzymatic Treatment, Protein Extraction, and Isoelectric Precipitation. The defatted and milled rapeseed press cake was treated with Pectinex Ultra SP-L at an enzyme dosage of 5 mg total protein/g dry substrate (Figure 1). The treatment was performed at 40 and 20% (w/v) solid content in water without pH adjustment at the natural pH of the substrate (pH 5.8−6.0). Reference treatments were carried out similarly but without enzyme addition. All treatments were performed in duplicate. After 2 h of enzyme or reference treatment at 40 °C in a closed 500 mL stirred tank reactor with 40 rpm mixing, the reaction suspension was thoroughly mixed and aliquoted into 50 mL extraction tubes. Water was added to obtain 10 and 20% (w/v) solid content. The subsequent water or alkaline extraction and precipitation steps were performed in duplicate for each replicate enzyme treatment or reference treatment (N = 4). Water extraction was performed without pH adjustment at pH 5.5−6.0. After agitation at 320 rpm and 30 °C for 1 h, the suspension was centrifuged for 15 min at 3220g and 4 °C. Both the liquid fractions (supernatant) and the residual solids (pellet) were freeze-dried and weighed before further analysis. The lyophilized liquid fraction from water extraction will be referred to as "water extract", "pH6 E" throughout the text.

For alkaline extraction, the pH of the initial aliquoted suspensions (10 or 20% solid content) was adjusted to 10 using 10 M NaOH. The suspensions were stirred at 320 rpm for 1 h at 30 °C and centrifuged for 15 min at 3220g. The residual solids were directly freeze-dried and weighed for further analysis. The liquid fractions (supernatant) from

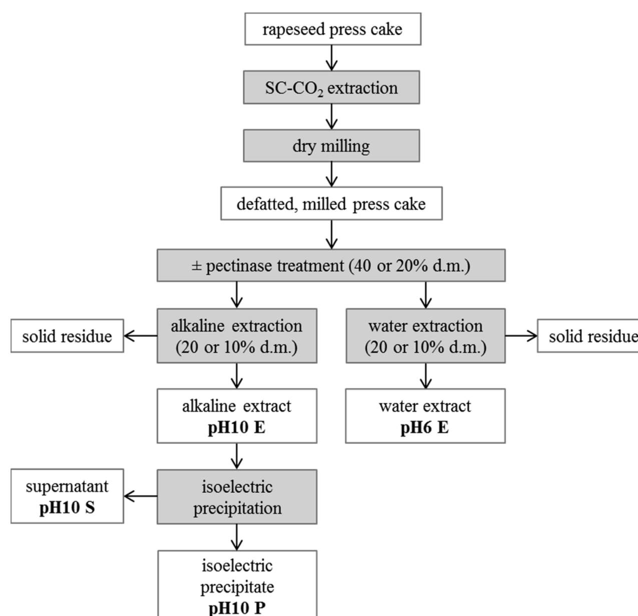

Figure 1. Pretreatment, enzymatic treatment, protein extraction, and protein precipitation schemes performed for rapeseed press cake. Pectinase treatment was performed at 40 or 20% total solid content (dm), and protein extractions were performed at 20 or 10% total solid content. Respective extraction and precipitation steps were performed after reference treatments of the defatted, milled press cake without enzyme addition. The isoelectric precipitates (pH10 P), water extracts (pH6 E), and extraction residues were obtained after centrifugation and freeze-dried before further analysis. Samples of the alkaline (pH10 E) and water extracts (pH6 E) and supernatants after precipitation (pH10 S) were stored at −20 °C.

alkaline extraction (referred to as "pH10 E") were subjected to isoelectric precipitation by adjusting the pH to 4 with 1 M HCl, mixing for 10 min, and centrifugation at 3220g for 20 min. The precipitate was recovered and freeze-dried without washing. The lyophilized precipitate will be referred to as "isoelectric precipitate", "pH10 P", throughout the text. All lyophilized samples as well as samples of unlyophilized water and alkali extracts (pH6 E and pH10 E) and supernatants remaining after isoelectric precipitation (referred to as "pH10 S") were stored at −20 °C until carbohydrate and protein analysis. Protein extraction yield was determined as the percentage of total nitrogen removed from the solids. The yield of protein precipitation was expressed as the percentage of total nitrogen or alkali-extracted nitrogen that was recovered in the precipitate. Extraction and precipitation yields were calculated as the average of four replicate experiments.

Protein Composition. Protein composition of the water and alkali extracts, isoelectric precipitates, and supernatants after isoelectric precipitation was analyzed by SDS-PAGE in reducing conditions using a Criterion TGX, Stain-Free Precast 18% gel (Bio-Rad, Hercules, CA, USA) and Precision Plus Protein standards (Bio-Rad). Water and alkali extracts and supernatants after isoelectric precipitation were diluted in 0.15% NaCl solution, mixed with loading buffer (0.1 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue), and heated at 98 °C for 5 min. Isoelectric precipitates were dissolved in the loading buffer by heating at 98 °C for 10 min. Samples (20−30 μg of protein/lane) were loaded onto the precast gel and subjected to electrophoresis at a constant voltage of 250 V. The gel was imaged using Criterion Stain Free Imager with UV-induced fluorescence detection of tryptophan residues and examined with Image Lab software (Bio-Rad). Cruciferin and napin polypeptides were identified on the basis of correspondence of their known molecular weights with the band molecular weights. Protein concentration in the residual solids, isoelectric precipitates (pH10 P), and lyophilized water extracts

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

(pH6 E) was determined as the total nitrogen content (N × 6.25) analyzed in duplicate by Kjeldahl, according to the method by Kane.[22]

Carbohydrate Composition. Free monosaccharide and sucrose concentrations in the water and alkali extracts were determined by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) using an ICS-3000 ion chromatography system equipped with a CarboPac PA1 column (Dionex, Sunnyvale, CA, USA) after filtration of the samples through a 0.45 μm syringe filter.[23] Total carbohydrate concentration was determined in duplicate by secondary hydrolysis of the extracts in 4% sulfuric acid for 1 h at 120 °C and analysis of the resulting monosaccharides by HPAEC-PAD after filtration of the hydrolysates through a 0.45 μm syringe filter.[23,24] Polysaccharide concentration was obtained by subtracting the free monosaccharides and sucrose from the total carbohydrates. To determine the total carbohydrate content of the isoelectric precipitates, the precipitates were hydrolyzed in triplicate with 70% sulfuric acid for 1 h at 30 °C followed by hydrolysis with 4% sulfuric acid for 50 min at 120 °C.[24] The released monosaccharides were analyzed by HPAEC-PAD as described above.[23]

Ash Content. The ash content of the lyophilized water extracts and isoelectric precipitates was determined in duplicate by combustion for 23 h at 550 °C in an N 11 muffle furnace (Nabertherm GmbH, Lilienthal, Germany) and weighing of the incombustible residue.

Solubility, Particle Size, and ζ-Potential. The amount of soluble protein in the lyophilized water extracts and isoelectric precipitates was determined at pH 4 and 8. The samples were dispersed in water to 2% (w/w) protein concentration on the basis of their total nitrogen content. The pH was adjusted to 4 and 8 using HCl or NaOH. The dispersions were stirred with a magnetic stirrer at 300 rpm and 40 °C for 2 h, after which they were centrifuged for 15 min at 10000*g*. The protein concentration of the supernatant was measured using a DC Protein Assay Kit (Bio-Rad) to determine the proportion of soluble protein in relation to the total nitrogen content of the dispersion.

Particle size distribution and ζ-potential of the supernatants were analyzed using Zetasizer nano ZS (Malvern Instruments, Malvern, UK). Two replicates with three consecutive measurements at 25 °C were performed. The samples were diluted 1:100 in MQ water and filtered through a 0.2 μm membrane (Millex Syringe Filter Unit, Millipore, Cork, Ireland). Disposable cuvettes and disposable folded capillary cells were used for particle size and ζ-potential measurements, respectively. Before the particle size measurement, the diluted and filtered samples were equilibrated in cuvettes for 2 h at 25 °C. For the intensity-based distribution of the particle sizes, the "general purpose" (non-negative least-squares analysis) algorithm in the Zetasizer Software was used. Volume size distributions were derived from the intensity distributions using the Mie theory by the software (refractive index $(RI)_{protein}$ = 1.450, RI_{water} = 1.330).

Statistical Analysis. Statistical analysis of the yield of extracted and precipitated protein from four replicate experiments was carried out by general linear model multivariate analysis using SPSS Statistics software (version 22, IBM, Armonk, NY, USA). The level of significance was set at $p < 0.05$ and assessed by Tukey's test.

RESULTS AND DISCUSSION

Effect of Total Solid Content, Enzymatic Treatment, and Extraction pH on Protein Extractability. The impact of solid content and pH on enzyme-aided recovery of protein from defatted rapeseed press cake was studied. Pectinase treatment was carried out at 20 and 40% total solid content to understand the influence of substrate concentration on the hydrolysis efficiency and subsequent protein release. As previously reported for nondefatted rapeseed press cake,[8] enzymatic treatment of the defatted press cake improved the protein recovery at pH 6 ($p \leq 0.05$ at 10% solid content, Tukey's test) (Figure 2). However, in alkaline extraction the preceding pectinase treatment did not have a clear benefit. Pectinase treatment at either 20 or 40% solid content resulted in similar protein extraction yields at 10% solid content, pH 6

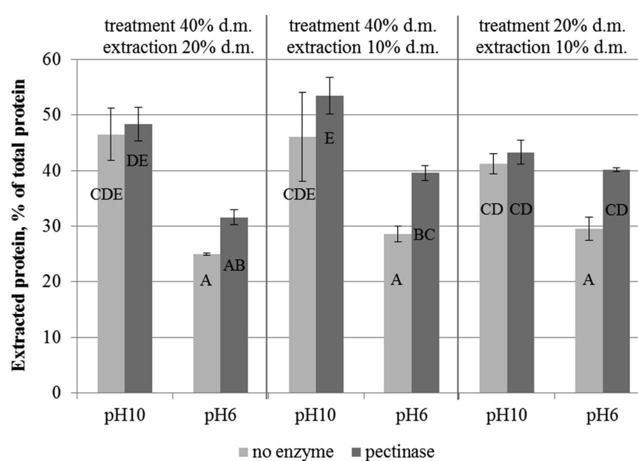

Figure 2. Yield of extracted proteins from defatted rapeseed press cake after pectinase treatment or reference treatment (no enzyme) at 40 and 20% solid content (dm) followed by alkaline (pH 10) or water extraction (pH 6) at 20 and 10% solid content. The yield was calculated on the basis of the loss of total nitrogen from the residual solids after extraction. Error bars represent standard deviations of two parallel extractions performed after each of the two replicate enzyme or reference treatments (N = 4). The sample points were categorized into subsets (A–E) on the basis of Tukey's test ($p < 0.05$).

(Figure 2, bars 8 and 12), indicating that the increase of solid content did not affect the hydrolysis efficiency. Interestingly, protein recovery in alkaline conditions seemed to increase when the enzyme or reference treatment was performed at higher solid content. Specifically after pectinase treatment at 40% solid content, alkaline extraction at 10% solid content was significantly improved ($p < 0.05$ Tukey's test) (Figure 2, bars 6 and 10). Although the enzyme itself did not clearly influence alkaline extraction, mixing at high solid content may have facilitated the release of additional protein that was extractable at pH 10.

Solid content of the extraction step (10 or 20%) did not significantly affect the protein yield (Figure 2), although at pH 6, somewhat less protein was extracted at 20% than at 10% solid content. The protein recovery was most probably limited by insufficient phase separation at increased solid content: after centrifugation of the 20% dm suspensions, a larger relative amount of liquid remained associated with the residual solids. Therefore, the residual solids presumably contained solubilized protein, which was not recovered in the extract. Protein recovery at low water content could be facilitated by more effective centrifugation practices that reduce the entrapment of soluble components in the sediment.

Overall, 41–53% of the total protein in the defatted rapeseed press cake was recovered in the alkaline extracts (pH10 E) and 25–40% in the water extracts (pH6 E) (Figure 2). In accordance with previous studies, protein recovery could be improved using either pectinase treatment or alkaline conditions.[8,16] When the extraction step was carried out at 10% solid content, enzyme-aided extraction at pH 6 resulted in the same protein yield as nonenzymatic alkaline extraction (Figure 2). This suggests that by facilitating protein recovery, pectinase treatment could allow protein extraction to be carried out without the use of alkali. However, enzyme-aided extraction at pH 6 was applicable only at relatively low (10%) solid content, whereas at 20% solid content, alkaline conditions

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

could more effectively improve protein recovery than pectinase treatment.

The highest protein yield (53% of total protein) was obtained by enzymatic treatment of the defatted press cake for 2 h at 40% solid content and subsequent alkaline extraction for 1 h at 10% solid content (Figure 2). A similar amount of protein was previously recovered during 48 h of pectinase treatment of a nondefatted rapeseed press cake at 10% solid content, pH 6.[8] At the highest, >80% of the total protein had been extracted at pH 11−12, ca. 5% solid content[19] and upon enzymatic hydrolysis of the proteins into peptides.[21] The effect of enzymatic hydrolysis was less profound than in the previous study,[8] in which pectinase treatment increased protein extractability by 73% in comparison to the reference treatment. In the present study, the enzymatic hydrolysis may have been less extensive due to reduced enzyme dosage and treatment time. According to protein patterns visualized by reducing SDS-PAGE, no selective extraction of the major rapeseed proteins occurred (Figure 3, lanes 2−5).

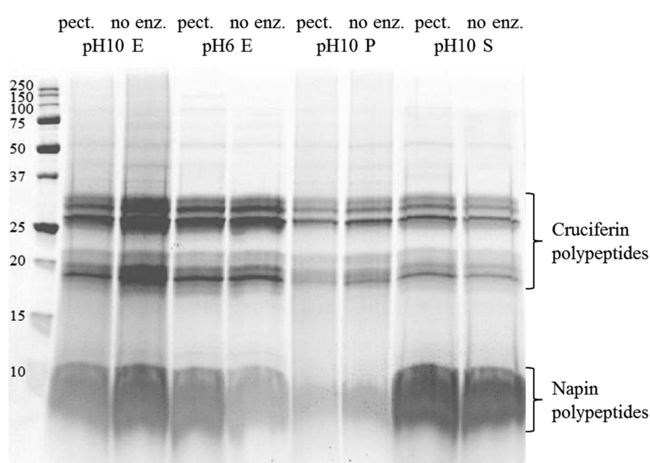

Figure 3. Protein patterns of the alkaline (pH10 E) and water extracts (pH6 E), isoelectric precipitates (pH10 P), and supernatants after isoelectric precipitation (pH10 S), as analyzed by SDS-PAGE in reducing conditions. The fractions were obtained after pectinase or reference (no enzyme) treatment of defatted rapeseed press cake at 40% solid content followed by extraction at 20% solid content and isoelectric precipitation of proteins from the alkaline extracts.

Effect of Enzymatic Treatment and Total Solid Content on Protein Precipitation. Precipitation of alkali-extracted proteins was carried out at pH 4, at which cruciferin (p*I* 4−7) was expected to have very low solubility.[25,26] However, upon acidification to pH 4, only 35−64% of the total extracted protein precipitated (Table 1). An expected factor for the limited precipitation yield was that napin, which constitutes 20% of rapeseed proteins, was positively charged and not prone to precipitation at pH 4. This was also shown in the SDS-PAGE patterns of the precipitates and the supernatants after isoelectric precipitation (Figure 3, lanes 5−8), where the precipitates (pH10 P) contained mainly cruciferin polypeptides and the supernatants (pH10 S) were enriched with napin polypeptides. Interference from nonprotein compounds in the extracts is another factor that probably contributed to the low protein precipitation yield. Anionic carbohydrates and proteins are known to interact through electrostatic interactions, and depending on the charge state of the proteins, either repulsion or formation of soluble or insoluble complexes can occur.[27] Isoelectric precipitation of cruciferin at pH 4 could have therefore been hindered by the formation of soluble complexes between negatively charged pectic carbohydrates and cruciferin, which, on the basis of its p*I* range (4−7), is suggested to have possessed a weak positive charge at pH 4.[5] The proposed cationic state of cruciferin is in accordance with a previous study on the surface charge of cruciferin-rich protein isolates as a function of pH.[28]

Proteins in the alkaline extracts were significantly less prone to precipitation after enzymatic treatment ($p < 0.05$ Tukey's test) (Table 1, precipitated protein/extracted protein). These extracts most probably contained partially hydrolyzed pectin with lower molecular weight, higher surface charge, and lower degree of esterification due to the pectin esterase activity of Pectinex.[29,30] Because the strength of pectin−protein complexes is affected by pectin molecular mass and degree of esterification,[31] the low-molecular-weight hydrolysis products of pectin may have formed stronger interactions with cruciferin at pH 4, limiting its aggregation and precipitation (Table 1).

Composition of Water Extracts and Isoelectric Precipitates. Water extracts and isoelectric precipitates (one representative from each of the four replicate samples) from extraction at 20% total solid content were characterized for chemical composition and solution behavior. In the water extracts (pH6 E), 16−29% of the total dry mass was recovered, comprising 38−47% protein and 48−52% carbohydrates (Table 2). By contrast, only 17−18% of the total dry mass was recovered in the isoelectric precipitates (pH10 P). In comparison to the extracts, the precipitates had a slightly higher protein content of 57−66% and contained 11−13% of carbohydrates and 5−6% of ash on dry matter basis. The composition is similar to that reported by Karaca et al.[18] The presence of carbohydrates and ash most probably results from

Table 1. Protein Yield in the Isoelectric Precipitates (pH10 P) Obtained after Pectinase and Reference (No Enzyme) Treatment of Defatted Rapeseed Press Cake Followed by Alkaline Extraction and Isoelectric Precipitation[a]

sample (pH10 P)	solid content in treatment (%)	solid content in extraction (%)	precipitated protein/total protein (%)	precipitated protein/extracted protein (%)
no enzyme	40	20	29.7 ± 3.2 (C)	63.9 ± 1.4 (E)
pectinase	40	20	25.6 ± 3.0 (BC)	53.1 ± 5.5 (CD)
no enzyme	40	10	28.0 ± 6.7 (BC)	60.3 ± 4.4 (DE)
pectinase	40	10	22.7 ± 2.5 (ABC)	42.4 ± 3.0 (AB)
no enzyme	20	10	20.3 ± 0.6 (AB)	49.4 ± 2.7 (BC)
pectinase	20	10	15.1 ± 1.3 (A)	34.9 ± 4.2 (A)

[a]Yields were calculated as the proportion of total or alkali-extracted nitrogen that was recovered in the precipitates. ± values represent standard deviations of two parallel extractions performed after each of the two replicate enzyme or reference treatments ($N = 4$). The sample points were categorized into subsets (A−E) on the basis of Tukey's test ($p < 0.05$).

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

Table 2. Protein, Carbohydrate, and Ash Concentrations of Rapeseed Isoelectric Precipitates (pH10 P) and Water Extracts (pH6 E)[a]

sample	protein (% dm)	total carbohydrates (% dm)	ash (% dm)
no enzyme pH10 P	65.6	10.7	10.1
pectinase pH10 P	57.3	13.1	10.1
no enzyme pH6 E	47.3	47.6	6.0
pectinase pH6 E	37.8	51.7	7.7

[a]The precipitates were obtained after pectinase or reference treatment (no enzyme) of rapeseed press cake at 40% solid content, followed by alkaline extraction at 20% solid content and isoelectric precipitation. The water extracts were obtained after extraction of protein from the pectinase and reference-treated samples without pH adjustment. The concentrations represent an average result from two parallel analyses.

Table 3. Solubility and ζ-Potential of the Supernatants Obtained after Dispersing the Isoelectric Precipitates and Dried Water Extracts in Water at pH 4 and 8[a]

sample	soluble protein/total protein (%)		zeta potential (mV)	
	pH 4	pH 8	pH 4	pH 8
no enzyme pH10 P	6.2 ± 0.1	25.1 ± 0.2	8.7 ± 0.2	−21.9 ± 1.9
pectinase pH10 P	6.4 ± 0.1	19.8 ± 0.1	10.2 ± 0.2	−21.3 ± 2.4
no enzyme pH6 E	13.4 ± 0.0	40.7 ± 0.1	−1.6 ± 0.6	−6.4 ± 0.5
pectinase pH6 E	10.2 ± 0.0	39.1 ± 0.1	−5.7 ± 0.6	−11.4 ± 0.3

[a]The isoelectric precipitates (pH10 P) were obtained after pectinase or reference treatment (no enzyme) of rapeseed press cake at 40% solid content followed by alkaline extraction at 20% solid content and isoelectric precipitation. The water extracts (pH6 E) were obtained after extraction of proteins from the pectinase and reference-treated samples without pH adjustment. ± values represent standard deviations of two parallel analyses for solubility and at least three parallel analyses for zeta potential.

the fact that the precipitates were not washed after centrifugation to prevent redissolution of proteins. The undefined 18−24% of dm was expected to include phenolic compounds such as condensed tannins and phenolic acids, which are strongly coextracted with rapeseed proteins in alkaline conditions.[32]

On the basis of the protein patterns visualized by SDS-PAGE, the isoelectric precipitates seemed more enriched in cruciferin, whereas no clear difference was observed in the intensities of the cruciferin and napin bands in the water extracts. After pectinase treatment, the majority of the extracted neutral carbohydrates were in the form of monosaccharides (mainly glucose, fructose, arabinose, and galactose), whereas without enzymatic treatment, mainly polysaccharides were present in the water extracts (data not shown). This is consistent with the results of our earlier study in which the pectinase and glucanase activities in Pectinex Ultra SP-L partially hydrolyzed rapeseed carbohydrates into monosaccharides and uronic acids.[8]

Solution Behavior of Water Extracts and Isoelectric Precipitates. Solubility of proteins in the isoelectric precipitates (pH10 P) and water extracts (pH6 E) was determined at pH 4 and 8. As expected, the solubility was pH-dependent: at pH 4, which is close to the p*I* range of cruciferin, only 6−13% of the protein among different samples was soluble, whereas at pH 8 the protein solubility varied between 20 and 41% (Table 3). The isoelectric precipitates generally contained less soluble protein in comparison to the water extracts. Similar solubility differences between rapeseed protein extracts and isoelectric precipitates were also reported by Karaca et al.[18] and Yoshie-Stark et al.[26] Lower protein solubility in the precipitates at pH 4 can be partially explained by the higher proportion of cruciferin in these samples, because the solubility of cruciferin was expected to be highly reduced at pH 4. In addition, it has been suggested that the lower solubility of oilseed isoelectric precipitates regardless of pH is related to possible partial denaturation of the protein during their production.[33] In the current study, SDS-PAGE analysis in nonreducing conditions (data not shown) revealed faint, smeared bands in the high molecular weight region of the isoelectric precipitates, indicating formation of non-native disulfide bonds as a result of partial polypeptide unfolding. Proteins are known to be prone to partial denaturation in alkaline conditions, and thus partial unfolding may have occurred during the alkaline extraction.[34] Because high protein solubility is usually associated with better technological functionality,[17] minimal processing in mild conditions can be favorable for the production of rapeseed protein ingredients for food and cosmetics applications.

The soluble fractions obtained from the protein dispersions were further characterized by ζ-potential (describing the net charge of the particles in the media) and particle size measurements to obtain insights on their dispersion stability. At acidic pH, ζ-potential values for the water extracts (pH6 E) obtained after pectinase and reference treatment were around −6 and −2 mV, respectively (Table 3). Isoelectric precipitates (pH10 P), on the other hand, attained positive ζ-potential values around +9 mV at pH 4, supporting the fact that cruciferin most probably possessed a weak positive charge at this pH. The proximity of the ζ-potentials to zero was in accordance with the low solubility of both products at pH 4. At pH 8, ζ-potentials of the water extracts were −11 and −6 mV with and without pectinase treatment, respectively, whereas isoelectric precipitates attained a higher negative charge around −21 mV. The net charge of isoelectric precipitates was not influenced by enzymatic treatment, whereas in the water extracts, the more negative charge of the enzymatically treated samples may be related to the de-esterification and depolymeration of pectin by the pectin esterase and polygalacturonase activities of Pectinex[29,30] and the resultant exposure of polar groups.[31]

Higher surface charge of proteins is generally associated with improved solubility.[35] However, the isoelectric precipitates, which attained stronger net charges, showed lower protein solubility than the water extracts, which attained net charges closer to zero (Table 3). This inverse relationship between solubility and net charge was more clearly seen at pH 8. Similar results were obtained by Karaca et al. when comparing rapeseed protein isolates prepared by isoelectric precipitation and salt extraction.[18] The low solubility of isoelectric precipitates may be related to partial denaturation during alkaline extraction, as indicated by the smears in nonreducing SDS-PAGE. Furthermore, soluble carbohydrates (Table 2) most probably contributed to the improved protein solubility of the water extracts. As previously discussed, protein solubility in the water extracts may have been increased by the formation of soluble complexes between oppositely charged proteins and pectic carbohydrates.[36] Similar mechanisms are well documented in

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

milk[37] and soy protein systems,[38] where attachment of pectin on protein particles rendered electrostatic and steric stabilization against sedimentation.

Particle size distribution in the supernatants of the protein dispersions was determined at pH 8, at which at least 20% of the protein was soluble. The water extract (pH6 E) samples contained mainly particles of 10 nm in diameter (constituting 15−20% of the sample volume), whereas the dominant particle size in the isoelectric precipitate (pH10 P) samples was remarkably higher and closer to 50 nm (Figure 4). Changes in particle size during storage were also monitored by carrying out measurements for the diluted, filtered samples, which were stored in the cuvettes at 25 °C for 24 h. A gradual increase in particle size of the isoelectric precipitate samples was observed, whereas absolutely no change occurred in the particle size distribution of the water extract samples (data not shown). The results support the proposed electrostatic and steric stabilization of protein particles by anionic carbohydrates in the water extracts.

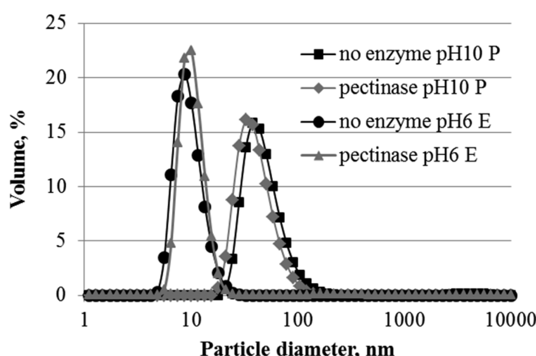

Figure 4. Volume-based particle size distribution in the supernatants obtained after dispersing the isoelectric precipitates and dried water extracts in water at pH 8. The isoelectric precipitates (pH10 P) were obtained after pectinase or reference (no enzyme) treatment of defatted rapeseed press cake at 40% solid content followed by alkaline extraction at 20% solid content and isoelectric precipitation. The water extracts (pH6 E) were obtained after extraction of proteins from the pectinase and reference-treated samples without pH adjustment.

The volume-based mean diameter of the protein particles in the water extract samples was around 10 nm, which corresponds to the reported size of rapeseed globulins measured by small-angle X-ray scattering.[39] By contrast, larger mean diameter in the isoelectric precipitate samples suggests that the precipitates contained aggregates of several monomers formed either via physical or chemical interactions. The formation of disulfide-linked soluble aggregates in the isoelectric precipitates was likewise observed by nonreducing SDS-PAGE (data not shown). Limited dispersion stability in the precipitate samples may have resulted from both partial protein denaturation and reduced concentration of stabilizing pectic carbohydrates. Additionally, it should be noted that apart from charged carbohydrates, other components in the water extracts and isoelectric precipitates may also have influenced the dispersion stability. Although not analyzed in this study, the aqueous extracts from rapeseed press cake commonly contain phytates and phenolics, which interact and form insoluble complexes with proteins.[18,32] Pectin has been reported to solubilize protein−phenolic aggregates, which may have further contributed to the better dispersion stability of the crude water extracts in comparison to the isoelectric precipitates.[31]

Our results suggest that alkaline extraction and isoelectric precipitation, which are commonly used to recover protein from rapeseed press cake, could be replaced by enzyme-aided aqueous extraction without pH adjustment in dilute conditions. Enzymatic hydrolysis of carbohydrates improved the recovery of protein extracts at pH 6 but did not introduce improvements in alkaline extraction. By contrast, enzyme treatment hindered the recovery of protein from alkaline extracts by isoelectric precipitation. Pectinase treatment was feasible also at high (40%) total solid content, which could open up possibilities for water-lean fractionation technologies. However, extraction of rapeseed protein at increased solid content is suggested to require the use of alkali due to the limited efficiency of water extraction in these conditions. Crude water extracts from pH 6 extraction had better protein solubility and dispersion stability than isoelectric precipitates from alkaline extraction. Different solution properties of these fractions are proposed to be caused by stabilizing electrostatic interactions of proteins and anionic pectic carbohydrates in the water extracts and partial denaturation of proteins in the isoelectric precipitates.

■ AUTHOR INFORMATION

Corresponding Author

*(K.R.) Phone: +358 20 722 4514. Fax: +358 20 722 7071. E-mail: katariina.rommi@vtt.fi.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under Grant Agreement 289170-APROPOS. In addition, we gratefully acknowledge funding from the Academy of Finland to Kaisa Poutanen.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Kankaisten Öljykasvit Oy for the rapeseed press cake, Tuija Kössö for the mechanical pretreatment and supercritical carbon dioxide extraction, and Atte Mikkelson for the carbohydrate analyses. We asknowledge with gratitude the experimental work performed by Mariitta Svanberg, Nina Vihersola, and Heljä Heikkinen at VTT Technical Research Centre of Finland.

■ REFERENCES

(1) Newkirk, R. W.; Classen, H. L.; Edney, M. J. Effects of prepress-solvent extraction on the nutritional value of canola meal for broiler chickens. *Anim. Feed Sci. Technol.* **2003**, *104*, 111−119.

(2) Bell, J. M.; Keith, M. O. A survey of variation in the chemical composition of commercial canola meal produced in western Canadian crushing plants. *Can. J. Anim. Sci.* **1991**, *71*, 469−480.

(3) Höglund, A.; Rödin, J.; Larsson, E.; Rask, L. Distribution of napin and cruciferin in developing rape seed embryos. *Plant Physiol.* **1992**, *98*, 509−515.

(4) Bérot, S.; Compoint, J. P.; Larré, C.; Malabat, C.; Guéguen, J. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J. Chromatogr., B* **2005**, *818*, 35−42.

(5) Wu, J.; Muir, A. D. Comparative structural, emulsifying, and biological properties of 2 major canola proteins, cruciferin and napin. *J. Food Sci.* **2008**, *73*, 210−216.

(6) Sawada, M. M.; Venâncio, L. L.; Toda, T. A.; Rodrigues, C. E. C. Effects of different alcoholic extraction conditions on soybean oil yield, fatty acid composition and protein solubility of defatted meal. *Food Res. Int.* **2014**, *62*, 662−670.

DOI: 10.1021/acs.jafc.5b01077
J. Agric. Food Chem. 2015, 63, 2997−3003

(7) Salleh, M. R. B. M.; Maruyama, N.; Adachi, M.; Hontani, N.; Saka, S.; Kato, N.; Ohkawa, Y.; Utsumi, S. Comparison of protein chemical and physicochemical properties of rapeseed cruciferin with those of soybean glycinin. *J. Agric. Food Chem.* **2002**, *50*, 7380−7385.
(8) Rommi, K.; Hakala, T. K.; Holopainen, U.; Nordlund, E.; Poutanen, K.; Lantto, R. Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (*Brassica rapa* L. and *Brassica napus* L.) press cakes. *J. Agric. Food Chem.* **2014**, *62*, 7989−7997.
(9) Schöne, F.; Kirchheim, U.; Schumann, W.; Lüdke, H. Apparent digestibility of high-fat rapeseed press cake in growing pigs and effects on feed intake, growth and weight of thyroid and liver. *Anim. Feed Sci. Technol.* **1996**, *62*, 97−110.
(10) Manamperi, W. a R.; Wiesenborn, D. P.; Chang, S. K. C.; Pryor, S. W. Effects of protein separation conditions on the functional and thermal properties of canola protein isolates. *J. Food Sci.* **2011**, *76*, 266−273.
(11) Moure, A.; Sineiro, J.; Domínguez, H.; Parajó, J. C. Functionality of oilseed protein products: a review. *Food Res. Int.* **2006**, *39*, 945−963.
(12) Catchpole, O. J.; Tallon, S. J.; Grey, J. B.; Fletcher, K.; Fletcher, A. J. Extraction of lipids from a specialist dairy stream. *J. Supercrit. Fluids* **2008**, *45*, 314−321.
(13) Sun, M.; Xu, L.; Saldaña, M. D. A.; Temelli, F. Comparison of canola meals obtained with conventional methods and supercritical CO_2 with and without ethanol. *J. Am. Oil Chem. Soc.* **2008**, *85*, 667−675.
(14) Kaukonen, O.; Sontag-Strohm, T.; Salovaara, H.; Lampi, A.-M.; Sibakov, J.; Loponen, J. Foaming of differently processed oats: role of nonpolar lipids and tryptophanin proteins. *Cereal Chem.* **2011**, *88*, 239−244.
(15) Konak, Ü. İ.; Ercili-Cura, D.; Sibakov, J.; Sontag-Strohm, T.; Certel, M.; Loponen, J. CO_2-defatted oats: solubility, emulsification and foaming properties. *J. Cereal Sci.* **2014**, *88*, 37−41.
(16) Ghodsvali, A.; Khodaparast, M. H. H.; Vosoughi, M.; Diosady, L. L. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Res. Int.* **2005**, *38*, 223−231.
(17) Aluko, R. E.; McIntosh, T. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *J. Sci. Food Agric.* **2001**, *81*, 391−396.
(18) Karaca, A. C.; Low, N.; Nickerson, M. Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Res. Int.* **2011**, *44*, 2991−2998.
(19) Klockeman, D. M.; Toledo, R.; Sims, K. A. Isolation and characterization of defatted canola meal protein. *J. Agric. Food Chem.* **1997**, *45*, 3867−3870.
(20) Tang, Q. N. Protein concentrates and isolates, and processes for the production thereof. Protein concentrates and isolates, and processes for the production thereof. US 2010/0136173 A1, 2010.
(21) Niu, Y. X.; Li, W.; Zhu, J.; Huang, Q.; Jiang, M.; Huang, F. Aqueous enzymatic extraction of rapeseed oil and protein from dehulled cold-pressed double-low rapeseed cake. *Int. J. Food Eng.* **2012**, *8*, 1−14.
(22) Kane, P. F. $CuSO_4$-TiO_2 as Kjeldahl digestion catalyst in manual determination of crude protein in animal feeds. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 664−666.
(23) Tenkanen, M.; Siika-Aho, M. An *alpha*-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. *J. Biotechnol.* **2000**, *78*, 149−161.
(24) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of structural carbohydrates and lignins in biomass. In *Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, USA, 2008; 18 pp.
(25) Wanasundara, J. P. D.; Abeysekara, S. J.; McIntosh, T. C.; Falk, K. C. Solubility differences of major storage proteins of Brassicaceae oilseeds. *J. Am. Oil Chem. Soc.* **2012**, *89*, 869−881.
(26) Yoshie-Stark, Y.; Wada, Y.; Wäsche, A. Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food Chem.* **2008**, *107*, 32−39.
(27) Klassen, D. R.; Elmer, C. M.; Nickerson, M. T. Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. *Food Chem.* **2011**, *126*, 1094−1101.
(28) Cheung, L.; Wanasundara, J.; Nickerson, M. T. The effect of pH and NaCl levels on the physicochemical and emulsifying properties of a cruciferin protein isolate. *Food Biophys.* **2014**, *9*, 105−113.
(29) Mikeš, O.; Sedláčková, J.; Rexová-Benková, L.; Omelková, J. High-performance liquid chromatography of pectic enzymes. *J. Chromatogr., A* **1981**, *207*, 99−114.
(30) Gonzalez, S. L.; Rosso, N. D. Determination of pectin methylesterase activity in commercial pectinases and study of the inactivation kinetics through two potentiometric procedures. *Cienc. Tecnol. Aliment.* **2011**, *31*, 412−417.
(31) Zhao, G.-Y.; Diao, H.-J.; Zong, W. Nature of pectin-protein-catechin interactions in model systems: pectin-protein-catechin interactions. *Food Sci. Technol. Int.* **2013**, *19*, 153−165.
(32) Xu, L.; Diosady, L. L. Interactions between canola proteins and phenolic compounds in aqueous media. *Food Res. Int.* **2000**, *33*, 725−731.
(33) Krause, J.-P.; Schultz, M.; Dudek, S. Effect of extraction conditions on composition, surface activity and rheological properties of protein isolates from flaxseed (*Linum usitativissimum* L). *J. Sci. Food Agric.* **2002**, *82*, 970−976.
(34) Ma, C.-Y.; Harwalkar, V. R.; Paquet, A. Physicochemical properties of alkali-treated oat globulin. *J. Agric. Food Chem.* **1990**, *38*, 1707−1711.
(35) Kramer, R. M.; Shende, V. R.; Motl, N.; Pace, C. N.; Scholtz, J. M. Toward a molecular understanding of protein solubility: increased negative surface charge correlates with increased solubility. *Biophys. J.* **2012**, *102*, 1907−1915.
(36) Stone, A. K.; Teymurova, A.; Dang, Q.; Abeysekara, S.; Karalash, A.; Nickerson, M. T. Formation and functional attributes of electrostatic complexes involving napin protein isolate and anionic polysaccharides. *Eur. Food Res. Technol.* **2014**, *238*, 773−780.
(37) Tromp, R. H.; de Kruif, C. G.; van Eijk, M.; Rolin, C. On the mechanism of stabilisation of acidified milk drinks by pectin. *Food Hydrocolloids* **2004**, *18*, 565−572.
(38) Tran, T.; Rousseau, D. Stabilization of acidic soy protein-based dispersions and emulsions by soy soluble polysaccharides. *Food Hydrocolloids* **2013**, *30*, 382−392.
(39) Plietz, P.; Damaschun, G.; Müller, J. J.; Schwenke, K.-D. The structure of 11-S globulins from sunflower and rape seed: a small-angle X-ray scattering study. *Eur. J. Biochem.* **1983**, *130*, 315−320.

PUBLICATION III

Impact of particle size reduction and carbohydrate-hydrolyzing enzyme treatment on protein recovery from rapeseed (*Brassica rapa* L.) press cake

Food and Bioprocess Technology 8: 2392–2399.
Copyright 2015 Springer Science+Business Media.
Reprinted with permission from the publisher.

PUBLICATION IV

Biocompounds from rapeseed oil industry co-stream as active ingredients for skin care applications

International Journal of Cosmetic Science 37: 496–505.
Copyright 2015 Society of Cosmetic Scientists and
the Société Française de Cosmétologie.
Reprinted with permission from the publisher.

International Journal of
Cosmetic Science

International Journal of Cosmetic Science, 2015, **37**, 496–505 doi: 10.1111/ics.12222

Biocompounds from rapeseed oil industry co-stream as active ingredients for skin care applications

D. Rivera*, K. Rommi[†], M. M. Fernandes*, R. Lantto[†] and T. Tzanov*

*Grup de Biotecnologia Molecular i Industrial, Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Rambla Sant Nebridi 22, Catalonia 08222, Spain and [†]VTT Technical Research Centre of Finland, P.O. Box 1000, Espoo FI-02044, Finland

Received 16 January 2015, Accepted 4 March 2015

Keywords: bioactive peptides, cell culture, chemical analysis, enzymatic hydrolysis, skin care, spectroscopy

Synopsis

OBJECTIVE: Despite the great number of substances produced by the skincare industry, very few of them seem to truly have an effect on the skin. Therefore, given the social implications surrounding physical appearance, the search for new bioactive compounds to prevent or attenuate skin ageing and enhance self-image is a priority of current research. In this context, being rich in valuable compounds, such as proteins, phenolics, lipids and vitamins, this study is focused on the potential activity of rapeseed press cake hydrolysates to be used as raw materials for skincare applications.

METHODS: In this study, the protein-rich press residue from the rapeseed oil industry was converted enzymatically into short-chain biologically active peptides using four protease products with varying substrate specificity – Alcalase 2.4L FG, Protex 6L, Protamex and Corolase 7089. The antioxidant, anti-wrinkle and anti-inflammatory activities of the obtained hydrolysates were evaluated *in vitro* while their biocompatibility with human skin fibroblasts was tested.

RESULTS: All hydrolysates were biocompatible with skin fibroblasts after 24 h of exposure, while the non-hydrolysed extract induced cell toxicity. Alcalase 2,4L FG and Protex 6L-obtained hydrolysates were the most promising extracts showing improved bioactivities suitable for skin anti-ageing formulations, namely antioxidant activity, inhibiting approximately 80% cellular reactive oxidative species, anti-inflammatory and anti-wrinkle properties, inhibiting around 36% of myeloperoxidase activity and over 83% of elastase activity.

CONCLUSION: The enzymatic technology applied to the rapeseed oil industry costream results in the release of bioactive compounds suitable for skincare applications.

Resume

OBJECTIF: Malgré le grand nombre de substances produites par l'industrie des soins de la peau, très peu d'entre elles semblent avoir vraiment un effet sur la peau. Par conséquent, étant donné les implications sociales entourant l'apparence physique, la recherche de nouveaux composés bioactifs pour prévenir ou atténuer le vieillissement de la peau et améliorer l'image de soi est une priorité de la recherche actuelle. Dans ce contexte, étant riche en composés de valeur tels que les protéines, les composés phénoliques, les lipides et vitamines, cette étude se concentre dans l'activité potentielle des hydrolysats de tourteaux de colza pour être utilisés comme matières premières pour les applications de soins de la peau.

MÉTHODES: Dans cette étude, les tourteaux de colza riche en protéines de l'industrie de l'huile de colza ont été convertis par voie enzymatique en peptides biologiquement actifs de courte chaîne utilisant quatre produits de protéase avec des spécificités différentes de substrat - Alcalase 2.4L FG, Protex 6L, Protamex et Corolase 7089. Les effets anti-oxydant, anti-rides et anti-inflammatoire des hydrolysats obtenus ont été évalués *in vitro* tandis que leur biocompatibilité avec fibroblastes de peau humaine a été testée.

RÉSULTATS: Tous les hydrolysats étaient biocompatibles avec des fibroblastes de peau après 24 h d'exposition, alors que l'extrait non hydrolysé induit une toxicité cellulaire. Les hydrolysats obtenues par Alcalase 2,4L FG et Protex 6L – s'avéraient comme les plus prometteurs qui montrent des activités biologiques améliorées appropriées pour les formulations anti-vieillissement, à savoir une activité anti-oxydante, d'inhibition d'environ 80% d'espèces oxydantes réactives cellulaires, des propriétés anti-inflammatoires et anti-rides, d'inhibition d'environ 36% de l'activité de la myéloperoxydase et plus de 83% de l'activité de l'élastase.

CONCLUSION: La technologie enzymatique appliquée aux coproduits de l'industrie de l'huile de colza résulte dans la libération de composés bioactifs appropriés pour des applications de soins de la peau.

Correspondence: Tzanko Tzanov, Grup de Biotecnologia Molecular i Industrial, Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, Rambla Sant Nebridi 22, Catalonia 08222, Spain. Tel.: +34 93 739 85 70; fax: +34 93 739 82 25;
e-mail: tzanko.tzanov@upc.edu

Introduction

Rapeseed is one of the leading oilseed crops, ranking the first with respect to oil production in the EU, and third after soybean and palm worldwide [1]. Processing rapeseeds into vegetable oil generates a residue which accounts for 60–70% of the total seed mass, reaching around 13.1 million tons of rapeseed press cake per year [2]. Currently, this coproduct is mainly used as an ingredient in animal feed and fertilizers [3–6]. To ensure an increased economic revenue in the production of rapeseed oil, the full potential of its costreams should be investigated [7,8]. Being rich in valuable compounds such as proteins, phenolics, essential amino acids, vitamins and lipids, rapeseed press cake is an excellent raw material for skin care ingredients. A common

 © 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

industrially used strategy to obtain value-added ingredients from protein-based co-streams is their enzymatic conversion into smaller, biologically active peptides [9]. Peptides obtained from the hydrolysis of rapeseed press cake proteins have shown high solubility, emulsifying and foaming capacity [10] as well as biological activities, including anti-hypertensive [6] and antioxidant [11] properties, exploited mainly in food applications [8]. Up to our knowledge, rapeseed hydrolysates have never been used as ingredients for skincare formulations. The only report found on their anti-ageing capacity relates to the application of a non-hydrolysed rapeseed meal extract [12].

One of the most important issues related to skin ageing and other conditions such as skin cancer is the exposure to UV radiation. Reactive oxygen species (ROS) and lipid peroxides formed under UV radiation damage cellular lipids, proteins and DNA, which finally leads to alteration of skin structure. Neutrophils production is also stimulated, increasing the activity of neutrophil-derived myeloperoxidase (MPO) – a ROS-generating enzyme, and proteases such as elastase and collagenase [13]. A chronic state of oxidative stress is thus caused by the elevated levels of these deleterious skin enzymes and ROS [14–16]. As a consequence, the regular skin function can be inhibited, reducing collagen and elastin production, decreasing fibroblasts activity, and inducing skin atrophy in both epidermis and dermis, as well as skin thinning [17]. These phenomena may cause different skin conditions: from the simple wrinkle formation and premature skin ageing to other complicated skin diseases such as pruritus, psoriasis and skin cancer [18]. Thus, anti-ageing ingredients should necessarily counteract ROS species and inhibit overexpressed MPO and elastase activity.

In this study, the protein-rich press residue from the rapeseed oil industry was converted enzymatically into short-chain biologically active peptides with potential for skin care applications. Proteolysis has potential to liberate bioactive peptides, otherwise inactive within the sequence of the parent protein. Furthermore, the peptides can be water-extracted to partially separate them from undesirable press cake components [19,20]. The efficiency of the obtained hydrolysates to target-specific mechanisms of the premature skin ageing was evaluated *in vitro*.

Materials and methods

Materials

Cold-pressed rapeseed (*Brassica rapa* ssp Oleifera) press cake was obtained from Kankaisten Öljykasvit Oy (Turenki, Finland). Four commercial endoproteases were used for proteolysis of the raw material – Protex 6L (Genencor International Oy, Jämsänkoski, Finland), Corolase 7089 (AB Enzymes GmbH, Darmstadt, Germany), Protamex and Alcalase 2.4L FG (Novozymes A/S, Bagsvaerd, Denmark)). Commercial rapeseed protein isolate, Isolexx $_{TM}$, (BioExx Proteins of Saskatoon Inc., Saskatoon, Canada). DC$_{TM}$ (detergent compatible) Protein Assay Kit was purchased from Bio-Rad Laboratories (Helsinki, Finland). AlamarBlue® Cell Viability Reagent and EnzChek® Gelatinase/Collagenase Assay kit were purchased from Invitrogen, Life Technologies Corp. (Madrid, Spain). Human foreskin fibroblasts cell line BJ-5ta (ATCC® CRL-4001$_{TM}$) and Hanks' Balanced Salt Solution (HBSS) were obtained from the American Type Culture Collection/LGC Standards S.L.U (Barcelona, Spain). All other reagents were from Sigma-Aldrich (Madrid, Spain).

Production of a protein-enriched fraction (PEF) from rapeseed press cake

A protein-enriched fraction (PEF) of rapeseed press cake was used as a raw material for the production of protein hydrolysates. For PEF production, the press cake was ground at 1000 min^{-1} using an SM 300 cutting mill (Retsch GmbH, Haan, Germany) and defatted by supercritical carbon dioxide (SC-CO_2) extraction in a Nova Swiss extraction vessel (Nova Werke AG, Illnau-Effretikon, Switzerland) with a Chematur Ecoplanning compressor (Chematur Engineering Ltd., Finland). The vessel was operated at 40°C, 300 bar for ca. 5 h. After defatting, the press cake was dry-milled twice in a 100 UPZ-II fine impact mill (Hosokawa Alpine Ag., Augsburg, Germany) with stainless steel pin discs at 17 800 min^{-1}. The milled material was air-classified in a Minisplit Air Classifier (British Rema Manufacturing Co. Ltd., Chesterfield, Derbyshire, UK) at 15 000 min^{-1}, 220 m^3 h^{-1} inlet air flow and 25 rpm feed rate to separate the fine kernel fragments, for further use as PEF sample, from the coarse hull particles.

Enzymatic hydrolysis of PEF to obtain rapeseed protein hydrolysates (RPH)

Four commercial endopeptidase preparations of microbial origin were used for PEF hydrolysis – Protex 6L (alkaline serine endopeptidase from *B. licheniformis*, dosed at 1634 nKat g^{-1} dry substrate), Protamex (alkaline serine and neutral metallo-endopeptidase from *B. licheniformis/B. amyloliquefaciens*, 2052 nKat g^{-1}), Corolase 7089 (neutral endopeptidase from *B. subtilis*, 2140 nKat g^{-1}) and Alcalase 2.4L FG (alkaline serine/glutamyl endopeptidase from *B. licheniformis*, 720.3 nKat g^{-1}). Their proteolytic activities in nKat g^{-1} of dry substrate were determined at pH 7.5, 30°C using casein as a substrate, and applying identical experimental conditions to allow for an activity comparison among the proteases. Commercial rapeseed protein isolate Isolexx$_{TM}$ was used as a reference material for protein hydrolysis and hydrolysates characterization. The protein concentration of Isolexx$_{TM}$ and PEF was determined as the total nitrogen content according to the Kjeldahl procedure [21]. Briefly, the proteins were digested with sulphuric acid at 420°C using $CuSO_4$ and TiO_2 as catalysts. The obtained NH_3 was distilled and titrated using HCl. Protein concentration was calculated from the total nitrogen content using a conversion factor of 6.25. The PEF and Isolexx$_{TM}$ were treated with proteases in a single- or two-step process as follows: (1) Protex 6L (sample code RPH_Px), (2) Protamex (RPH_Pm), (3) Corolase 7089 (RPH_Co), (4) Alcalase 2.4L FG (RPH_Al), (5) Protex 6L followed by Protamex (RPH_Px-Pm), (6) Protex 6L followed by Corolase 7089 (RPH_Px-Co), and (7) Protex 6L followed by Alcalase 2.4L FG (RPH_Px-Al). Additionally, Isolexx$_{TM}$ and the PEF were subjected to aqueous treatment without enzyme to obtain non-hydrolysed reference protein extracts (Ixx_NH and RP_NH, respectively). The enzyme and reference treatments were performed at the natural pH of the substrate (ca. pH 6) without pH adjustment. Each step was carried out for 2 h at 50°C, 10% (w/v) consistency in water, with 10 mg total enzyme protein per g dry substrate. Before addition of the proteolytic enzymes, the substrate-water suspensions were boiled for 10 min to inactivate endogenous enzymes. The enzymatic hydrolysis was stopped by boiling the suspensions for 10 min. After the final treatments, the suspensions were centrifuged for 15 min at 15 281 *g* and the supernatants were freeze-dried to obtain RPH in the form of fine powders. The samples were kept at −20°C until required for further use.

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

Free amino nitrogen (FAN) content and molecular weight (MW) distribution in RPH

Liberation of peptides and amino acids during protease treatment of rapeseed materials was quantified by determining the free amino nitrogen (FAN) ends in the hydrolysates, according to the method described in Analytica-EBC [22]. The degree of hydrolysis (DH) was calculated as the proportion of FAN out of total raw material nitrogen. MW range of proteins and peptides in Isolexx$_{TM}$, PEF and in the non-hydrolysed extracts and hydrolysates from these substrates was determined by reducing SDS-PAGE. The samples were dissolved in 1 M Tris–HCl buffer pH 6.8 containing 40% glycerol, 2% SDS and 1% mercaptoethanol by heating at 100°C for 5 min, and run on a 16.5% Tris–Tricine Precast peptide gel Bio-Rad Laboratories (Helsinki, Finland) at 125 V for 95 min in Tris-Tricine buffer pH 8.3. The MW of the protein and peptide bands was determined based on the migration of 1400–26 600 Da polypeptide standards Bio-Rad Laboratories (Helsinki, Finland).

Chemical composition of the RPH

The chemical composition of the RPH was analysed in terms of protein, sugar and phenolic content. To analyse protein content, the Lowry method was performed using DC$_{TM}$ Protein Assay Kit. Briefly, the dried RPH were dissolved in water to 40 mg mL^{-1}, and alkaline copper tartrate solution and Folin reagent were added. Colour development upon 20-min incubation was determined by measuring absorbance at 750 nm. BSA 0.2–1.5 mg mL^{-1} (from Sigma) was used to plot a standard curve from which the protein concentration of the hydrolysates was determined.

The total sinapic and ferulic acid content of the RPH was determined by liquid chromatography according to the method by Vuorela *et al.* [23]. The samples were hydrolysed with 2 M NaOH to de-esterify bound phenolics, and phenolic acids were extracted with 0.6 M ethyl acetate. The extracts were analysed by ultra-performance liquid chromatography with diode array detection (UPLC-DAD) using an Acquity$_{TM}$ Ultra Performance LC unit equipped with an Acquity UPLC BEH C18 column (Waters Corporation, Milford, MA, USA). Sinapic and ferulic acid concentrations were calculated on the basis of their respective standard curves (0.1–1.0 mg mL^{-1}).

Neutral sugar (glucose, fructose, rhamnose, galactose, arabinose, mannose, xylose and sucrose) concentrations in the RPH were determined by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) using a ICS-3000 ion chromatography system equipped with a CarboPac PA1 column (Dionex, Sunnyvale, CA) based on their respective standard curves [24].

Antioxidant activity of RPH *in vitro*

DPPH radical scavenging activity assay

DPPH radical scavenging activity of RPH was determined using a modified method described by Badakhshan *et al.* [25]. Briefly, 50 μL of RPH water solution (0.5 mg mL^{-1}) and 5 mL of 0.004% (w/v) DPPH solution in methanol were incubated in dark during 30 min at room temperature. The absorbance values were then measured at 517 nm using a microplate reader TECAN infinite M200 (Tecan Austria GmbH, Grödig, Austria). Methanol was used as a blank and ascorbic acid (0.5 mg mL^{-1}) and sinapic acid were used as a positive control.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to the method described by Alashi *et al.* [26] with modifications. Briefly, RPH were dissolved in 75 mM sodium phosphate buffer pH 7.4. The samples (final peptide concentration 0.5 mg mL^{-1}) were then mixed with 300 nM fluorescein in a 96-well tissue culture-treated polystyrene black plate (Nunc) followed by incubation of the mixture in the dark at 37°C for 15 min. Thereafter, 50 μL (80 mM) 2, 2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added to the mixture and the change in fluorescence due to AAPH-induced oxidation of fluorescein was measured at 1-min intervals during 90 min at 485–535 nm excitation and emission wavelengths, respectively, using a fluorescence microplate reader.

Cell culture

To determine the biocompatibility of RPH with skin, BJ-5ta cells were used. The cells were maintained in four parts Dulbecco's Modified Eagle's Medium (DMEM) containing 4 mM L-glutamine, 4500 mg L^{-1} glucose, 1500 mg L^{-1} sodium bicarbonate, 1 mM sodium pyruvate and 1 part of Media 199, supplemented with 10% (v/v) of foetal bovine serum (FBS), and 10 g mL^{-1} hygromycin B at 37°C, in a humidified atmosphere with 5% CO_2, according to the recommendations of the manufacturer. The culture media was replaced every 2 days. At pre-confluence, cells were harvested using trypsin-EDTA (ATCC-30-2101, 0.25% (w/v) trypsin/0.53 mM EDTA solution in HBSS without calcium or magnesium).

Cellular oxidative stress

Cellular antioxidant activity (CAA) was determined as described by Wolfe *et al.* [27]. Growth media per well, containing BJ-5ta cells, was seeded at a density of 1×10^5 per well on a 96-well tissue culture-treated polystyrene black plate clear flat bottom (Sigma-Aldrich, Madrid, Spain). The growth media was removed 24 h after seeding and the wells were washed with 100 μL of PBS. The cells were then treated for 1 h with 100 μL of 1% FBS media containing RPH (2 mg mL^{-1}) plus 25 μM DCFH-DA. After incubation, the wells were washed again with 100 μL of PBS followed by the application of 100 μL of HBSS containing 600 μM AAPH and fluorescence was immediately measured in a microplate reader at 37°C. Emission at 538 nm was measured with excitation at 485 nm every 5 min during 1 h. Negative control wells contained cells treated with DCFH-DA and AAPH. Ascorbic acid (1 mg mL^{-1}) was used as positive control.

Anti-inflammatory activity of RPH

The effect of RPH on MPO activity was detected spectrophotometrically using guaiacol as a substrate. The samples were previously diluted in 50 mM PBS pH 6.6 at 0.2 mg mL^{-1} and were further incubated for 1 h at 37°C, with 0.24 units MPO and 10 mM guaiacol to a final volume of 180 μL. Thereafter, the reaction was initiated by adding 1 mM H_2O_2 (20 μL). The activity was determined by the increase of the absorbance rate per min at 470 nm and expressed as a percentage of MPO inhibition.

Anti-ageing activity of RPH *in vitro*

The inhibitory effect of biopeptides on the HNE was determined according to the method described by Vasconcelos *et al.* [28] with modifications. Briefly, 15 μL of HNE (20 μg mL^{-1}) was incubated with 15 μL of RPH (20–80 μM) for 30 min at 25°C. The final

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

volume of each sample was adjusted to 300 μL with 0.1 M HEPES, 0.5 M NaCl pH 7.5 assay buffer. 5 mM of N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide solution was prepared in DMSO. The reaction was initiated with the addition of the substrate previously diluted in the assay buffer (200 μM). Negative control was performed in the same conditions without inhibitor, and 100 μM phenylmethylsulfonyl fluoride (PMSF) was used as a positive control. The residual activity was determined by the increase of the optical density at 410 nm over 10 min using a microplate reader.

Biocompatibility of RPH

Cell viability was monitored using AlamarBlue® (Resazurin as an active compound) assay. Briefly, cells in growth media were seeded at a density of 1×10^5 cells per well into a 96-well tissue culture-treated polystyrene plate (Nunc). 24 h after seeding, cells were exposed to 150 μL of growth media containing RPH (0.2 and 2 mg mL^{-1}) and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 24 h of contact with cells, the RPH were removed and the cells washed twice with PBS. Subsequently, 100 μL of 10% (v/v) AlamarBlue® in grow media was added to each well as a reagent for detecting cell viability. The absorbance at 570 nm was measured after 4 h of incubation at 37°C using 600 nm as a reference wavelength, in a microplate reader. Wells with media were used as the blank, wells with 500 μM H_2O_2 were used as a positive control of cell death, and those seeded with BJ-5ta in media were the negative control. All tests were performed in duplicate. BJ-5ta cells relative viability percentage was determined for each concentration of RPH based on the proportionality of resorufin formation and the number of viable cells.

Statistical analysis

The experiments were performed in triplicate (MPO, DPPH, ORAC and CAA) or duplicate (HNE and biocompatibility), and results are presented as means with error bars representing the SD. The data were analysed using the statistical software graph pad prism version 5.04 for windows (Graph Pad Software, San Diego, CA). Differences between treatments were analysed with one-way analysis of variance (ANOVA). Significant differences among means were determined by Dunnett's *post hoc* test with a significance level of $P < 0.05$.

Results and discussion

RPH generation by enzymatic hydrolysis of crude intermediate PEF

The commercial proteases Protex 6L, Protamex, Alcalase 2.4 FG and Corolase 7089 were used to hydrolyse rapeseed PEF and a commercial rapeseed protein isolate (Ixx_{TM}) containing ~46% and ~90% protein, respectively. The latter was used as a reference material to compare the efficacy of the enzymes for RPH production from a rapeseed protein isolate and a crude rapeseed PEF. Endopeptidases were selected due to their capability to cleave peptide bonds in the middle parts of the polypeptide chains, generating small and medium-size peptides, the expected size range for optimal bioactivity [29]. The hydrolysates were analysed for free amino nitrogen content to monitor the efficiency of the enzymatic hydrolysis, and using a SDS-PAGE gel electrophoresis to monitor the breakdown of the proteins.

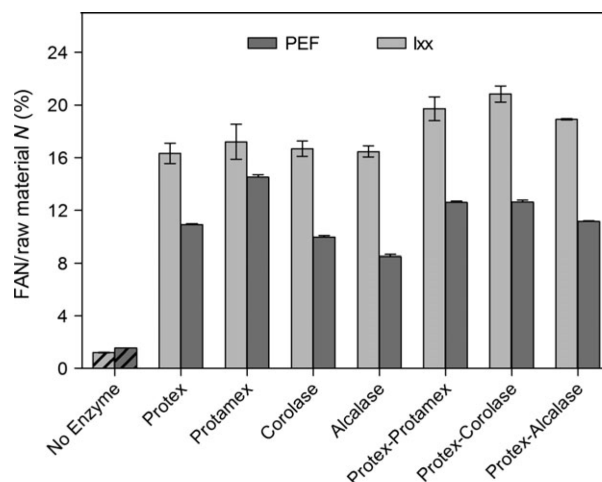

Figure 1 Degree of enzymatic hydrolysis in the hydrolysates of commercial rapeseed protein isolate $Isolexx_{TM}$ (Ixx_{TM}) and crude rapeseed protein-enriched fraction (PEF) determined as the proportion of FAN out of total raw material nitrogen (N) in the hydrolysates. Error bars represent standard deviations of three independent treatments ($N = 3$).

The increase of the FAN levels in the hydrolysates (Fig. 1), in comparison with the non-hydrolysed extracts, indicated the liberation of peptides or amino acids during the protease treatments. The hydrolysis was more pronounced on the commercial substrate $Isolexx_{TM}$ due to its higher protein content. Two-stage enzyme treatments (Px-Pm, Px-Co and Px-Al) hydrolysed both substrates to a larger extent than single-enzyme treatments, releasing more FAN in the form of peptides and most probably free amino acids.

Protex 6L and Protamex applied individually were also efficient on both substrates. This might be explained by their specificity towards hydrophobic amino acids which are the predominant residues in the major rapeseed proteins: cruciferins, napins and lipid transfer proteins [30,31].

Alcalase 2.4L FG and Corolase 7089 caused low DH (9.96% and 8.48%, respectively) towards PEF in comparison with other enzymes. Although majority of rapeseed protein bioactivities have been reported for small molecular weight peptides and hydrolysates with relatively high DH [29,32,33], a low DH may be favourable for certain functional properties. Partial DH (3–10%) has been found to result in improved angiotensin converting enzyme-inhibitory activity and functional properties of the resulting hydrolysates in comparison with non-hydrolysed protein [34]. In addition to the choice of enzyme, the selected hydrolysis conditions are expected to have influenced the DH. In this study, all protease treatments were carried out in water without pH adjustment to avoid introduction of ions into the hydrolysates. Modification of the hydrolysis conditions towards the pH optimum of each enzyme preparation would most probably increase the hydrolysis efficiency, especially in case of the alkaline enzyme preparations Alcalase 2.4L FG, Protamex and Protex 6L.

The proteolysis generated small molecular weight peptides from both $Isolexx_{TM}$ and PEF as confirmed by SDS-PAGE (Fig. 2). As a result of protease treatment, most of the bands with MW above 26.6 kDa and between 26.6 and 6.5 kDa disappeared and a large and clear band in the region below 1.4 kDa appeared, confirming

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

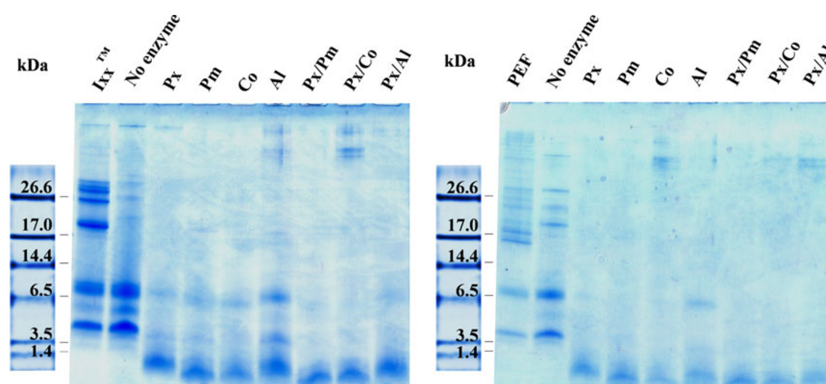

Figure 2 SDS-PAGE in reducing conditions of Isolexx$_{TM}$ (Ixx$_{TM}$) (left side) and PEF (right side) and of extracts obtained from these materials under different enzymatic treatments: No enzyme: non-hydrolysed extract, Px: Protex 6L, Pm: Protamex, Co: Corolase 7089, Al: Alcalase 2.4L FG, Px-Pm: Protex 6L followed by Protamex Px-Co: Protex 6L followed by Corolase 7089 and Px-Al: Protex 6L followed by Alcalase 2.4L FG. Proteins were dissolved in SDS buffer, and disulphide bonds in proteins were reduced with 1% mercaptoethanol.

that all enzymes and combinations thereof were able to hydrolyse the original fractions (Ixx$_{TM}$ and PEF) to low-MW peptides.

The overall protein composition of Isolexx$_{TM}$ and PEF was determined by SDS-PAGE after dissolution of the proteins in SDS-PAGE sample buffer (2% SDS and 1% mercaptoethanol). Some of the bands with MW above 14.4 kDa present in the raw materials ('Ixx$_{TM}$' and 'PEF' columns in Fig. 2) were not present in the non-hydrolysed extracts ('No enzyme' columns in Fig. 2), suggesting that the corresponding proteins (cruciferins) remained partially insoluble without enzymatic treatment and were thus not recovered in the non-hydrolysed extracts.

The gel profile of the extract obtained from hydrolysis of PEF with Corolase 7089 showed attenuated bands with MW above 26.6 kDa, which were not found in the original fractions (PEF and Ixx$_{TM}$ column) or in the respective non-hydrolysed extracts ('No enzyme' column). Similar results were observed after hydrolysis of Isolexx$_{TM}$ with Alcalase. This behaviour suggests the breakdown of high MW proteins (e.g. cruciferins), not observed in the reference gel profile due to range of the used standard marker, into proteins around 26.6 kDa.

The bands around 6.5 kDa in PEF, Isolexx$_{TM}$ and the Alcalase-obtained hydrolysates, corresponding to napin subunits [30], were attenuated or not present in the hydrolysates obtained after other enzyme treatments. This suggests that albumins remained partly undigested after Alcalase treatment. In comparison with the other enzymes, Alcalase 2.4L FG may have possessed a weaker catalytic efficiency towards albumins which are stabilized by their rigid structure with four disulphide bonds and have been reported to be more resistant to hydrolysis by Alcalase than rapeseed cruciferins [35].

Table I Chemical composition of Isolexx$_{TM}$ and PEF-derived RPH in terms of protein, neutral sugars and sinapic acid content after enzymatic treatment

Hydrolysate	Protein (% d.m.)	Neutral Sugars (% d.m.)	Sinapic Acid (% d.m.)
Ixx_NH	68.8	2.1	Na
RP_NH	38.1	31.2	3.5
RPH_Px	68.5	23.1	2.0
RPH_Pm	59.1	21.7	1.6
RPH_Co	57.3	24.1	1.6
RPH_Al	63.2	25.5	2.2
RPH_Px-Pm	54.8	17.8	1.4
RPH_Px-Co	59.2	21.9	1.5
RPH_Px-Al	65.8	16.3	1.7

Ixx_NH, Non-hydrolysed extract from Isolexx$_{TM}$; RP_NH, Non-hydrolysed extract from protein-enriched fraction; RPH, Rapeseed protein hydrolysates from protein-enriched fraction; Px, Protex 6L; Pm, Protamex; Co, Corolase 7089; Al, Alcalase 2.4L FG; Na, not analysed.
Results are expressed as dry matter percentage (% d.m.)

Chemical composition of the RPH

The chemical properties of Isolexx$_{TM}$ and PEF-derived RPH samples were assessed in terms of protein, neutral sugars and sinapic acid content (Table I). The non-hydrolysed extract (NH) and hydrolysates from Isolexx$_{TM}$ contained generally more protein per dry matter than the respective extracts obtained from PEF.

Due to the presence of carbohydrates, lignin, lipids, ash and phenolic compounds in rapeseed press cake [36], the PEF-derived extracts were expected to contain a larger share of cosolubilized non-protein components than Isolexx$_{TM}$-derived extracts. This was accordingly shown as the higher content of neutral sugars in the PEF-derived extracts.

As a result of enzymatic hydrolysis, the obtained RPH were enriched in protein content while the content of neutral sugars and sinapic acid was significantly reduced (Table I). The improved protein recovery is considered a common feature of the enzymatic hydrolysis, resulting from the higher solubility of peptides in comparison with intact rapeseed protein.

Effect of RPH on the antioxidant activity in vitro

As the largest organ in the body, skin provides a barrier against UV radiation, chemicals, microbes and physical pollutants [16]. Nevertheless, with advancing age, the cellular antioxidant potential as well as the absorption of nutrients, including scavengers of harmful free radicals, gradually diminishes [37]. The incorporation

 © 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

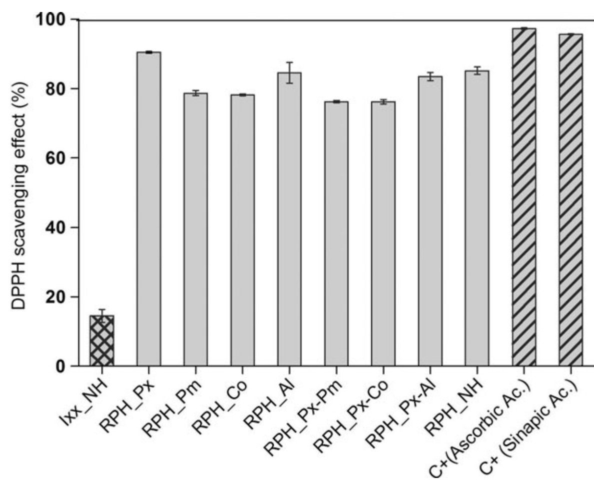

Figure 3 DPPH radical scavenging effect of RPH and non-hydrolysed extracts from Isolexx$_{TM}$ (Ixx_NH) and PEF (RP_NH). Error bars represent standard deviations of three independent treatments (N = 3).

of antioxidants into skin-conditioning products enhances the ability of other antioxidants within the skin to protect skin tissues, hinders the UV-induced immunosuppression, and could be used for the treatment and prevention of oxidative stress-mediated alterations [38]. The obtained RPH were studied for their antioxidant capacity using three different methods: (i) DPPH radical scavenging activity assay using the non-biological radical DPPH was performed as a preliminary test, (ii) ORAC assay, based on a hydrogen atom transfer (HAT) reaction mechanism, was carried out as it better mimics human biology than DPPH, and finally (iii) a cell-based antioxidant activity that better represents the complexity of biological systems Figs. 3–5.

The antioxidant efficacy of RPH obtained from the CAA and ORAC assays was in good agreement with that measured in the DPPH radical scavenging activity assay. In general, all RPH showed remarkably higher radical scavenging ability when compared with the non-hydrolysed reference extract from Isolexx$_{TM}$ (Ixx_NH).

Interestingly, the non-hydrolysed extract from PEF showed a similar level of DPPH activity as the hydrolysates. Knowing that this sample contained negligible amounts of low-Mw peptides (Fig. 2) and had a high content (3.5% d.m.) of sinapic acid (Table I) in comparison with the hydrolysates, the presence of phenolic compounds, is suggested to play an important role in its antioxidant activity. Phenolics have been extensively reported to have good antioxidant properties neutralizing free radicals by acting as donors of a hydrogen atom to radicals [39], and sinapic acid, the main phenolic compound in rapeseed press cake, is a well-known antioxidant [40]. Hydrolysates from RPH_Px and RPH_Al treatments showed the highest antioxidant activities in CAA assay (~80%) at a concentration of 2 mg mL^{-1}, while the same samples reached 50–60% antioxidant activity in the ORAC assay at 0.5 mg mL^{-1} (Fig. 5). These results correlate well with a number of studies reporting potent antioxidant activities of Alcalase-derived hydrolysates when compared to other enzyme samples. The hydrolysates obtained using a combination of these two alkaline serine endopeptidases (Protex and Alcalase) also contain a higher sinapic acid content (Table I) which might have enhanced the overall antioxidant activity of the hydrolysates [9].

On the other hand, the two-stage protease treatments showed lower radical scavenging activity. This was probably due to the extensive proteolysis, as observed in Fig. 1, which might have resulted in a decrease of the amount of active peptides and an increase of free amino acids, which are considered ineffective as antioxidants [41].

By analysing the antioxidant properties of the obtained hydrolysates, it could be concluded that the presence of phenolic acids, found in both hydrolysed and non-hydrolysed PEF samples, boosts the antioxidant properties of the proteins/peptides. Especially in the non-hydrolysed extracts, antioxidant activity can be assigned to the higher content of phenolic compounds. After proteolysis, the antioxidant activity is believed to be mainly due to the presence of low-MW peptides that act synergistically with sinapic acid for antioxidant activity as observed for the RPH_Px and RPH_Al hydrolysates. The differences in the antioxidant properties of the other hydrolysates are suggested to be affected mainly by the specificity of the enzyme preparation used and the extent of hydrolysis, influencing the nature and the composition of the obtained peptides and consequently their functional properties.

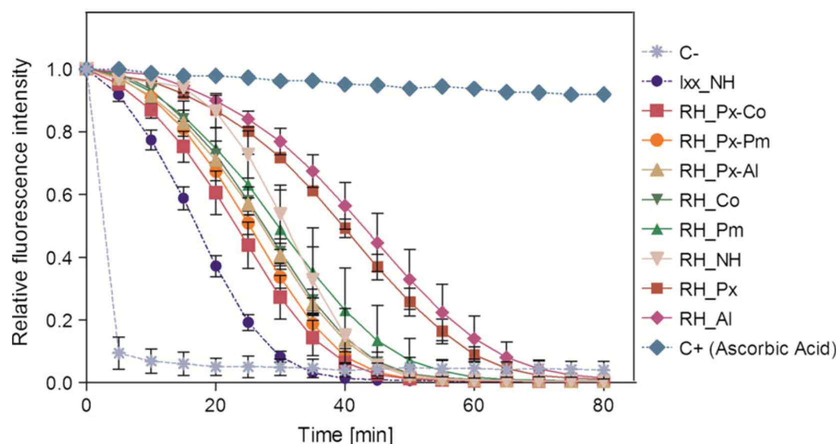

Figure 4 Oxygen radical absorbance capacity (ORAC) of RPH and non-hydrolysed extracts from Isolexx$_{TM}$ (Ixx_NH) and PEF (RP_NH) at 0.5 mg mL^{-1}. Error bars represent standard deviations of three independent treatments (N = 3).

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

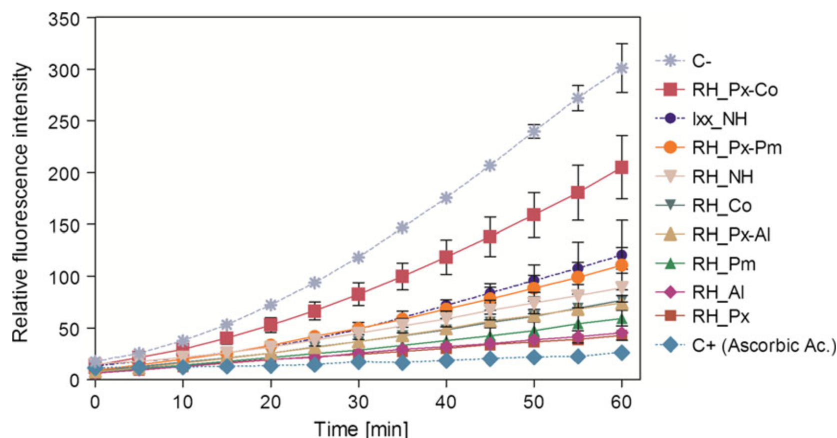

Figure 5 Cellular antioxidant activity of RPH at a concentration of 2 mg mL^{-1} assessed in skin fibroblasts (BJ-5ta) cells. Peroxyl radical-induced oxidation of DCFH to DCF (% of dichlorofluorescein released) in skin fibroblasts cells, without antioxidant (C-), upon contact with RPH, 1 mg mL^{-1} ascorbic acid and non-hydrolysed sample. Error bars represent standard deviations of three independent treatments (N = 3).

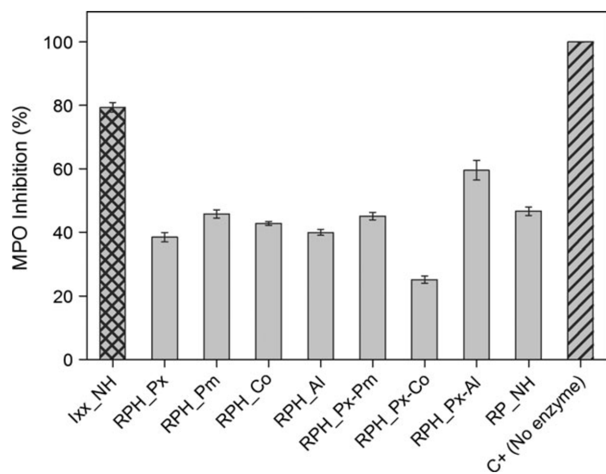

Figure 6 Inhibition of MPO activity by RPH at 0.2 mg mL^{-1}. Error bars represent standard deviations of three independent treatments (N = 3).

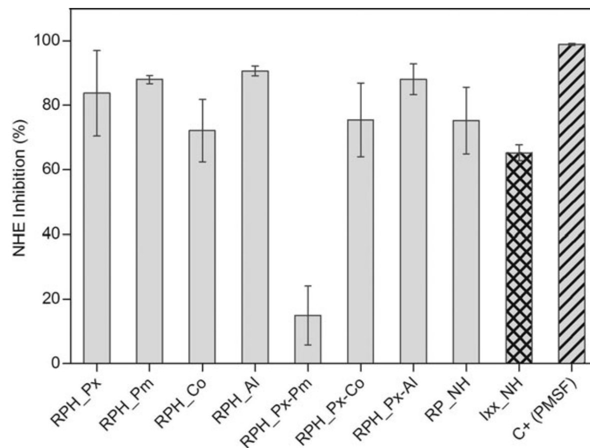

Figure 7 Inhibition of human neutrophil elastase by the RPH (0.5 mg mL^{-1}). Error bars represent standard deviations of two independent treatments (N = 2).

Anti-inflammatory activity – MPO activity inhibition

Skin damage is a cosmetic issue but also a medical problem. Severely damaged skin is prone to bruising and chronic inflammation, which together with UV exposure and other environmental factors may trigger an imbalance between ROS and endogenous antioxidant systems, stimulation the production of neutrophils and thus increasing the activity of myeloperoxidase (MPO). This enzyme is involved in a wide range of body-regulating activities, including infection protection, but when overexpressed increases the susceptibility of skin to inflammation [42]. Previous studies have suggested that the presence of MPO in inflamed skin was much higher compared with normal skin, providing evidence for its role in the inflammatory process [13]. Moreover, the MPO activity is commonly used as a measure of total infiltrating neutrophil content found in inflamed UV-irradiated skin [43,44]. Therefore, a moderated MPO inhibition is desired to provide inflammation control, without inducing unbalanced skin functions.

In general, all RPH inhibited moderately the MPO activity (Fig. 6), showing potential in counteracting skin inflammation. The RPH_Px and RPH_Al hydrolysates inhibited MPO by 36%, which indicates that apart from being efficient antioxidants they could act as anti-inflammatory agents as well. Surprisingly, the RP_NH inhibited around 80% of the MPO activity, suggesting that the phenolic compounds present in high concentration in this non-hydrolysed sample may act as competitive substrates for MPO.

The propensity of plant polyphenolic extracts to bind proteins presumably accounts for the fact that polyphenols inhibit virtually every enzyme tested *in vitro* [45]. However, such strong inhibition is not desired due to the biological functions of MPO, and thus moderate inhibition by peptides is considered more favourable. Out

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

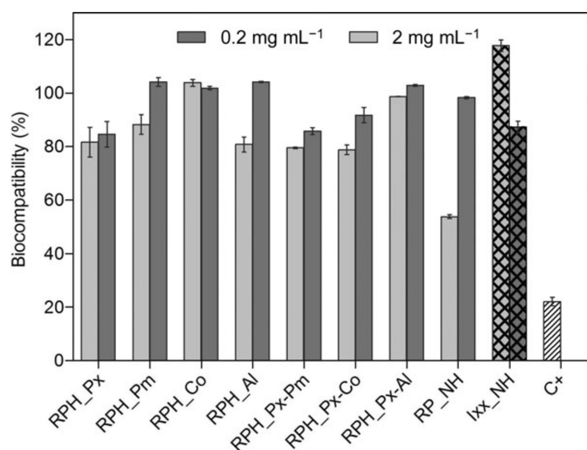

Figure 8 Relative cell viability in the presence of 0.2 and 2 mg mL^{-1} of RPH after 24-h incubation. Error bars represent standard deviations of two independent treatments (N = 2).

of the hydrolysates, the RPH_Px-Al-treated sample showed strongest activity inhibiting the MPO enzyme up to 65%, a level which could be considered a balanced MPO inhibition for treatment of skin irritation.

Effect of RPH on human neutrophil elastase (HNE) activity in vitro

The process of skin ageing induces changes in the elasticity and thickness of the skin over time due to the degradation of collagen and elastin components of the extracellular matrix (ECM). These changes result in visible alterations such as wrinkles, pigmentation and skin thickness [39]. In normal conditions, the presence of proteolytic enzymes such as elastase and collagenase on skin is necessary to maintain skin balance [37]. Nevertheless, UV exposure and other environmental conditions can over-induce their activity causing irreversible skin damage and premature ageing [13]. In terms of anti-ageing, finding inhibitors of these proteolytic enzymes could prevent the loss of skin elasticity and thus skin ageing. Therefore, the ability of natural sources such as RPH to act as anti-ageing and skin repairing agents by blocking their activity and altering or inhibiting their metabolic pathways was evaluated. RPH_Al, RPH_Pm and RPH_Px hydrolysates inhibited the elastase activity by 88%, 87% and 83%, respectively (Fig. 7). Nevertheless, good inhibitory activities were also found for the other enzyme-obtained hydrolysates, including the non-hydrolysed sample (~65%). The low-MW peptides generated by the hydrolysis may act as competitive substrates for elastase, deviating its activity from the elastin substrate.

During the last decades, an intense effort has been directed towards the development of inhibitors to supplement the body's elastase inhibitory capacity. The most pursued approach was the development of low MW recombinant endogenous or synthetic elastase inhibitors that act as competitive substrates [46,47]. Recently, a large number of natural compounds, comprising phenolics, terpenoids, fatty acids and carbohydrates have also been reported as elastase inhibitors [48]. Therefore, the higher inhibitory activity found for the reference treatment might be related to the higher amount of phenolic acids compared to the hydrolysates. On the other hand, RPH_Px-Pm hydrolysate induced only ~15% elastase inhibition, possibly due to its high DH (Fig. 1) which may have resulted in generation of free amino acids unable to act as competitive substrates.

Biocompatibility of RPH with human foreskin fibroblasts

Aiming at cosmetic applications, the biocompatibility of RPH is an essential parameter to be assessed. It is important to ensure that they do not cause adverse effects upon interaction with human cells. The biocompatibility of the obtained peptides was assessed in human skin fibroblasts. Two different RPH concentrations (0.2 and 2 mg mL^{-1}) were used in the biological activity assays performed after 24 h contact with the cells (Fig. 8).

The results confirmed that both the lowest and highest sample concentrations used in the assay did not damage the cells except for the non-hydrolysed sample RP_NH. This one induced toxicity to the cells at 2 mg mL^{-1} after 24 h contact (53%), probably due to the relatively high concentration of sinapic acid (3.5% d.m.) or the expected presence of other phenolic compounds deriving from PEF. In all other cases, the cell viability was above 80% indicating that at these concentrations the RPH are safe for skin-conditioning purposes.

Conclusions

In the present work, bioactive peptide-rich extracts suitable for skincare applications were obtained from a coproduct of rapeseed oil processing industry using enzyme technology. Microbial endoproteases with different substrate specificity, that is Alcalase 2.4L FG, Protex 6L, Protamex and Corolase 7089 were applied individually or in combination to hydrolyse proteins in the rapeseed press cake. All enzymes liberated peptides from the rapeseed press cake.-The DH was generally higher when a combination of two enzymes, namely Protex 6L followed by another enzyme, was used. Such extensive hydrolysis, however, resulted in less bioactive extracts when compared with the hydrolysates obtained using a single enzyme, most probably due to the generation of non-active free amino acids rather than peptides. One-step single-enzyme hydrolysis was thus found to generate peptides with better antioxidant, anti-wrinkle and anti-inflammatory properties suitable for skin anti-ageing. The dry fractionation (i.e. SC-CO_2 extraction, milling and air classification) and hydrolysis process eliminated any potential toxic compounds encountered in rapeseed press cake, as confirmed by the high biocompatibility of the hydrolysates with skin fibroblasts. Enrichment of protein content and reduction of the amount of phenolic acids and neutral sugars were observed after the proteolytic treatment.

Hydrolysates obtained with either Alcalase 2.4L FG or Protex 6L showed potential as antioxidants scavenging the cellular free radicals. This was most probably related to the substrate specificity of these alkaline serine endopeptidases, promoting the liberation of antioxidant peptides. The peptides may in addition act synergistically with the antioxidant phenolic acids present at higher concentration in these extracts than in the other hydrolysates. Alcalase 2.4L FG and Protex 6L hydrolysates also showed anti-inflammatory and anti-wrinkle properties by inhibiting myeloperoxidase and elastase activity, most probably acting as competitive substrates. Ruled by the same mechanism, the non-hydrolysed extracts showed good anti-inflammatory and anti-wrinkle properties as well. However, toxicity of the non-hydrolysed extracts from the protein-enriched fraction of rapeseed press cake (PEF) towards human cells makes it unsuitable for skin care applications. This study presents the rare example of an

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

underutilized natural product, obtained from a rapeseed oil pressing coproduct, and subsequently enzymatically transformed into suitable bioactive ingredients that have shown interesting activities and can be used in topical skin care applications for improving its properties.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement No. 289170 – APROPOS.

References

1. Fahs, A. and Louarn, G. Plant protein interactions studied using AFM force spectroscopy: nanomechanical and adhesion properties. *Phys. Chem. Chem. Phys.* **15**, 11339–11348 (2013).
2. Ferchau, E. Equipment for decentralised cold pressing of oil seeds. Folkecenter for Renewable Energy. 1–64 (2000).
3. Avramidou, P., Evangelou, A. and Komilis, D. Use of municipal solid waste compost as a growth media for an energy plant (rapeseed). *J. Environ. Manage.* **121**, 152–159 (2013).
4. He, R., Ju, X., Yuan, J., Wang, L., Girgih, A.T. and Aluko, R.E. Antioxidant activities of rapeseed peptides produced by solid state fermentation. *Food Res. Int.* **49**, 432–438 (2012).
5. Nyambura, M.B. Valorisation of fish waste biomass through recovery of nutritional lipids and biogas. 137 (2011).
6. Yoshie-Stark, Y., Wada, Y., Schott, M. and Wäsche, A. Functional and bioactive properties of rapeseed protein concentrates and sensory analysis of food application with rapeseed protein concentrates. *LWT - Food Sci. Technol.* **39**, 503–512 (2006).
7. Chakrabarti, S., Forough, J. and Wu, J. Food-derived bioactive peptides on inflammation and oxidative stress. *Biomed Res. Int.* **2014**, 1–11 (2014).
8. Pedroche, J., Yust, M.M., Megías, C. *et al.* Utilisation of rapeseed protein isolates for production of peptides with angiotensin I-converting enzyme (ACE)-inhibitory activity. *Grasas Aceites* **55**, 354–358 (2004).
9. Power, O., Jakeman, P. and FitzGerald, R.J. Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids* **44**, 797–820 (2013).
10. Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J. and Millán, F. Partially hydrolyzed rapeseed protein isolates with improved functional properties. *J. Am. Oil Chem. Soc.* **77**, 447–450 (2000).
11. McCarthy, A., O'Callaghan, Y. and O'Brien, N. Protein hydrolysates from agricultural crops—bioactivity and potential for functional food development. *Agriculture* **3**, 112–130 (2013).
12. Kim, S.M. and Na, M.S. A study on skin care effects of rapeseed meal extract. *Korean Soc. Biotechnol. Bioeng.* **28**, 177–184 (2013).
13. Kim, K.H., Park, S.J., Lee, Y.J. *et al.* Inhibition of UVB-induced skin damage by exopolymers from *Aureobasidium pullulans* SM-2001 in hairless mice. *Basic Clin. Pharmacol. Toxicol.* **116**, 73–86 (2014).
14. Aslam, M.N., Lansky, E.P. and Varani, J. Pomegranate as a cosmeceutical source: pomegranate fractions promote proliferation and procollagen synthesis and inhibit matrix metalloproteinase-1 production in human skin cells. *J. Ethnopharmacol.* **103**, 311–318 (2006).
15. Ruiz, M.A., Clares, B., Morales, M.E. and Gallardo, V. Evaluation of the anti-wrinkle efficacy of cosmetic formulations with an anti-aging peptide (Argireline®). *ARS Pharm.* **50**, 168–176 (2010).
16. Thring, T.S., Hili, P. and Naughton, D.P. Antioxidant and potential anti-inflammatory activity of extracts and formulations of white tea, rose, and witch hazel on primary human dermal fibroblast cells. *J. Inflamm.* **8**, 1–7 (2011).
17. Ramesh, T., Yoo, S.K., Kim, S.W., Hwang, S.Y., Sohn, S.H. and Kim, I.W. Cordycepin (3′-deoxyadenosine) attenuates age-related oxidative stress and ameliorates antioxidant capacity in rats. *Exp. Gerontol.* **47**, 979–987 (2012).
18. Fuchs, J., Zollner, T., Kaufmann, R. and Podda, M. Redox-modulated pathways in inflammatory skin diseases. *Free Radic. Biol. Med.* **30**, 337–353 (2001).
19. Korhonen, H. and Pihlanto, A. Food-derived bioactive peptides – opportunities for designing future foods. *Curr. Pharm. Des.* **9**, 1297–1308 (2003).
20. Samaranayaka, A.G.P. and Li-Chan, E.C.Y. Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *J. Funct. Foods* **3**, 229–254 (2011).
21. Kane, P.F. CuSO4-TiO2 as Kjeldahl digestion catalyst in manual determination of crude protein in animal feeds. *J. Assoc. Off. Anal. Chem.* **69**, 664–666 (1986).
22. Analytica-EBC Free Amino Nitrogen of Malt by Spectrophotometry. *Analytica-EBC* (1997).
23. Vuorela, S., Meyer, A.S. and Heinonen, M. Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. *Eur. Food Res. Technol.* **217**, 517–523 (2003).
24. Hausalo, T. Analysis of wood and pulp carbohydrates by anion exchange chromatography with pulsed amperometric detection. In: Proceedings of the 8th International Symposium on Wood and Pulping Chemistry, June 6–9, Helsinki, Finland. 131–136 (1995).
25. Mahdi-Pour, B., Jothy, S.L., Latha, L.Y., Chen, Y. and Sasidharan, S. Antioxidant activity of methanol extracts of different parts of Lantana camara. *Asian Pac. J. Trop. Biomed.* **2**, 960–965 (2012).
26. Alashi, A.M., Blanchard, C.L., Mailer, R.J. *et al.* Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chem.* **146**, 500–506 (2014).
27. Wolfe, K.L. and Liu, R.H. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **55**, 8896–8907 (2007).
28. Vasconcelos, A., Azoia, N.G., Carvalho, A.C., Gomes, A.C., Güebitz, G. and Cavaco-Paulo, A. Tailoring elastase inhibition with synthetic peptides. *Eur. J. Pharmacol.* **666**, 53–60 (2011).
29. Cumby, N., Zhong, Y., Naczk, M. and Shahidi, F. Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chem.* **109**, 144–148 (2008).
30. Bérot, S., Compoint, J.P., Larré, C., Malabat, C. and Guéguen, J. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J. Chromatogr. B* **818**, 35–42 (2005).
31. Chabanon, G., Alves da Costa, L., Farges, B. *et al.* Influence of the rapeseed protein hydrolysis process on CHO cell growth. *Bioresour. Technol.* **99**, 7143–7151 (2008).
32. He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *J. Funct. Foods* **5**, 219–227 (2013).
33. Wu, J., Aluko, R.E. and Muir, A.D. Purification of angiotensin I-converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. *Food Chem.* **111**, 942–950 (2008).

 © 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

34. Yoshie-Stark, Y., Wada, Y. and Wäsche, A. Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food Chem.* **107**, 32–39 (2008).
35. Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S. and Marc, I. Hydrolysis of rapeseed protein isolates: kinetics, characterization and functional properties of hydrolysates. *Process Biochem.* **42**, 1419–1428 (2007).
36. Rommi, K., Hakala, T.K., Holopainen, U., Nordlund, E., Poutanen, K. and Lantto, R. Effect of enzyme-aided cell wall disintegration on protein extractability from intact and dehulled rapeseed (*Brassica rapa* L. and *Brassica napus* L.) press cakes. *J. Agric. Food Chem.* **62**, 7989–7997 (2014).
37. Giacomoni, P.U., Declercq, L., Hellemans, L. and Maes, D. Aging of human skin: review of a mechanistic model and first experimental data. *IUBMB Life* **49**, 259–263 (2000).
38. Schueller, R. and Romanowski, P. Substantiating cosmetic product claims. *Cosmet. Toilet.* **113**, 73–78 (1998).
39. Alhakmani, F., Kumar, S. and Khan, S.A. Estimation of total phenolic content, in-vitro antioxidant and anti-inflammatory activity of flowers of *Moringa oleifera*. *Asian Pac. J. Trop. Biomed.* **3**, 623–627 (2013).
40. Jalaludeen, A.M. and Pari, L. Studies on the antioxidant and free radical-scavenging effect of sinapic acid: an in vivo and in vitro model. *J. Pharm. Sci. Res.* **3**, 1447–1455 (2011).
41. Gallegos Tintoré, S., Guerrero, L.C., Corzo Ríos, L.J. and Martínez Ayala, A.L. Péptidos con actividad antioxidante de proteínas Vegetales. In: *Bioactividad de péptidos derivados de proteínas alimentarias*, Vol. 1 (Segura-Campos, M., Guerrero, L.C. and Betancur Ancona, D., eds), pp. 111–122. OmniaScience, Mexico (2013).
42. Díaz-González, M., Francesko, A., Fernandes, M.M. and Tzanov, T. Myeloperoxidase – a paradoxical enzyme – from host defense to disease. In: *Enzymes and Enzyme Activity: Structure, Biology and Clinical Significance*, Vol. 1 (Lashinski, E.M., ed.), pp. 1–38. Nova Science Publishers, ISBN: 978-1-62417-697-5 (2013).
43. Casagrande, R., Georgetti, S.R., Verri, W.A. Jr, Dorta, D.J., dos Santos, A. and Fonseca, M.J. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. *J. Photochem. Photobiol. B Biol.* **84**, 21–27 (2006).
44. Katiyar, S.K. and Mukhtar, H. Green tea polyphenol (-)-epigallocatechin-3-gallate treatment to mouse skin prevents UVB-induced infiltration of leukocytes, depletion of antigen-presenting cells, and oxidative stress. *J. Leukoc. Biol.* **69**, 719–726 (2001).
45. Haslam, E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* **59**, 205–215 (1996).
46. Bernstein, P.R., Edwards, P.D. and Williams, J.C. Inhibitors of human leukocyte elastase. *Prog. Med. Chem.* **31**, 59–120 (1994).
47. Ohbayashi, H. Current synthetic inhibitors of human neutrophil elastase. *Expert Opin. Ther. Pat.* **12**, 65–84 (2002).
48. Siedle, B., Hrenn, A. and Merfort, I. Natural compounds as inhibitors of human neutrophil elastase. *Planta Med.* **73**, 401–420 (2007).

© 2015 Society of Cosmetic Scientists and the Société Française de Cosmétologie

Series title and number

VTT Science 130

Title

Enzyme-aided recovery of protein and protein hydrolyzates from rapeseed cold-press cake

Author(s)

Katariina Rommi

Abstract

New protein sources are needed to fulfil the growing global demand for food protein. The co-stream from cold pressing of rapeseed oil, press cake, is a rich source of protein (32–26%) with good nutritional value. Several technologies based on alkaline or saline extraction have been developed for enrichment of rapeseed protein, but high energy and water consumption due to dilute conditions and multiple processing steps limit their sustainability and profitability.

In the present study, enzyme-aided methods for extraction of protein from rapeseed cold-press cake were developed, and factors influencing protein extractability and properties of the obtained protein-rich fractions were elucidated. Pretreatments and carbohydrate-hydrolyzing enzyme treatment were used to facilitate extraction at reduced water content and without chemicals such as alkali or salt. Particularly a pectinase preparation with -glucanase side activity enabled effective hydrolysis of rapeseed cell wall polysaccharides, increasing protein recovery by aqueous extraction. Similar improvement of protein extractability was, however, not achieved by mechanical cell wall disruption, suggesting that protein release was not to a major extent physically hindered by the residual cell structures in the press cake. Instead, enzyme treatment reduced the water holding capacity of the press cake and released hydrolysis products which are suggested to have enhanced the solubility of rapeseed protein during water extraction.

At 10% solid content, enzyme-aided water extraction produced protein-enriched fractions with a similar yield and estimated production costs as conventional alkaline extraction (pH 10) and isoelectric precipitation. Techno-economic evaluation of different extraction schemes also suggested substantial reduction of energy costs when the extraction was carried out at 20% solid content. Due to the co-extraction of carbohydrates, enzyme-aided protein extraction was less selective than non-enzymatic alkaline extraction and isoelectric precipitation. On the other hand, carbohydrates are suggested to have improved the solubility and dispersion stability of protein extracts by electrostatic interactions. The results indicate that enzyme-aided fractionation methods are suitable for rapeseed protein production and may offer a techno-economically feasible alternative to alkaline or saline extraction.

Additionally, protein hydrolyzates were extracted from dry-fractionated rapeseed press cake by proteolytic enzyme treatment. The hydrolyzates showed high inhibition of radical-induced oxidation in vitro, and their observed antioxidativity correlated with their protein and sinapic acid concentration. The hydrolyzates were also able to inhibit the myeloperoxidase and elastase enzymes involved in inflammation and skin aging, therefore having novel application potential as active ingredients or natural preservatives in skin care products.

ISBN, ISSN, URN

ISBN 978-951-38-8433-8 (Soft back ed.)
ISBN 978-951-38-8434-5 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8434-5

Date

June 2016

Language

English, Finnish abstract

Pages

108 p. + app. 44 p.

Name of the project

APROPOS - Added value from high protein & high oil industrial co-streams

Commissioned by

European Commission

Keywords

rapeseed, press cake, protein extraction, bioactive peptides, pectinase, dry fractionation

Publisher

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Julkaisun sarja ja numero

VTT Science 130

Nimeke	**Proteiinin ja proteiinihydrolysaattien entsyymiavusteinen rikastus rypsin kylmäpuristeesta**
Tekijä(t)	Katariina Rommi
Tiivistelmä	Maapallon kasvavan proteiinintarpeen tyydyttämiseksi tarvitaan uusia elintarvikkeisiin sopivia proteiinilähteitä. Rypsiöljyn kylmäpuristuksen sivuvirrassa, ns. puristeessa, on 32–36 % ravitsemuksellisesti hyvälaatuista proteiinia. Rypsiproteiinin rikastukseen puristeesta on kehitetty useita emäs- ja suolauuttomenetelmiä, mutta niiden kannattavuutta heikentää suuri veden- ja energiankulutus, joka johtuu useista prosessivaiheista ja laimeista uutto-olosuhteista. Tässä työssä kehitettiin entsyymiavusteisia menetelmiä proteiinin rikastamiseen rypsin kylmäpuristeesta ja selvitettiin, mitkä tekijät vaikuttavat proteiinin uuttuvuuteen sekä tuotettujen proteiinirikkaiden jakeiden ominaisuuksiin. Tavoitteena oli esikäsittelyn ja hiilihydraatteja hydrolysoivien entsyymien avulla tehostaa rypsiproteiinin uuttumista tavanomaista alhaisemmassa vesipitoisuudessa sekä ilman kemikaaleja, kuten emästä tai suolaa. Erityisesti pektinaasituote, joka sisälsi sivuaktiivisuutena -glukanaasia, hajotti tehokkaasti soluseinien hiilihydraatteja. Pektinaasikäsittelyllä saatiin myös merkittävästi parannettua proteiinin talteenottoa. Soluseinien mekaanisella hajotuksella ei kuitenkaan ollut proteiinisaantoa vastaavasti lisäävää vaikutusta, mikä viittaa siihen, etteivät puristeessa osittain säilyneet siementen solurakenteet merkittävässä määrin fyysisesti rajoittaneet proteiinin uuttumista. Tulosten perusteella entsyymikäsittely vähensi puristeen vedensitomiskykyä ja paransi siten neste-kiintoaine-erotuksen tehokkuutta ja uutteen talteenottoa. Lisäksi entsyymikäsittelyn hydrolyysituotteet näyttivät lisäävän proteiinin liukoisuutta vesiuutossa suolan tai emäksen tavoin. Entsyymiavusteinen vesiuutto kymmenen prosentin kuiva-ainepitoisuudessa osoittautui yhtä tehokkaaksi ja kuluiltaan samantasoiseksi rypsiproteiinin rikastusmenetelmäksi kuin perinteinen emäsuuton (pH 10) ja happosaostuksen yhdistelmä. Teknistaloudellinen tarkastelu lisäksi osoitti, että uuttosakeuden nostaminen 20 prosenttiin laskee selvästi prosessin energiakuluja. Entsyymiavusteinen proteiiniuutto oli hiilihydraattien samanaikaisen liukenemisen takia vähemmän valikoiva kuin ilman entsyymiä tehty emäsuutto ja happosaostus, mutta toisaalta hiilihydraatit paransivat proteiiniuutteiden liukoisuutta ja dispersio-ominaisuuksia sähköisten vuorovaikutusten kautta. Tulosten perusteella entsyymiavusteiset menetelmät soveltuvat rypsiproteiinin rikastukseen ja voivat tuoda teknistaloudellisesti kilpailukykyisen vaihtoehdon emäs- ja suolauuttomenetelmille. Kuivafraktioidusta rypsipuristeesta tuotettiin proteolyyttisen entsyymikäsittelyn avulla myös proteiinihydrolysaatteja, joiden todettiin ehkäisevän radikaalien indusoimia hapetusreaktioita in vitro. Hydrolysaattien antioksidatiivisuus korreloi niiden proteiinipitoisuuden ja fenolisen sinappihappopitoisuuden kanssa. Lisäksi hydrolysaatit inhiboivat myeloperoksidaasi- ja elastaasientsyymejä, joilla on rooli tulehdusreaktioissa ja ihon kimmoisuuden heikkenemisessä. Näin ollen rypsipuristeesta rikastetuilla proteiinihydrolysaateilla on myös uudenlaisia sovellusmahdollisuuksia ihonhoitotuotteiden hapettumista estävinä ja ihoa hoitavina ainesosina.
ISBN, ISSN, URN	ISBN 978-951-38-8433-8 (nid.) ISBN 978-951-38-8434-5 (URL: http://www.vtt.fi/julkaisut) ISSN-L 2242-119X ISSN 2242-119X (Painettu) ISSN 2242-1203 (Verkkojulkaisu) http://urn.fi/URN:ISBN:978-951-38-8434-5
Julkaisuaika	Kesäkuu 2016
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	108 s. + liitt. 44 s.
Projektin nimi	APROPOS - Added value from high protein & high oil industrial co-streams
Rahoittajat	Euroopan komissio
Avainsanat	rypsipuriste, proteiini, bioaktiiviset peptidit, pektinaasi, kuivafraktiointi
Julkaisija	Teknologian tutkimuskeskus VTT Oy PL 1000, 02044 VTT, puh. 020 722 111

VTT SCIENCE 130

Enzyme-aided recovery of protein and protein...

Enzyme-aided recovery of protein and protein hydrolyzates from rapeseed cold-press cake

New protein sources are needed to fulfil the growing global demand for food protein. The co-stream from cold pressing of rapeseed oil, press cake, is a rich source of protein with good nutritional value. Several technologies based on alkaline or saline extraction have been developed for enrichment of rapeseed protein, but high energy and water consumption due to dilute conditions and multiple processing steps limit their sustainability and profitability.

In the present study, enzyme-aided methods were developed for extraction of protein and protein hydrolyzates from rapeseed cold-press cake. Carbohydrate-hydrolyzing enzymes facilitated protein extraction at reduced water content and without chemicals such as alkali or salt. Additionally, protein hydrolyzates exhibiting bioactive properties were extracted from dry-fractionated press cake by proteolytic enzyme treatment. Results of the study indicate that enzyme-aided fractionation methods are suitable for rapeseed protein production and may offer a techno-economically feasible alternative to current technologies such as alkaline or saline extraction.

ISBN 978-951-38-8433-8 (Soft back ed.)
ISBN 978-951-38-8434-5 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8434-5