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In memory of my grandparents

TABLE OF CONTENTS

ABSTRACT

Consumers' increasing awareness of healthiness and sustainability of food presents a great challenge to food industry to develop healthier, biologically active and sustainable food products. Bioactive peptides derived from food proteins are known to possess various biological activities. Among the activities, the most widely studied are antioxidant activities and angiotensin I converting enzyme (ACE) inhibitory activity related to blood pressure regulation and antihypertensive effects. Meanwhile, vast amounts of by-products with high protein content are produced in food industry, for example potato and rapeseed industries. The utilization of these by-products could be enhanced by using them as a raw material for bioactive peptides.

The objective of the present study was to investigate the production of bioactive peptides with ACE inhibitory and antioxidant properties from rapeseed and potato proteins. Enzymatic hydrolysis and fermentation were utilized for peptide production, ultrafiltration and solid-phase extraction were used to concentrate the active peptides, the peptides were fractionated with liquid chromatographic processes, and the peptides with the highest ACE inhibitory capacities were putified and analyzed with Maldi-Tof/Tof to identify the active peptide sequences. The bioavailability of the ACE inhibitory peptides was elucidated with an *in vitro* digestion model and the antihypertensive effects *in vivo* of rapeseed peptide concentrates were investigated with a preventive premise in 2K1C rats.

The results showed that rapeseed and potato proteins are rich sources of ACE inhibitory and antioxidant peptides. Enzymatic hydrolysis released the peptides effectively whereas fermentation produced lower activities.The native enzymes of potato were also able to release ACE inhibitory peptides from potato proteins without the addition of exogenous enzymes. The rapeseed peptide concentrate was capable of preventing the development of hypertension *in vivo* in 2K1C rats, but the quality of rapeseed meal used as raw material was found to affect considerably the antihypertensive effects and the composition of the peptide fraction.

SUOMENKIELINEN ABSTRAKTI

Bioaktiiviset peptidit ovat ruoan proteiinien rakenneosia, joilla tiedetään olevan biologisia vaikutuksia. Eniten tutkittuihin vaikutuksiin lukeutuu Angiotensiini I:ä muuntelevan entsyymin (ACE) toimintaa estävä vaikutus, joka liittyy verenpaineen säätelyyn ja verenpaineen laskuun. Peptideillä on laajalti todettu myös antioksidatiivisia vaikutuksia, joilla voidaan osaltaan pystyä edistämään verisuoniterveyttä ja toisaalta tarjota luonnollinen vaihtoehto elintarvikkeiden säilyvyyden parantajina. Samaan aikaan elintarviketeollisuudessa, mm. rypsi- ja perunateollisuudessa, muodostuu sivutuotteena suuria määriä proteiinipitoisia jakeita, joiden käyttöä voitaisiin tehostaa hyödyntämällä niitä bioaktiivisten peptidien raaka-aineena.

Tämän työn tavoitteena oli tutkia mahdollisuutta tuottaa perunan ja rypsin proteiineista bioaktiivisia peptidejä, joilla on ACE esto aktiivisuutta ja antioksidatiivisia vaikutuksia. Peptidejä tuotettiin entsymaattisen hydrolyysin ja fermentoinnin avulla, tuotettujen peptidien ACE esto ja antioksidatiiviset vaikutukset määritettiin *in vitro*, aktiiviset peptidit konsentroitiin kalvosuodatuksella ja kiinteäfaasiuutolla, jonka jälkeen aktiivisimmat peptidit puhdistettiin nestekromatografisin menetelmin ja analysoitiin massaspektrometrisesti aktiivisten peptidisekvenssien tunnistamiseksi. Rypsistä tuotettujen ACE esto aktiivisten peptidien kykyä säilyä aktiivisessa muodossa ihmisen ruuansulatuksessa arvioitiin *in vitro* ruuansulatusmallin avulla käyttämällä ihmisestä eritettyjä ruuansulatusnesteitä ja rypsin peptidikonsentraatin kykyä ehkäistä verenpaineen nousua *in vivo* tutkittiin eläinmallissa ns. 2K1C rotilla.

Tulokset osoittivat, että rypsi- ja perunaproteiineista voidaan entsymaattisen hydrolyysin avulla tuottaa peptidejä, joilla on tehokasta ACE esto ja antioksidatiivista aktiivisuutta. Fermentointikäsittelyillä tuotettujen peptidien aktiivisuudet olivat heikompia entsyymikäsittelyillä tuotettuihin verrattuna. Tulokset osoittivat myös, että perunan luontaiset entsyymit pystyvät tuottamaan perunan proteiineista ACE esto aktiivisia peptidejä ilman ulkopuolisen entsyymin lisäystä. Rypsin peptidikonsentraatti pystyi estämään verenpaineen nousua *in vivo* 2K1C rotilla, mutta peptidien valmistuksessa raaka-aineena käytetyn rypsirouheen laadulla todettiin olevan merkittävä vaikutus verenpainevaikutuksiin ja peptidifraktion koostumukseen.

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	angiotensin I-converting enzyme
ACN	acetonitrile
bw	body weight
CVD	cardiovascular diseases
DM	dry matter
dpp	days post planting
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
HDJ	human duodenal liquid
HGJ	human gastric liquid
MAP	mean arterial pressure
MWCO	molecular weight cutoff
ORAC	oxygen radical absorbance capacity
RAS	renin-angiotensin system
ROS	reactive oxygen species
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
TBARS	thiobarbituric acid reactive sustances
TE	Trolox eqvivalent
TFA	trifluoro acetic acid
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TRAP	total reactive antioxidant potential
TOSC	total oxyradical scavenging capacity
2K1C	two kidneys, one clip model

LIST OF ORIGINAL PUBLICATIONS

I. Pihlanto, A.; Akkanen, S.; Korhonen, HJ. ACE-inhibitory and antioxidant properties of potato (*Solanum tuberosum*). *Food Chem.* **2008,** *109,* 104-112.

II. Mäkinen, S.; Kelloniemi, J.; Pihlanto, A.; Mäkinen, K.; Korhonen, H.; Hopia, A.; Valkonen, JPT. Inhibition of angiotensin converting enzyme I caused by autolysis of potato proteins by enzymatic activities confined to different parts of the potato tuber. *J. Agric. Food Chem.* **2008**, *56*, 9875–9883.

III. Mäkinen, S.; Johansson, T.; Vegarud, GE.; Pihlava, J-M.; Pihlanto, A. Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *J. Funct. Foods* **2012**, *4*, 575-583.

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1 INTRODUCTION

Cardiovascular diseases (CVD) represent the main cause of death in the world. Over 17 million people globally are estimated to die from CVD every year, and in Europe these diseases are responsible for more than half of all deaths (World Health Organization, 2011). Primary prevention has an increasing importance as part of public health strategies to reduce societal burden due to CVD-related morbidity and mortality worldwide. The World Health Organization emphasizes the importance of improved nutrition as means of controlling the expected rise in global CVD incidence over the coming decades.

The renin-angiotensin system (RAS) is a major regulator of blood pressure and fluid homeostasis; dysregulation of the RAS can lead to blood pressure elevation with ensuing cardiovascular disease, chronic kidney disease and diabetes. The regulatory mechanism takes place in the kidneys, where the hydrolytic enzyme renin is secreted. Renin cleaves plasma angiotensinogen to release the inactive decapeptide called Angiotensin I, which is subsequently hydrolyzed by Angiotensin Converting Enzyme (ACE: EC 3.4.15.1) to form Angiotensin II, which is a prohypertensive vasoconstrictor (Cheung *et al.*, 1980). ACE is a metallopeptidase membrane protein and it also degrades bradykinin, which regulates different physiological processes and excerts antihypertensive effects through the kinin-nitric oxide system. Therefore, ACE and renin are two key enzymes that regulate RAS operation and ACE inhibitors have been widely used as antihypertensive agents (Guang *et al.,* 2012). Meanwhile, there is strong evidence that reactive oxygen species (ROS) generated from sequential reduction of oxygen during the normal respiration of aerobic organisms may cause cellular damage leading to a number of pathological conditions, including atherosclerosis, arthritis, diabetes, and carcinoma (Beckman and Ames, 1998). The natural endogenous enzymatic and non-enzymatic antioxidant system suppresses ROS in order to maintain the balance called redox homeostasis. An imbalance in the redox homeostasis leads to excessive levels of ROS and oxidative stress (Valko *et al.,* 2007). Therefore, dietary antioxidants can supplement the antioxidant system and help to reduce the degenerative oxidative damage (Fang *et al.*, 2002). On the other hand, oxidation is also a critical mechanism also in deterioration during food processing and storage. Synthetic food antioxidants have been widely applied in the food industry for food preservation, but the use of natural antioxidants as food preservatives is of increasing interest. For these reasons dietary antioxidant compounds, food-derived bioactive peptides among them, have gained a great attention.

The role of dietary compounds in the prevention of CVD is of increasing importance in tackling this major health problem. Increased protein consumption, particularly from plant sources, has been related to reduced blood pressure and prevention of CVD in recent observational and clinical studies. Meanwhile, interest has been emerging in identifying and characterizing bioactive peptides with cardiovascular health-enhancing properties. Bioactive peptides are considered specific protein fragments that are inactive within the sequence of the parent protein. After they are released they may exert various physiological functions, such as antihypertensive, antioxidant, opioid, immunomodulatory and antimicrobial effects (Pihlanto and Korhonen, 2003; Guang and Phillips, 2009). Most widely studied protein sources of bioactive peptides are of animal origin, such as milk proteins and egg (Pihlanto and Korhonen, 2003; Majumder and Wu, 2009), but plant protein sources have attracted increasing attention in the recent years. ACE inhibitory and antioxidative activities *in vitro* have been reported for several plant protein-derived enzymatic hydrolysates, for example for rapeseed (Mäkinen *et al.*, 2012; He *et al.*, 2013a), flaxseed (Marambe *et al.*, 2011), mung bean (Li *et al.*, 2006) and potato (Pihlanto *et al.*, 2008) protein-derived hydrolysates. Recently, antihypertensive effects *in vivo* of plant protein-derived hydrolysates have also been demonstrated. For example, sweet potato protein hydrolysate (Ishiguro *et al.*, 2012) and rapeseed hydrolysate (Alashi *et al.*, 2014) have been shown to possess antihypertensive effects in spontaneously hypertensive rats.

Rapeseed (*Brassica napus*) is the third most important oilseed crop worldwide and also represents the world's second leading source of protein meal (Fediol, 2014). The protein content of defatted rapeseed meal is high, approximately 32% (Klockeman *et al.*, 1997), making it a potential food ingredient instead of being used as animal feed or wasted. The nutritive and functional properties of rapeseed are characterized by three main protein families: storage proteins cruciferin and napin, and oleosin, which is a structural protein associated with the oil fraction (Bos *et al.*, 2007). Yet, there are only a few studies examining rapeseed meal as a source of bioactive peptides to enhance the value of this rapeseed industry by-product. These studies include the use of enzymatic hydrolysis to produce antihypertensive and antioxidative peptides from rapeseed (Marczak *et al*., 2003; He *et al.*, 2013a; Alashi *et al.*, 2014) and production of antithrombotic peptide fractions with Alcalase hydrolysis (Zhang *et al.*, 2008). At the same time, potato is one of the main vegetables consumed in European and American diets, and the consumption is increasing strongly in Asia, Africa and Latin America. The content of protein in potato is around 3%; however the nutritional value of proteins is high. The production of starch from potatoes - 1.4 million tons in EU during the year 2012 - produces huge quantities of residual by-products:

potato pulp consisting of remnants of the cell walls of potato tubers, including the skin and residual intact cells containing starch, and potato fruit liquid. The potato fruit liquid is characterized by a high content of proteins, free amino acids and salts, whereas the pulp fraction contains mainly starch, cellulose, hemicelluloses, pectin and fewer proteins (Kempf, 1980). Potato fruit liquid is primarily used as a source for enriching proteins and amino acids, and also as a fertilizer due to its high nitrogen content. However, the high quality protein component of the potato fruit liquid could be a potential source material of bioactive peptides for production of health promoting ingredients to enhance the value of this by-product.

2 REVIEW OF THE LITERATURE

2.1 Potato

The potato has achieved worldwide prominence as a food item, in part, due to its tremendous yield per unit area compared to many other food crops. World potato growing areas are in a state of dynamic change. The production levels in the developed nations of Europe, North America, and the former Soviet Union have declined by 20 million metric tons (mmt) within the years 2003 and 2012. During the same interval, production has doubled in countries of the developing world including Asia, Africa and Latin America. More than a billion people globally eat potato and total potato production exceeds 300 million metric tons worldwide. Almost half of the global potato supply is now consumed in Asia (FAO, 2008). Potato increasingly contributes to world security and has a critical role to play in developing countries facing hunger. Potatoes supplement or replace grain-based diets where rice, wheat or corn availability has lessened or price has become unaffordable. The potato now ranks third, behind rice and wheat for human food as the use of corn for biofuels and animal feeds has lessened its human food applications. Considered from environmental aspects, potatoes produce more food per unit of water than any other major crops. In comparison to cereals, potatoes produce greater dry matter and protein per unit of growing area (FAO, 2014).

2.1.1 Characteristics of potato tuber

The potato is a common staple food and is available in many forms. The protein content of potato is around 3%; however the nutritional value of these proteins is high and in the dry matter the protein content is about 10% which is at the same level as most cereals, including rice and wheat (Friedman, 1996). Potato tuber proteins are classified into three major groups: patatins, protease inhibitors, and other proteins (Pots *et al.*, 1999). Patatin is the major storage protein in potato tubers and also an allergen for some people. This allergenicity is significantly reduced by heating. The second major potato tuber storage protein is a diverse group of low molecular-weight protease inhibitors. The patatins and protease inhibitors comprise about 40% of the total potato tuber protein. The majority of the patatin and proteinase inhibitor isoforms also possess enzymatic and inhibitory activities, respectively, which might be of physiological relevance. A summary of the nutritive value of potato protein was produced in 1975 by Markakis and it showed that the amino acid composition is nutritionally comparable to whole egg protein; the calculated protein quality is about 70% that of whole egg protein. The lysine content of

potatoes is excellent, but they contain fewer sulfur amino acids that limit the nutritive value. On the other hand, human feeding trials have suggested that the nutritional quality of potato proteins may be higher than the amino acid composition indicates. It needs to be taken into account that about 50% of the total nitrogen of potato is derived from free amino acids and amide nitrogen of asparagine and glutamine (38%) as well as nonprotein nitrogen associated with glycoalkaloids and secondary metabolites (12%) and these components may enhance the utilization of proteins (Markakis, 1975). When the nutritional quality of potato concentrate was measured in terms of biological value (BV), net protein utilization (NPU), nitrogen retention and protein efficacy ratio (PER), the nutritional quality was found to be very high with a PER-value of 2.90. PER-value was measured as a weight gain of a test group/total protein consumed (Nestares *et al.*, 1993). An earlier human trial by Kies and Fox (1972) showed that the physiological nitrogen balance increased when the potato protein was supplemented with methionine (0.3%). Recently Bartova and Barta (2009) isolated protein concentrates from industrial potato juice and measured the nutritional value. The protein concentrates exhibited high nutritional value as the essential amino acid index was over 80%.

Potato is also an important source of vitamins and minerals, such as calcium, potassium, phosphorus and ascorbic acid. Studies have indicated that potato tubers contain phenolic compounds, for example chlorogenic acid, which have been shown to possess free radical scavenging activity *in vitro* (Friedman, 1996). Due to the large amounts consumed, they may be a significant source of health-promoting phenolic compounds. The nutrient content of potatoes is favourable; therefore, using potatoes moderately as a part of a meal should be encouraged. A medium-size 150 g potato with the skin provides 27 mg of vitamin C (45% of the daily value (DV)), 620 mg of potassium (18% of DV), 0.2 mg of vitamin B6 (10% of DV) and trace amounts of thiamin, riboflavin, folate, niacin, magnesium, phosphorus, iron, and zinc (Camire *et al.*, 2009). Potatoes contain toxic compounds known as glykoalkaloids, of which the most prevalent are solanine and chaconine. Exposure to light, physical damage and age increase glykoalkaloid content within the tuber. Although glykoalkaloids are perceived as potentially toxic, studies during the past 10 years suggest that they may also possess beneficial effects, depending on dose and conditions of use (Friedman, 2006).

In the current literature, only rather scattered information concerning the potato proteome is available. Most of the data is related to the resistance of potato plants to pathogens, and the regulation of pathogenesis-related and stress-responsive proteins in potato (Lim *et al*., 2013). Studies have consistently shown that the developmental stage and physiological age of potato tubers are associated with significant alterations in the protein

composition of tubers. This fact is presumably a notable factor for the production of bioactive peptides (Mäkinen *et al*., 2008). Lehesranta *et al.* (2006) and DelaPlace *et al.* (2009) observed significant changes in the potato tuber proteome during tuber development and physiological age and qualitative and quantitative changes have been detected in protein content during the development of tubers (Borgmann *et al.*, 1994), dormancy (Espen *et al.*, 1999) and after dormancy (Desires *et al.*, 1995a; Desires *et al.*, 1995b). The differentiation in the potato protein isoforms and protein expression levels between the potato cultivars has recently been linked to techno-functional properties of potato, such as the tuber's susceptibility to bruising upon mechanical impact (e.g. an enzymatic browning reaction) (Urbany *et al.*, 2012) and to the cold-induced sweetening (Fischer *et al.*, 2013; Folgado *et al.*, 2014). Differentiations between patatin and lipoxygenase isoforms have been suggested to play an important role in these aspects. Moreover, patatins have been reported to possess enzymatic activities, in particular, broad phospholipase activity has been characterized and documented (Bauw *et al.*, 2006; Scherer *et al.*, 2010). The lipid acyl hydrolase activity of patatins has also been detected in industrial potato fruit juice and this enzyme activity may be utilizable even after concentration of proteins by precipitation (Bartova and Barta, 2009). The patatin group consists of a number of glycoprotein isoforms that differ between potato cultivars as well as in physiological state (DelaPlace *et al.*, 2009; Hoehenwarter *et al.*, 2011). A high degree of homology between the patatin isoforms has been observed by N-terminal amino acid sequence analysis (Shewry, 2003). Protease inhibitors are a diverse group of proteins that differ widely in their amino acid sequence (Heibges *et al.*, 2003). The protease inhibitors have various physiological activities associated with the defense mechanisms of potato against pathogens and they inhibit a variety of proteases and furthermore, also some other enzymes, for example invertase (Heibges *et al.*, 2003).

Studies have reported that potatoes are one of the most glycaemic and insulinogenic foods. However, in most meals, potatoes are accompanied by other foods and glycaemic and insulinaemic responses are modified when ingested as part of a mixed meal: the co-ingestion of fat and protein reduced the glycaemic responses and the co-ingestion of protein increased the insulinaemic responses in subjects with type 2 diabetes (Gulliford *et al.*, 1989). A recent study investigated how glycaemic and insulinaemic responses to a mashed potato meal changed when a high-fat food (rapeseed oil), a high-protein food (chicken breast) and/or salad were added to the meal. The results indicated the protein, fat and salad contents of a meal exert considerable influence on the glycaemic and insulinaemic responses to mashed potatoes (Hätönen *et al.*, 2011).

In summary, the results indicate potential for potato protein to be utilized in food formulations with enhanced protein quality. It would be useful to combine diets consisting of potatoes (high in lysine and low in sulfur amino acids) with cereals (high in sulfur amino acids and low in lysine) to achieve a well-balanced protein composition. As potatoes have to be processed before eating it's important to notice, that the losses in the nutritive value of potato protein during boiling and frying are minor (Friedman, 1997), whereas canning and chipping cause remarkable losses (Jaswal, 1973).

2.1.2 Potato starch production

Over the last 20 years there has been a threefold worldwide increase in starch production and in the EU the production figures have increased by 2.5. In the year 2012 the worldwide starch production totalled 75 million tons of which the production in the EU was 10 million tons. Potato is the third most important raw material for industrial starch production after maize and wheat; potato accounts for 33% of raw material within the EU (Figure 1) (AAF European Starch Industry Association, 2014). In particular, potatoes are a fundamental European crop for starch and it is extremely important to keep potato starch competitive with maize starch made from imported maize.

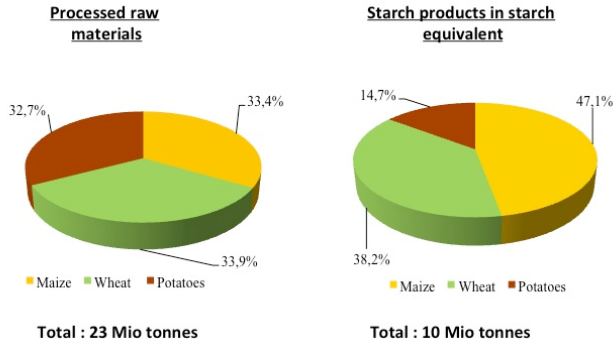

Figure 1 Raw materials used for starch production in the EU 2012 (AAF European Starch Industry Association, 2014)

The production of starch from potatoes - 1.4 million tons in EU 2012 - produces huge quantities of residual by-products (AAF European Starch Industry Association, 2014): particulate fraction called potato pulp and the potato fruit liquid characterized by high protein content (Kempf, 1980). The current use of potato fruit liquid for enriching proteins and for fertilization could be enhanced by utilizing the proteins as potential raw material for value-added ingredients such as bioactive peptides. Potato pulp is applied as cattle

feed, in an enzymatically treated and condensed form as syrup for crisps and chips and as a substrate for the cultivation of fungi that may be used for degradation of soil contaminants and in addition, for the production of cosmetics.

2.1.3 Potential health-promoting properties of potato proteins and peptides

2.1.3.1 Prevention of cardiovascular diseases

Although many factors affect the status of the heart and circulatory system, maintenance of normal serum lipids and blood pressure is essential for health. Potato contains several compounds beneficial to cardiovascular health. The lipid content is low (0.10 g/100 g FW), and dietary fiber is especially found in peels. With regard to the mineral composition, relatively high potassium levels are needed to counteract the effect of sodium and protect against hypertension. Reactive oxygen species are shown to be the key modulating factors responsible for the development of many age-related disease conditions including cardiovascular diseases. Inflammation is another risk factor for cardiovascular disease. Although many biomarkers for inflammation exist, high-sensitivity C-reactive protein (CRP) has received most attention as a predictor for cardiovascular disease. Potatoes contain a diverse mixture of antioxidants - mainly phenolic compounds but also peptides - which exhibit multiple antioxidant activities *in vitro* and antioxidant effect of potato peel extract has been demonstrated also in rats (Singh *et al.*, 2008). Results from clinical trials have indicated that phenolic compounds of potato tubers may also lower inflammation (Kaspar *et al.*, 2011). In addition, peptides can inhibit the Angiotensin I-converting enzyme (ACE) which is one of the key factors in the control of blood pressure. The ACE inhibitory and antioxidant effects of potato peptides have been shown *in vitro* and the effects *in vivo* remain to be investigated. The antioxidant and ACE inhibitory activities of potato proteins and peptides are summarized in Table 1. The ACE inhibition activities are expressed as protein concentrations needed to inhibit 50% of the activity of ACE (IC50 mg protein/ml), and antioxidant activities are represented as Trolox equiivalents and inhibition percentages describing the efficacy of samples for reducing the formation of oxidation products in lipid oxidation.

Table 1 Antioxidant and ACE inhibitory properties of potato protein-derived hydrolysates

Sample	*ACE inhibition IC50 protein mg/ml*	*Radical scavenging activity (ABTS) µM Trolox*	*Inhibition of lipid oxidation (inhibition of TBARS formation % of control)*	*Peptides identified*
Hydrolysates of potato protein				
Esperase[a]	0.08	8	-	-
Neutrase[a]	0.09	8	-	-
Alcalase[a]	0.04	16	-	ACE inhibitory RP-HPLC fractions rich in alanine
Autolysate[b]	0.36	-	-	-
Fractions of Alcalase hydrolyzed potato protein				
Precipitate, 50% saturated ammonium sulphate[c]	-	2 700	60 %	TSNLLT, SSGFTY, SSGFTMQ, IYLGQ, KPYVFRATGAL, LMRWMR
Size exclusion fraction, 709 Da[d]	-	2 300	70 %	TY, VY, NYKQM, YSTA, YFE, SWN, HVCYMF, MWL

[a]Pihlanto *et al.*, 2008; [b]Mäkinen *et al.*, 2008; [c]Cheng *et al.*, 2010a; [d]Cheng *et al.*, 2010b

Cheng *et al*. (2010a and 2010b) recently identified several low molecular-weight antioxidative peptides from the Alcalase hydrolysate of potato protein. The peptides enhanced the oxidative stability of soybean oil in water emulsion and possessed radical scavenging activity *in vitro* against ABTS-radical. Peptides consisting predominantly of hydrophobic amino acid residues are known to exert high radical scavenging activity. The results indicated that the protective effect of potato peptides against lipid oxidation is probably due to other reaction mechanisms than radical scavenging as the hydrophobicity of peptides was not the dominating factor of the protective effects. In fact, the results indicated that the partitioning of the peptides on the membrane would have a critical role in the protection against oxidation (Cheng *et al.*, 2010a; Cheng *et al.,* 2010b). The results of Elias *et al.* (2008) support the partitioning theory as they reported that the partitioning of less polar peptides in the membrane is more extensive and this provides a stronger protection of fat globules from free radical and peroxidant attack. Liu *et al*. (2003) found that purified patatin exerts antioxidant or antiradical activity in *in vitro* tests, such as DPPH radical and hydroxyl radical scavenging activity, and protection against hydroxyl radical-induced calf thymus DNA damage. DelaPlace *et al.* (2009) studied the changes in the potato tuber antioxidant system during the post-harvest ageing of the tubers and found out that the changes in the proteome allowed potato tubers to maintain their radical scavenging activity until the end of the rather long storage period (+4 °C 270 d). Studies by Singh *et al*. (2008) indicated that potato peel extract treatment had a potent protective effect against oxidative stress and acute liver damage induced by CCl4 in rats, as revealed by a remarkable decrease in hepatic MDA content accompanied by enhanced GSH and SOD activities. Human studies in healthy men showed that consumption of pigmented potatoes (150 g/day) lowered plasma IL-6 and DNA damaged compared to white potatoes. Moreover, the purple flesh potato group tended to have lower plasma C-reactive protein concentration than the white potato group (Kaspar *et al.*, 2011).

Hydrolysis of protein isolates from potato tubers has also been found to enhance ACE inhibition (Table 1: Pihlanto *et al.*, 2008). In further studies it was shown that autolysis with native potato proteases also enhanced ACE inhibition capacity (Mäkinen *et al.*, 2008). The physiological age of the tubers had a significant effect on the production of ACE inhibition during the autolysis, the rate of ACE inhibition formation and the tuber tissue where ACE inhibition was most pronounced. Differences in enzymatic activities were confined to different parts of the potato tuber at different physiological stages. Many of the tuber proteins were seen to degrade during the autolysis including two aspartic protease inhibitors which were degraded in the highest concentrations. Enrichment of recombinant potato tuber protein to the autolysis

enhanced the production of activity significantly, which suggests the possibility of enhancing potato tuber ACE inhibitory potential by means of biotechnological tools (Mäkinen *et al.*, 2008). According to the current literature summarized in Table 1, Alcalase would be the most effective enzyme for the production of ACE inhibitory and antioxidative peptides from potato proteins (Pihlanto *et al*., 2008; Cheng *et al*., 2010a; Cheng *et al*., 2010b). Moreover, results indicate that the peptide sequences of ACE inhibitory and antioxidative peptides are different as the ACE inhibitory peptides are rich in alanine (Pihlanto *et al.*, 2008) whereas antioxidative peptides contain very few alanine residues (Cheng *et al.*, 2010a; Cheng *et al.*, 2010b). In any case, it has to be underlined that rather few potato peptides or peptide fractions with antioxidant or ACE inhibitory properties have been characterized thus far and more research is needed to characterize and identify the ACE inhibitory potato peptides and to evaluate the *in vivo* antihypertensive and antioxidant potential.

2.1.3.2 Antimicrobial and antifungal properties

Antimicrobial peptides are a group of peptides that have a broad activity against a diverse group of microorganisms. Due to their action mechanisms these peptides are potential molecules that could help to overcome the problems of the increasing resistance of bacteria to conventional antibiotics. Antimicrobial proteins/peptides in plants have a major role in the innate host defense mechanisms. Innate immunity is an ancient defense system of multicellular organisms to control natural flora and combat pathogens. Antimicrobial peptides have been detected in a wide variety of plants and their potent *in vitro* antimicrobial activity indicates that they may play a general protective role against pathogens. These peptides act through various mechanisms, eg. altering cell membrane permeability and liposome interactions (Garcia-Olmedo *et al.*, 1998), but many of the mechanisms are not clear thus far.

Several antibiotic peptides/proteins have been purified from potato. These peptides and proteins isolated from potato tubers have been classified into three classes: globulins, defensins and protease inhibitors. The globulins include the major proteins/peptides, patatins, in potato tubers. Patatins have been reported recently to possess phospholipase activity on phospholipid and lysophospholipid substrates and also esterase activity. Patatin have also been found to have hydrolytic activity as an acidic β-1,3-glucanase. This glucanase is involved in the pathogenesis-related protein response (Van Loon *et al.*, 1999). Defensins were isolated during the extraction of the insoluble proteins of potato tubers and they include, for example, the antifungal peptides Pseudothionin *Solanum tuberosum* (Pth-St1) (Moreno *et al.*, 1994), snaking-1 and snaking-2

(Segura *et al.*, 1999; Berroca-Lobo *et al.*, 2002). The snaking/GASA (gibberellic acid-stimulated *Arabidopsis*) family peptides are basic and rich in Cys residues and possess almost identical antibacterial activity with aggregation of bacteria (Berroca-Lobo *et al.*, 2002). The third class of antibiotic peptides/proteins of potato, protease inhibitors, also plays an important role in the defense mechanism of plants. Several peptides with antibiotic properties have been purified and identified as being homologous with protease inhibitors (Feng *et al.*, 2003).

Many of the isolated antimicrobial peptides of potato that are supposed to be antibiotic against bacterial and fungal pathogens of potato (such as *Clavibacter michiganensis* subspecies and *Fusarium solani*) have not been tested against human pathogens. However, a few rather low molecular-weight antimicrobial potato peptides belonging to the protease inhibitor Kuniz family have also recently been reported to exhibit antibimicrobial activity against human pathogenic fungal and microbial strains (Table 2). Kim *et al*. (2006) isolated a small, 5 578.9 Da, antimicrobial peptide from a water-soluble protein fraction of potato tubers (*Solanum tuberosum* L cv. *Golden valley*). This peptide, Potide-G, was found to inhibit the growth of a variety of bacterial (*Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Clavibacter michiganense* subsp. *michiganinse*) and fungal (*Candida albicans* and *Rhizoctonia solani*) strains. Potide-G was reported to be heat stable and suppress the proteolytic activity of trypsin, chymotrypsin and papain. Potamin-1 peptide, 5 600.4 Da, a trypsin-chymotrypsin protease inhibitor was also isolated from the water-soluble fraction of potato tubers (*Solanum tuberosum* L cv. *Gogu*) (Kim *et al.*, 2005). Potamin-1 inhibited several pathogenic microbial and fungal strains of which *Candida albicans* is the most common cause of oral, esophageal, vaginal and urinary candidiasis in humans (Kim *et al.*, 2005). Also an antifungal protein, AFP-J, has been purified from potato tubers (*Solanum tuberosum* L cv. *Jopung*) (Park *et al.*, 2005). This peptide, AFP-J, 13 500.5 Da, is produced in potato upon fungal attack (Kim *et al.*, 2005) and was shown to exhibit effective antifungal activity against several human pathogenic fungi (*Candida albicans*, *Trichosporon beigelii* and *Saccharomyces cerevisiae*). Recently, the AFP-J peptide was characterized further and a novel antimicrobial peptide against *Candida albicans* was identified as Ala-Val-Cys-Glu-Asn-Asp-Leu-Asn-Cys-Cys with MW of 1 083.1 Da (Lee *et al.*, 2012). Moreover, Jin *et al.* (2009) discovered that refined potato protein was able to reduce the total bacteria, coliforms and *Staphylococcus* spp in the feces and contents of the colon and rectum in weanling pigs.

Table 2 Antibiotic activities of potato peptides against human pathogens

Inihibited microbial strain	***Minimal inhibition concentration (μg/mL)***			
	Potide-G[a]	**AFP-J[b]**	**Potamin- 1[c]**	**Potide-J[d]**
T. beigilii	ND	6.25	-	-
C. albicans	<30	6.25	100 μM	6.25
S. cerevisiae	<30	6.25	-	-

[a]Kim *et al*., 2006; [b]Park *et al.*, 2005; [c]Kim *et al.*, 2005; [d]Lee *et al.*, 2012

2.1.3.3 Satiety-promoting properties, antitumor effects and some other potential health-promoting properties of potato proteins/peptides

Obesity-related health problems are generally known to be a rapidly escalating issue globally. Diet and lifestyle are important factors in the prevention and treatment of obesity and obesity-related diseases. The current literature includes indications that potatoes may have a role in controlling appetite and therefore weight gain, by contributing to satiety (Foltz *et al*., 2008; Nakajima *et al*., 2011). The satiety-promoting and other bioactivities reported in the literature for potato proteins and hydrolysates are summarized in Table 3. Gastrointestinal hormones such as cholecystokinin (CCK) are key factors in the regulation of food intake and maintaining energy homeostasis (Cummings and Overduin, 2007). CCK is known to be a major endocrine determinant in food intake regulation and proteins have been reported to be the most satiating of all macronutrients (Cummings and Overduin, 2007). Studies in animals and humans using different protein sources suggest that in order to stimulate CCK secretion efficiently, the protein needs to be hydrolyzed to short-chain peptides and amino acids through processing or gastrointestinal degradation (Schwartz, 2000). As obesity is a major global health problem the prevalence of which is increasing alarmingly, discovering functional food products for weight management purposes is of a great interest. Either specific protein hydrolysates or other food-grade bioactive compounds with optimized CCK-releasing properties are potential target molecules for this purpose. Protease inhibitors with a variety of physiological roles comprise about 40% of potato tuber proteins (Pots *et al*., 1999). Hill *et al*. (1990) reported reduced energy intake and increased CCK release, when protease inhibitors extracted from potatoes were tested in 11 lean subjects. More recent results have reinforced the potential of potato peptides as satiety-promoting agents as potato protein hydrolysate was found to induce CCK release and stimulate CCK1R expression in enteroendocrine cells (Foltz *et al.*, 2008). Potato protein extract has also been found to suppress food intake dose dependently in rats after oral administration (Nakajima *et al*., 2011). Also in this study, the results indicated that food intake

suppression was controlled through the induced CCK secretion and inhibition of luminal trypsin. However, Peters *et al.* (2011) found that potato protease inhibitors in a minidrink at a dose of 30 mg, as commercially used, had no effect on a range of behavioral and physiological appetite and intake control measures in humans.

The incidence of diabetes is increasing enormously throughout the world and type -2 diabetes involves 90% of diabetics. Diabetes is often associated with obesity and dyslipidemia and the development of diabetes and its progressive complications is characterized by hyperglycemia meaning unregulated elevated blood sugar levels (Hätönen *et al.*, 2011). Hyperglycemia induces oxidative stress; biomarkers of free radical-induced damage are increased and antioxidant defenses are reduced. The role of potato in the prevention of diabetes is complicated due to the high content of carbohydrates and on the other hand, antidiabetic factors such as antioxidants. Potato peel extracts, which contain a rich content of phenolic antioxidants have been demonstrated to reduce hyperglycemia, oxidative stress and overall food consumption in diabetic rodents (Singh *et al.*, 2005). The observed effects are presumably mainly due to the phenolic compounds and the potential effects of potato proteins/peptides on these issues remains to be investigated.

Table 3 Other bioactive properties of potato proteins and peptides

Sample	***Outcome***
Potato protein preparate[a]	Cholesterol-lowering effects in rats
Potato peptide fraction[b]	LDL-lowering, HDL-increasing and fecal neutral steroid excretion properties
Potato protease inhibitor extract[c]	Reduced energy intake and increased CCK release in human subjects
Potato protein extract[d]	Suppressed food intake in rats
Potato serine and carboxypeptidase inhibitors[e; f; g]	Tumor cell growth inhibition

[a]Morita *et al*., 1997; [b]Liyanage *et al*., 2008; [c]Foltz *et al*., 2008; [d]Nakajima *et al*., 2011; [e]Blanco-Aparicio *et al*., 1998; [f]Kennedy, 1998a; [g]Huang *et al*., 1997

The role of vegetable consumption in the prevention of cancer has not reached consensus in the scientific community. Protease inhibitors have received new interest as they have been shown to have potent ability to prevent carcinogenesis in various *in vivo* and *in vitro* systems (Kennedy, 1998a;

Kennedy, 1998b). Tumor cell growth has also been reported to be inhibited by serine protease inhibitors and carboxypeptidase inhibitors of potato (Huang, *et al.*, 1997; Blanco-Aparicio *et al.*, 1998; Kennedy, 1998a). The antitumor properties related to potato proteins are summarized in Table 2. Anticancer effects of purified sweet potato protein on human colorectal cancer cells have also been shown (Li *et al.*, 2013). The role of phenolic compounds of potato in cancer prevention also needs to be taken into account. Potato tuber extracts and the anthocyanin fraction has been reported to reduce cell growth and induce apoptosis in prostate cancer cell lines (Reddivari *et al.*, 2007). Likewise, intake of anthocyanins from purple and red potatoes might play a protective role against stomach cancer (Hayashi *et al.*, 2006). Isolated anthocyanins induced apoptosis in human cancer cell lines as well as suppressing mouse stomach cancer proliferation, indicating that anthocyanins were bioactive antitumor components. Potato glykoalkaloids inhibited the growth of human colon and liver cancer cells (Lee *et al.*, 2004). In summary, the published data regarding the cancer-related health effects of potato in the diets of consumers is limited; however, cell line studies indicate potato protease inhibitors and phenolic compounds as antitumor agents.

2.2 Rapeseed

Rapeseed (*Brassica napus*) is grown mainly as a raw material for oil production. Rapeseed is related to mustard, cabbage, broccoli, cauliflower and turnip. Rapeseed is the third most important oilseed crop worldwide after sunflower and palm, and it represents the world's second leading source of protein meal after soybean meal (Fediol, 2014). Global rapeseed production has grown rapidly over the past 40 years, rising from the sixth largest oil crop to the second largest. Rapeseed production represented 10 - 15 percent of the world oil crop production between years 2000 and 2009. During the last decade, European rapeseed production increased sharply from 12 million tons in 2000 to 20 million tons in 2010 (FAOSTAT, 2014), mainly due to higher demands for biofuel.

Usage of the terms rapeseed and canola is sometimes confusing, and thus, needs to be noted briefly. Canola seeds, which are mainly produced in Canada, Australia and the US, represent double 00-spring varieties such as *Brassica napus L.*, *Brassica campestris L.* and *Brassica Rapa var.*, and are characterized by low levels of erucic acid (<20 g/kg in the oil fraction) and low levels of glucosinolates (<30 μmol/g in toasted oil-free meal; Canola Council of Canada, 2014). In comparison, contents of erucic acid and total glucosinolates in double 00-winter rapeseed varieties grown in Europe must not exceed 20 g/kg in the

oil fraction and 27 μmol/g dry matter (DM) in the rapeseed (European Food Safety Authority, 2008), respectively.

The small, round rapeseed seed contains 38 to 45 percent oil. In addition to high oil content, rapeseed seeds contain approximately 17 - 26% protein (Uppstrom, 1995). In the food sector the importance of rapeseed is mainly related to oil composition; it contains both omega-6 and omega-3 fatty acids in a ratio of 2:1. Due to the high protein content in the seeds the presence of traces of protein in the final oil is extremely difficult to prevent. It is well recognized that the role of proteins in the allergenicity of edible oils is a growing problem. Among all edible oil sources, it has been reported that olive and rapeseed/canola are nonallergenic (Hidalgo and Zamora, 2006).

2.2.1 Production of milled rapeseed

Rapeseed seeds are crushed at processing facilities into oil and meal. They are first pressed to extract the bulk of the oil, and afterward, most crushing facilities add a chemical solvent to remove the remaining oil. The supplies of canola meal and oil are linked through the crushing process even though demand for these products is independent. Rapeseed oil production increased globally by 10 million tons between the years 2000 and 2010 (Fediol, 2014). The majority of the global rapeseed oil supply is produced in the EU, China, Canada and India. In EU countries the annual production is estimated to reach 10 million tons in 2014 (USDA, 2014). Rapeseed meal is generated as a by-product of industrial oil extraction and has been widely used as a protein supplement in the formulation of livestock feed (Aidera and Barbanab, 2011). In 2012, global canola meal production was 33.8 million metric tons (Carré and Pouxet, 2014). Rapeseed meal is primarily used as protein-rich feed ingredient for livestock due to its relatively high content of indispensable amino acids, particularly sulphur amino acids, in comparison to other protein ingredients, including soybean meal (Mansour *et al.*, 1993; Woyengo *et al*., 2010; USDA, 2012). However, high protein content makes it a potential food ingredient instead of being used as animal feed or wasted.

2.2.1.1 Characterisrics of rapeseed meal

Rapeseed meal contains up to 50% protein on a dry basis (USDA, 2012). The amino acid compositions of the rapeseed protein isolates have been studied and reported by several researchers and the results indicate that the amino acid composition is altered depending on the industrial oil extraction process, and particularly depends on the process used for protein extraction from the rapeseed meal residue. Usually, up to 30% of the total protein initially present in rapeseed meal is extracted in an alkaline medium, and large-scale

purification of rapeseed proteins significantly affects the final protein composition (Chabanon *et al.*, 2007).

The use of rapeseed meal in diets for monogastric animals such as pigs is often limited due to its high fiber content (Grala *et al*., 1998; Mailer *et al*., 2008) and the presence of several antinutritional factors, including different glucosinolates, phenolic compounds and phytates (de Lange *et al*., 1998). These compounds are responsible for the toxic, antinutritional and undesirable coloration capacity of canola proteins. In particular, free and esterified phenolic acids have been reported to cause the undesirable taste of rapeseed products (Naczk *et al*., 1998). Condensed tannins are also found in rapeseed meal residue and proteins extracted from it (Clandinin and Heard, 1968; Shahidi and Naczk, 1989). Proteins may interact with flavoring compounds such as phenolics and these interactions can influence the flavor properties (Hagerman and Butler, 1981; Shahidi and Naczk, 1995; Naczk *et al*., 1998). Another limiting factor is the rather high content, up to 4%, of phytates in rapeseed proteins (Naczk *et al.,* 1986). Phytates are antinutritional compounds reducing the bioavailability of divalent cations such as Ca, Mg, Zn, Cu and Fe and inhibiting the digestion of starch. On the other hand, phytates at low concentrations, may possess antioxidative and anticarcinogenic effects (Rickard and Thompson, 1997). Canola meal also contains indigestible fibers that are, however, diminished after protein extraction (Lacki and Duvnjak, 1998). The glucosinolates can be eliminated to a certain extent for example, through hydrothermal treatment (Grala *et al*., 1998). In particular, the composition of rapeseed has been significantly improved by developing low glucosinolate and low erucic acid rapeseed cultivars.

The underutilized protein component of rapeseed has potential properties for human nutrition that remain to be explored. The nutritional and functional properties of rapeseed protein are characterized by the major protein constituents of rapeseed meal: the storage proteins napin and cruciferin, and oleosin, which is a structural protein associated with the oil fraction (Uppstrom, 1995). Rapeseed protein is a potential ingredient for use in the food industry as many characteristics of rapeseed protein are favorable for human nutrition. Rapeseed protein has recently been demonstrated to be of high nutritional value in human subjects (Bos *et al.*, 2007). The amino acid composition of rapeseed meal is well balanced (Ohlson and Anjou, 1979; Mariscal-Landin *et al.*, 2008) and the protein efficiency ratio of rapeseed meal is rather high, at 2.64, in comparison for example to soybean, at 2.19 (Delisle *et al*., 1984). Rapeseed proteins have shown interesting and promising functional properties and could be potentially used in various food matrices (Khattab and Arntfield, 2009; Xu and Diosady, 1994a and 1994b). The fat absorption, emulsifying activity and

foaming properties of rapeseed protein concentrates were superior in comparison to soybean protein (Ghodsvali *et al.*, 2005).

Efforts are focusing on developing canola protein products for food applications. Recently, the European Food Safety Authority has regarded rapeseed protein isolate as a safe novel food ingredient (European Food Safety Authority, 2013). Also, canola protein isolate and hydrolysate have received GRAS (Generally recognized as safe) notification in the USA by the Center for Food Safety and Applied Nutrition (U.S. Food and Drug Administration, 2011).

2.2.2 Rapeseed as a source of bioactive peptides

The increase in consumer awareness about healthy foods has encouraged researchers to identify bioactive natural components in different products (Murty *et al.*, 2010). Rapeseed protein is suggested to be an attractive source of bioactive compounds. Recently, various different methods have been utilized to produce active peptides from rapeseed proteins. Most of the studies concern ACE inhibitory and antioxidant activity *in vitro* and antihypertensive effects in animal studies, but also other activities such as antithrombotic activity and cell growth effects have been demonstrated as bioactive characteristics of rapeseed proteins. The bioactive properties of rapeseed and canola protein-derived hydrolysates and peptides are summarized in Table 4 and Table 5.

Table 4 Antioxidant, ACE inhibitory, renin inhibitory and hypotensive properties of rapeseed protein-derived hydrolysates and peptides

Source material	*Enzyme/microbial strain*	*Treatment/peptide property/sequence*	*Outcome*	*Reference*
Rapeseed meal, canola meal	Pepsin, Trypsin, Alcalase, Subtilisin, Thermolysin, Pancreatin, Chymotrypsin *Bacillus subtilis*, *Lactobacillus helveticus*	Protein hydrolysates, membrane ultrafiltration fractions (mostly <3 kDa), gel exclusion chromatography fractions (mostly 300-500 Da), peptides Arg-Ile-Tyr, Ile-Tyr, Val-Trp, Val-Trp-Ile-Ser, Val-Ser-Val, Phe-Leu, Leu-Tyr, Thr-Phe, Arg-Ala-Leu-Pro	High ACE inhibition *in vitro*	Marczak *et al.*, 2003; Wu *et al.*, 2008; Mäkinen *et al.*, 2012; Pihlanto *et al.*, 2012; He *et al.*, 2013a
Rapeseed meal, canola meal	Alcalase, Thermolysin, Pepsin, Pepsin+Pancreatin, Chymotrypsin, Flavourzyme, Proteinase K	Protein hydrolysates, membrane filtration fractions (1-3 kDa and 5-10kDa depending on the enzyme used), peptides Leu-Tyr, Thr-Phe, Arg-Ala-Leu-Pro, Gly-His-Ser	Renin inhibition *in vitro*	He *et al.*, 2013a; Alashi *et al.*, 2014
Rapeseed meal, canola meal	Subtilisin, Pepsin, Alcalase, Thermolysin, Chymotrypsin, Pancreatin, Pepsin+Pancreatin, Flavourzyme, Proteinase K	Protein hydrolysates, membrane filtration fractions, peptides Gly-His-Ser, Val-Trp, Val-Trp-Ile-Ser, Ile-Tyr, Arg-Ile-Tyr, Leu-Tyr, Thr-Phe, Arg-Ala-Leu-Pro	Hypotensive effects *in vivo* on spontaneously hypertensive rats (SHR)	Marczak *et al.*, 2003; He *et al.*, 2013a; He *et al.*, 2013b Alashi *et al.*, 2014,
Rapeseed meal	Pepsin, Pepsin+Pancreatin, Trypsin, Alcalase, Subtilisin, Thermolysin, Proteinase K, Flavourzyme *B. subtilis*, *L.helveticus*	Protein hydrolysates, membrane filtration fractions (mostly <1 kDa), gel exclusion chromatography fractions (mostly 1200-1300 Da), peptide Pro-Ala-Gly-pro-Phe	Radical scavenging activity, inhibition of lipid peroxidation in liposome micelles, inhibition of linoileic acid oxidation, inhibition of autohemolysis of rat red blood cells, inhibition of the malonyldialdehyde generation in blood serum	Zhang *et al.*, 2008; Xue *et al.*, 2009a; Xue *et al.*, 2008b; Mäkinen *et al.*, 2012; Pihlanto *et al.*, 2012; He *et al.*, 2013a; He *et al.*, 2013b

2.2.2.1 Prevention of cardiovascular diseases

2.2.2.1.1 Hypotensive properties

Rapeseed meal as a source of bioactive peptides with ACE inhibitory properties has received increasing attention in the recent years. Most of the studies concern the utilization of various proteolytic enzymes to liberate ACE inhibitory rapeseed peptides (Marczak *et al.*, 2003; Mäkinen *et al*., 2012; He *et al*., 2013c; He *et al*., 2013a; He *et al*., 2013b; Alashi *et al.,* 2014), but also fermentation with lactic acid bacteria has been shown to release *in vitro* ACE inhibitory peptides from rapeseed (Pihlanto *et al*., 2012). Enzymatic hydrolysates of rapeseed meal and protein extracts have shown high ACE inhibitory potencies *in vitro*; the most active hydrolysates have been produced by Alcalase with 50% ACE inhibition concentrations of 15 µg protein/mL (Wu *et al.*, 2008) and 20 µg protein/mL (Mäkinen *et al*., 2012). Wu and co-workers utilized consecutive hydrolysis with Alcalase and ENZECO Alkaline Protease L-FG resulting in a 50% ACE inhibitory value of 18 µg protein/mL (Wu *et al.*, 2008). Also, He and co-workers have reported high ACE inhibitory capacities *in vitro* for rapeseed meal hydrolysates and found Alcalase (~ 80% inhibition with 1 mg protein/mL) and Thermolysin (~ 85% inhibition with 1mg protein/mL) to be the most effective enzymes (He *et al.*, 2013a). Rapeseed protein hydrolysates have also been shown to possess inhibitory capacity against renin, the other key enzyme in the regulation of blood pressure through the RAS system. In particular, the rapeseed protein Alcalase hydrolysate and Pancreatin hydrolysate have been reported to possess high renin inhibitory capacity *in vitro*, over 80% and 63%, respectively, with a protein concentration of 1 mg/mL (He *et al*., 2013a; Alashi *et al.*, 2014). Rapeseed protein Subtilisin digest and Alcalase digest have been subjected to simulated gastrointestinal digestion *in vitro* in order to elucidate the ability of ACE inhibitory peptides present in these digests to resist gastrointestinal digestion and the possibilities of reaching the site for absorption (Marczak *et al*., 2003; Mäkinen *et al*., 2012). The Alcalase hydrolysate was treated with human gastric juices in laboratory conditions simulating human gastric and duodenal digestion, and the ACE inhibitory activity was retained at the same level through the digestion treatment, indicating that ACE inhibitory peptides present in the Alcalase digest are relatively resistant (Mäkinen *et al*., 2012). The Subtilisin digest was subjected to hydrolysis with different proteases to simulate gastrointestinal digestion *in vitro*, and the ACE inhibitory activity was changed only slightly, indicating that ACE inhibitory peptides present in the Subtilisin digest possess a relatively high ability to resist further hydrolysis into inactive degradation products (Marczak *et al*., 2003).

Relatively few ACE inhibitory peptides have been identified thus far and all of the identified peptides have been produced by enzymatic hydrolysis either with Subtilisin or Alcalase. Four ACE inhibitory peptides were isolated from the rapeseed Subtilisin digest: Arg-Ile-Tyr (IC50, 0.028 mM), Ile-Tyr (IC50, 0.037 mM), Val-Trp (IC50, 0.0016 mM) and Val-Trp-Ile-Ser (IC50, 30mM) (Marczak *et al*., 2003). Peptides Ile-Tyr and Arg-Ile-Tyr can be found in the primary structure of napin and Val-Trp and Val-Trp-Ile-Ser exist in the primary structure of cruciferin and ribosomal protein, respectively. Altogether five peptide sequences with high ACE inhibitory capacity have been identified from rapeseed meal hydrolyzed with Alcalase: Val-Ser-Val (IC50, 0.15 µM), Phe-Leu (IC50, 1.33 µM), Leu-Tyr (IC50, 0.11 mM), Thr-Phe (IC50, 0.81 mM) and Arg-Ala-Leu-Pro (IC50, 0.97 mM). These peptides are located in the primary structure of rapeseed napin and cruciferin proteins (Wu *et al.*, 2008). In this study, the peptides Leu-Tyr, Thr-Phe and Arg-Ala-Leu-Pro were shown to possess dual inhibition potential against both ACE and renin activity. In contrast to ACE inhibitory activity, peptide sequence Arg-Ala-Leu-Pro (IC50, 0.97 mM) was reported to possess the highest capacity against renin activity in comparison to Leu-Tyr (IC50, 1.87 mM) and Thr-Phe (IC50, 3.1 mM). A novel ACE inhibitory peptide Gly-His-Ser was identified from the 3 kDa membrane ultrafiltration permeate of a Pepsin+Pancreatin rapeseed protein digest (He *et al.*, 2013c). The IC50 value was rather weak (520 µg protein/mL) when compared to the other identified ACE inhibitory rapeseed peptides, but Gly-His-Ser was also shown to possess renin inhibitory activity with an IC50 value of 320 µg protein/mL.

Recently, rapeseed hydrolysates, fractions and peptides have been reported to have antihypertensive effects on spontaneously hypertensive rats (SHR). The hypotensive effects of rapeseed hydrolysates and peptides are presented in Table 5 on page 27. Subtilisin digest of rapeseed protein has shown a dose-dependent antihypertensive effect after oral administration to SHR with a significant decrease in blood pressure even at a single dose of 0.15 g/kg body weight (bw). Rapeseed protein-derived Alcalase and Pepsin hydrolysates have also shown appreciable antihypertensive effects, with Alcalase hydrolysate producing the greatest (−34 mm Hg) and fastest (4 h) decrease in systolic blood pressure (SBP). Permeate of rapeseed protein Pepsin+Pancreatin digest (<3 000 Da) has induced a corresponding hypotensive effect (−21 mmHg) after oral dosage on SHRs. In comparison, the nonhydrolyzed canola protein isolate had a significant and more prolonged (24 h) SBP-reducing effect, which is attributable to the extensive protein hydrolysis in the gastrointestinal tract (Alashi *et al*., 2014). In contrast, trypsin hydrolysate has been reported to be ineffective at reducing hypertension in SHR after oral administration (Marczak *et al.*, 2003). The current literature also includes information on

antihypertensive effects for rapeseed protein-derived pure peptides. Marczak and colleagues (2003) measured the antihypertensive activities of Val-Trp, Val-Trp-Ile-Ser, Ile-Tyr and Arg-Ile-Tyr following oral administration to SHR. All peptides displayed a dose-dependent antihypertensive effect. Hypotensive activity of the peptides was compared between young (19 - 20 weeks) and old (28 - 30 weeks) SHRs. Usually the hypotensive effect of ACE inhibitors in old SHR is lower than in young SHR, but Arg-Ile-Tyr (rapakinin) showed a similar effect both in old and young rats. Thus, it was suggested that another mechanism besides ACE inhibition may be involved in the hypotensive effect of rapakinin. Subsequently, the hypotensive effect of rapakinin was found to be mediated mainly by the prostaglandin-cholecystokinin-dependent vasorelaxation (Yamada *et al.*, 2010). Recently, the antihypertensive effects of the peptides Leu-Tyr, Thr-Phe and Arg-Ala-Leu-Pro from Alcalase digest of rapeseed protein were tested on SHR (He *et al*., 2013b). Single oral administration to SHR showed Leu-Tyr and Arg-Ala-Leu-Pro to be the more effective hypotensive agents with a maximum blood pressure reduction of -26 and -16 mmHg, respectively, when compared to Thr-Phe (-12 mmHg). It was suggested that the higher number of hydrophobic amino acid residues of Leu-Tyr and Arg-Ala-Leu-Pro contributed to their higher *in vitro* and *in vivo* activities when compared to Thr-Phe (He *et al.*, 2013b). Oral administration of Gly-His-Ser to SHR showed a maximum blood pressure reduction of −17 mmHg after 6 h. The *in vitro* ACE inhibitory effects of Gly-His-Ser were significantly weaker in comparison to the previously mentioned peptides and the hypotensive effects of Gly-His-Ser was suggested to be controlled mainly through renin inhibition (He *et al.,* 2013b). The administration doses of single, pure rapeseed peptides reviewed above varied between 7.5 and 30 mg/kg bw, and the crude hydrolysates and fractions were administered in higher doses varying between 100 and 500 mg/kg bw.

2.2.2.1.2 Antioxidant properties

Rapeseed meal has been used as a source material for the production of peptides with antioxidant activities in several studies. Antioxidant effects of rapeseed hydrolysates and peptides have been observed with various different *in vitro* methods, either related to scavenging free radicals or inhibition of lipid peroxidation and formation of malondialdehyde (MDA). Most of the studies concern the utilization of commercial proteases for the production of antioxidant peptides, in particular, Alcalase has produced highly effective antioxidant capacity from rapeseed protein. Xue *et al.* (2009a) investigated the digestion of insoluble rapeseed meal protein with Alcalase and Flavorzyme to produce peptides with antioxidant properties and observed dose-dependent reducing antioxidant activities and hydroxyl radical scavenging abilities. The hydrolysate also inhibited malonyldialdehyde (MDA) generation by 50% in

blood serum at 150 mg/ml. The hydrolysate was separated into three fractions by size-exclusion chromatography and the fractions were found to inhibit the autohemolysis of rat red blood cells and MDA formation in a rat liver tissue homogenate (Xue *et al.*, 2009b). Radical scavenging activity against DPPH radical and inhibition capacity against lipid peroxidation in a liposome system have also been demonstrated for rapeseed protein-derived Alcalase hydrolysate with comparable effects to those of ascorbic acid at 5 mg/mL (Zhang *et al.*, 2008; Zhang *et al.*, 2009). In addition, two other studies have reported high antioxidant activities of rapeseed protein hydrolysates against free radicals, and in particular Alcalase hydrolysate showed high potential with the oxygen radical absorbance capacity (ORAC) method (He *et al*., 2013a; He *et al*., 2013b). Antioxidant capacity against lipid peroxidation in a liposome system for rapeseed protein-derived hydrolysates has also been demonstrated by Mäkinen *et al.* (2012), and the highest antioxidant activity was obtained with Alcalase consistently with the other published data.

Results have demonstrated a notable inverse relationship between radical scavenging activity (ORAC) and peptide molecular weight; the ORAC values were highest for the <1 kDa peptides but decreased as peptide size increased to 5 - 10 kDa indicating that large-size peptides may not interact efficiently with the free radical (He *et al*., 2013a). On the other hand, Pihlanto and co-workers (2012) investigated the inhibition of lipid peroxidation of fermented rapeseed meal and different molecular weight fractions using a liposome model. Fermentation with *L. helveticus* and *B. subtilis* strains produced the capacity to inhibit lipid oxidation and fractionation with size exclusion chromatography separated the inhibition activity against lipid peroxidation in the 1 200 – 1 300 Da fraction. This indicates that the peptides with lipid peroxide inhibitory properties are relatively higher in molecular mass than peptides with efficient radical scavenging properties. The difference could be due to the ability of larger peptides to bind into the liposome micelle structure.

Relatively few rapeseed protein-derived antioxidant peptide sequences have been identified. Zhang and co-workers (2009) identified the peptide Pro-Ala-Gly-Pro-Phe with high radical scavenging activity from the rapeseed protein Alcalase hydrolysate. This peptide corresponds to amino acid residues 38 - 42 of napin. Antioxidative peptides with larger molecular sizes have been identified, for example from whey with 1 200 Da (Hernandez-Ledesma *et al*., 2005), giant squid muscle with 747 and 1 307 Da (Rajapakase *et al*., 2005), and chicken essence with 1 400 Da (Wu *et al.*, 2005).

Altogether, due to the various different methods used for antioxidant determination, comparison of the reported results is not straightforward, but on the other hand, results show that rapeseed is a diverse source of different types of antioxidant peptides as the hydrolysates and fractions have shown the

capacity to prevent degenerative oxidative damage through different mechanisms. Also, the published data demonstrate that rapeseed hydrolysates have potentially high antioxidant potency as compared to other hydrolysates found in literature. For example, soy protein hydrolysates with Alcalase yielded ORAC values between 23 and 83.8 μM TE/g protein (Zhang *et al.*, 2010) while amaranth protein hydrolysate produced by PP had a value of about 75 μM TE/g protein (Orsini Delgado *et al.*, 2011), which are substantially lower than the values obtained with rapeseed hydrolysates. However, the nonprotein compounds, such as phenols and pigments need to be noted when evaluating the rapeseed samples. Rapeseed meal is known to be rich in phenolic compounds, most importantly sinapic acid derivatives (Vuorela *et al*., 2004). In the published data Alcalase has been consistently shown to be an excellent protease for releasing antioxidant peptides from rapeseed proteins; however, another report indicated that Flavourzyme should be selected to produce antioxidant peptides from rapeseed-like proteins (Cumby *et al.*, 2008).

2.2.2.1.3 Other bioactivities

In addition to antioxidant and antihypertensive properties, some other potential health-enhancing properties have also been reported for rapeseed protein-derived peptides (Table 5). Rapakinin (Arg-Ile-Tyr) derived from rapeseed napin, has been reported to possess food intake-suppressing properties, in addition to the hypotensive properties. Rapakinin dose-dependently decreased food intake and gastric emptying after oral administration at a dose of 150 mg/kg in mice (Marczak *et al.*, 2006). Rapeseed protein has also been linked to preventing insulin resistance. Mariotti and others (2008) demonstrated that substituting rapeseed protein for milk protein prevented the early onset of insulin resistance, and the effects corresponded to those achieved by manipulating dietary fat and carbohydrates in a rat model.

Hypercholesterolemia is considered to be a major cause of heart diseases and atherosclerosis (World Health Organization, 2011). Healthy diet, exercise and consumption of bile acid sequestrants, which are better known as hypolipidemic agents, are important in the prevention of hyperlipidemia (Anderson and Siesel, 1990). Bile acid-binding promotes the consumption of cholesterol in the liver, which therefore reduces the level of cholesterol in the blood (Anderson and Siesel, 1990). The *in vitro* hypocholesterolemic properties of rapeseed proteins have been measured by determining the bile acid-binding capacity. Yoshie-Stark *et al.* (2008) demonstrated the bile salt-binding effects for rapeseed protein isolates and their hydrolysates, which were obtained by Pepsin and Pepsin/Pancreatin digestion. The concentration of bile salts used in this study corresponded to the physiological concentrations of bile acids in the human body (1.5–7 mM). Nevertheless, the hydrolysis treatments did not affect the bile acid-binding capacities indicating that some polypeptides present

already in the rapeseed protein isolates are able to bind the ligands (Yoshie-Stark *et al.,* 2008). Bile acid-binding properties of some other plant proteins and hydrolysates have also been reported with corresponding observations that hydrolysis does not necessarily enhance the bile acid-binding capacity (Yoshie-Stark and Waesche, 2004; Ma and Xiong, 2009).

Thrombosis is an anomaly in blood coagulation and leads to severe symptoms, heart injury among them. The blood-clotting process is related to the interaction between thrombin and fibrinogen to form the fibrin clot. Antithrombotics can reduce platelet aggregation and enhance fibrinolysis and thus, reduce the risk of thrombosis (Scheraga, 2004; Erdmann *et al.*, 2008). Bioactive peptides have been demonstrated to possess antithrombotic activity, for example milk κ-casein-derived peptides and egg white protein hydrolysate (reviewed by Silva and Malcata, 2005). Recently, antithrombotic activity has also been demonstrated for rapeseed peptides (Zhang *et al.*, 2008). The rapeseed peptide fractions produced with Alcalase hydrolysis had significant inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen. The maximum inhibitory effect was 90% at peptide concentrations between 30 and 50 mg/mL. The observed values were superior to those reported for egg white hydrolysate at the same concentration, but weaker than the antithrombotic activity of heparin, which is commonly used as an antithrombotic drug (Zhang *et al.*, 2008).

Table 5 Other potential health enhancing properties of rapeseed protein-derived hydrolysates and peptides

Source material	***Treatment***	***Sample***	***Outcome***	***Reference***
Rapeseed meal	Subtilisin	Peptide Arg-Ile-Tyr (rapakinin)	Suppressed food intake and increased CCK release in SHR, Vasorelaxing activity in mesenteric artery isolated from SHR	Marczak *et al.*, 2006
Rapeseed	Pepsin, Pepsin+Pancreatin	Protein isolates obtained by ultrafiltration or iso-precipitation, protein hydrolysates	Hypocholesterolemic activity *in vitro*: bile acid-binding capacity	Yoshie-Stark *et al.*, 2008
Dehulled rapeseed	Alcalase	Protein fraction obtained after aqueous enzymatic extraction of rapeseed, protein hydrolysate, peptide fractions obtained after stepwise desorption with 25 and 55% ethanol from a nonpolar styrene-based macroporous adsorption resin	Inhibitory activity on the thrombin-catalyzed coagulation of fibrinogen *in vitro*	Zhang *et al*., 2008
Rapeseed meal	Alcalase+Flavourzyme	Protein hydrolysate	Antitumor and antioxidant effect *in vivo* in a murine model: decreased tumor weight in tumor-bearing female mice, enhanced superoxide dismutase activity and reduced serum level of thiobarbituric acid reactive substances	Xue *et al.*, 2009b

Rapeseed protein hydrolysate has also been shown to have antitumor effects *in vivo* on a tumor-bearing Kunming mice model (Xue *et al*., 2009b). Rapeseed protein hydrolysate significantly decreased the tumor weight by 44% and 53% with the gastric perfusion at 100 and 150 mg/kg/d, respectively, without causing mortality or growth retardation. Furthermore, rapeseed protein hydrolysate was found to enhance the superoxide dismutase activity and reduce the serum level of thiobarbituric acid reactive substances. The results suggested that an oral rapeseed protein hydrolysate administration may have an antitumor protective effect and may improve the immune function by reducing free radical formation and oxidative stress (Xue *et al.*, 2009b).

On the other hand, there is an increasing need for nonanimal-derived ingredients for culture medias used in the production processes of recombinant therapeutics due to the risk of contamination by animal-derived products. In this respect, the effects of rapeseed meal protein hydrolysates on cell growth and metabolism have been studied in animal cell line models. Chabanon *et al*. (2008) observed cell growth-increasing properties with rapeseed protein-derived hydrolysates produced with various commercial proteases (Chabanon *et al*., 2008). Moreover, a peptide fraction was purified from the Alcalase-hydrolyzed rapeseed protein with ultra- and nanofiltration, and the fraction stimulated significantly the growth rates of several animal cells and was observed to possess anti-apoptotic activity (Farges *et al.*, 2008). The high nutritional value of rapeseed protein hydrolysates explains partly the positive effects on cell growth; however, the role of peptides in the anti-apoptotic activity has also been suggested (Chabanon *et al.*, 2008; Farges *et al.*, 2008).

2.3 Targets for bioactive peptides used in this study

As mentioned previously in the introduction, bioactive peptides may affect various molecular disease targets related to human health. The background for selecting ACE inhibitory activity and antioxidant activity amongst the multitude of activities as target properties is given in this section, as well as an overview of peptides' structural characteristics related to these activities based on current literature. Also the methods used for assessing these bioactivities are briefly reviewed.

2.3.1 Hypertension

Hypertension constitutes a high risk factor for the development of cardiovascular diseases, stroke and end-stage renal disease (Daien *et al.*, 2012). Figure 2 illustrates the key roles of renin and ACE in the physiological control of blood pressure through the RAS and the kinin-nitric oxide system. In pathological conditions there is an excessive level of angiotensin II, which

causes severe blood vessel contractions and limited relaxation to produce high blood pressure (Ibrahim, 2006). ACE has a dual effect preventing vasodilation by inactivating bradykinin, and activating vasoconstriction by converting angiotensin I into angiotensin II (Figure 2).

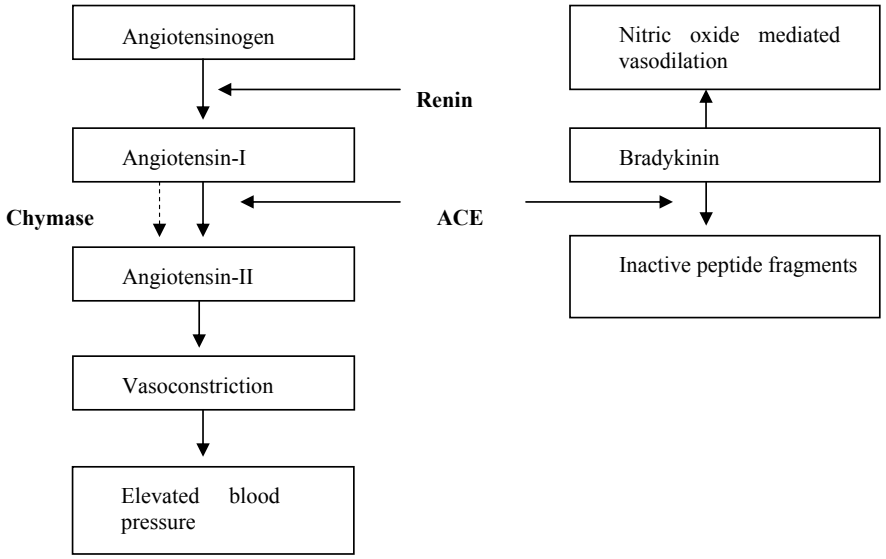

Figure 2 The blood pressure-regulating renin-angiotensin system (RAS) pathway and the potential molecular targets (renin and ACE) for bioactive peptides.

ACE inhibitors are widely used as antihypertensive agents and several drugs (captopril, enalapril, lisinopril etc.) are available as effective ACE inhibitors. Although there are various mechanisms involved in the regulation of blood pressure, inhibition of ACE still remains one of the crucial strategies in the treatment of hypertension (Aleixandre and Miguel, 2012). However, the synthetic ACE inhibitory drugs are not without side effects, such as a dry cough and angiodema. Nutraceuticals, bioactive peptides among them, provide the potential to avoid undesirable side effects and can decrease the growing costs of drug therapy (Shahidi and Zhong, 2008; Guang *et al.*, 2012). Therefore, finding new sources of ACE inhibitors, especially in foodstuff, is of great interest.

ACE inhibitory peptides are generally short sequences as the active site of ACE cannot accommodate large peptide molecules. Current literature on molecular mechanism of ACE inhibition by peptides indicate that C-terminal tri-peptide residues play a predominant role in binding to the active site of ACE, and hydrophobic (aromatic or branched side chains) amino acid residues of peptides correlate with enhanced inhibitory potential. In addition, it has been shown that single amino acid substitution, even with isomers, can greatly

influence the nature of interactions between peptides and ACE (Sato *et al.*, 2002). Recently, the mechanism of ACE inhibition by peptides was reviewed (Phelan and Kerins, 2011). The most effective ACE inhibitory peptides identified contain Tyr, Phe, Trp and/or Pro at the C-terminal. In addition, structure-activity data suggests that the positive charge of Lys and Arg as the C-terminal residue may contribute to the inhibitory potency (Ondetti *et al.*, 1977; Cheung *et al.*, 1980; Ariyoshi, 1993). Quantitative structure-activity relationship (QSAR) modelling studies support the previous findings that C-terminal bulky hydrophobic amino acids (for example Trp, Phe, Pro and Tyr) and N-terminal aliphatic amino acids (such as Leu, Ile and Val) are necessary features for ACE inhibition in the case of di- and tri-peptides (Wu *et al.*, 2006a). On the other hand, QSAR studies indicate that in the case of 4-10 amino acids containing peptides the important determinant for ACE inhibition is that the last four C-terminal amino acids are predominantly hydrophobic (Wu *et al.*, 2006b).

2.3.2 Oxidation

Oxidative stress is both a cause and a consequence of hypertension; it induces cardiovascular and renal damage with associated increase in blood pressure. It has been shown, for example, that Angiotensin II-dependent hypertension is particularly sensitive to reactive oxygen species (ROS) (Uzuner *et al.*, 2010). Therefore, therapies based on scavenging of ROS could have potential effects in the management of CVD, and food supplements containing antioxidants could be used to reduce the physiological oxidative damage. On the other hand, lipid oxidation is one of the most important oxidation processes in foods and it generates toxic compounds and undesirable odors and flavors (Laguerre *et al.*, 2007). The potential health hazards of the synthetic antioxidants have caused widespread public concern, and thus there is a growing interest in natural antioxidants.

The current literature offers abundant information on various food protein hydrolysates and peptides with antioxidant properties, e.g. from milk (Pihlanto, 2006), egg (Davalos *et al.*, 2004), barley hordein (Chiue *et al.*, 1997), soybean (Pyo and Lee, 2007), canola (Cumby *et al.*, 2008; He *et al.*, 2013a) and potato (Wang and Xiong, 2005; Pihlanto *et al.*, 2008). The oxidative reaction systems used for assessing antioxidant activities of the protein hydrolysates and peptides include scavenging of ROS or other free radicals and inhibition of ROS-induced oxidation of biological macromolecules such as lipids, proteins and DNA. An interesting phenomenon is that many of the antioxidative hydrolysates and peptides possess multifunctional properties, such as ACE inhibitory properties together with antioxidant capacity.

The antioxidative properties of the peptides are dependent on their structural and physicochemical characteristics, such as molecular size, hydrophobicity and amino acid composition (Pihlanto, 2006). The mechanisms of antioxidant activity of peptides are not fully understood, although different mechanisms have been demonstrated. The radical quenching activities have been suggested to be due to the ability of peptides to participate in single electron transfer reaction (Huang *et al.*, 2005), and the other known mechanisms of antioxidant activity of peptides include metal-chelating activity and ferric-reducing power. Amino acids with electron-dense aromatic rings, Phe, Tyr and Trp can donate protons to electron deficient radicals. This property improves the radical scavenging properties of the amino acid residues (Chen *et al.*, 1998; Pihlanto, 2006). Hydrophobic amino acids are important for enhancement of antioxidant properties of peptides since they can increase the accessibility of the antioxidant peptides to hydrophobic cellular targets such as the polyunsaturated chain of fatty acids in biological membranes (Chen *et al.*, 1998). Moreover, His residue of peptides can chelate metal ions, quench active oxygen and scavenge lipid peroxyl radical; these abilities were attributed to the imidazole group of His (Chan and Decker, 1994; Rajapakse *et al.*, 2005). On the other hand, the sulfhydryl group of Cys has an important antioxidant activity due to its direct interaction with radicals (Qian *et al.*, 2008). Cys residues of peptides can also serve as precursors for the synthesis of glutathione, a universal cellular antioxidant tripeptide, and thus Cys can contribute to the regeneration of the physiological antioxidant defense system (Meisel, 2005). In any case, in addition to the particular effects of the mentioned amino acids, the correct position of amino acids in the peptide sequence plays an important role in the antioxidant properties of peptides. It has also been reported that antioxidative peptides keep cells safe from damage by ROS through the induction of genes (Erdmann *et al.*, 2008). Recently, casein hydrolysate has been shown to posess antioxidant activities in human Jurkat T cells (Lahart *et al.*, 2011). Although the antioxidant activities of peptides have been studied *in vitro* quite extensively and the results are promising, it would be necessary to target the focus more on the *in vivo* effects of these peptides to evaluate the mechanisms of action more precisely and to enable application of these peptides in formulating health-promoting food products.

2.3.3 Methods for assaying hypotensive and antioxidant properties *in vitro* and *in vivo* of food protein-derived bioactive peptides

ACE inhibitory activity *in vitro* is generally evaluated by measuring the effect of potential inhibitors on the conversion of an appropriate substrate by ACE. There are several methods, and those based on spectrophotometric (Cushman and Cheung, 1971; Vermeirssen *et al.*, 2002; Li *et al.*, 2005) and

high-performance liquid chromatography (HPLC) assays are the most commonly utilized (Doig and Smiley, 1993; Hyun and Shin, 2000; Shalaby *et al.*, 2006). The most commonly utilized substrate for ACE is Hippuryl-His-Leu (HHL), and the methods measure the catalytic activity of ACE to produce hippuric acid from the substrate (Cushman and Cheung , 1971; Doig and Smiley, 1993; Li *et al.*, 2005; Shalaby *et al.*, 2006). In the widely used spectrophotometric method of Cushman and Cheung (1971) the hippuric acid released is measured after its extraction with ethyl acetate. The hippuric acid can also be measured by HPLC assays avoiding the extraction step (Vermeirssen *et al.*, 2002). However, 2-furanacryloyl-phenylalanylglycyl-glycine (FAPGG) can also be used as substrate for ACE (Holmquist *et al.*, 1979). This method has also been applied in microtiter plates (Vermeirssen *et al.*, 2002). Moreover, substrates such as o-aminobenzoylglycyl-p-nitrophenylalanylproline are designed for fluorometric assays (Carmel and Yaron, 1978; Sentandreu and Toldrá, 2006).

On the other hand, another strategy for the selection of potential food-derived antihypertensive peptides is to search for *in vitro* renin inhibitory activity. Renin inhibition could provide a more complete blockade of the RAS, since renin catalyzes the rate-determining step in RAS (Fitzgerald *et al.*, 2011). Recent studies have reported renin inhibition capacity, for example from rapeseed protein-derived hydrolysates and peptides (He *et al.*, 2013a; He *et al.*, 2013b; Alashi *et al.*, 2014). The *in vitro* renin inhibitory capacities have been measured with a fluorometric method using a human recombinant renin inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, USA). Interestingly, several peptides have shown dual inhibition capacity both for ACE and renin (He *et al.*, 2013b).

In vitro cultured cell model systems are used for screening of compounds for their bioavailability, metabolism and bioactivity to obtain valuable information before expensive and time-consuming *in vivo* studies. Endothelial cell models are commonly utilized, particularly in angiogenesis research. Endothelial dysfunction initiates atherosclerosis and the dysfunction is characterized by alteration in nitric oxide and endothelin-1 homeostasis (Verma *et al.*, 2003). There are several assays available to analyze the effects of bioactive compounds, such as bioactive peptides, on the endothelial function *in vitro*. For example, expression and activation of endothelial nitric oxide synthase – the enzyme responsible for the release of the vasodilator compound nitric oxide – can be analyzed in endothelial cells *in vitro* to elucidate the effects of the compounds of interest on the potential release of nitric oxide in the vascular endothelium.

Methods for assaying antioxidant capacity *in vitro* are various. Oxidative processes occurring in food or biological systems are complex, and thus the

antioxidative mechanisms by which bioactive compounds may act are various. Therefore, finding one method for characterizing the overall antioxidative potential of food is not relevant. Nevertheless, the current literature describes methods such as the Trolox equivalent antioxidant capacity (TEAC) assay, total radical-trapping antioxidant parameter (TRAP) assay, and oxygen radical absorbance capacity (ORAC) assay for measuring the antioxidative capacity of food and biological samples (Cao and Prior, 1998; Re *et al.*, 1999). Commonly used assays include measuring the inhibition of lipid peroxidation in a linoleic acid model system and in the liposome model, and the capacity to scavenge the ABTS and DPPH radicals. In particular, the antioxidant mechanisms in human health promotion go beyond the antioxidant activity of scavenging or quenching free radicals, and thus the use of cell culture models for antioxidant research is particularly important (Liu and Finley, 2004). Intracellular oxidative stress, for example in endothelial cells, can be induced with a peroxy generator (Elisia and Kitts, 2008), and subsequently the extent of intracellular radical formation can be measured in the presence and absence of antioxidative compounds (Wolfe and Liu, 2007).

The antihypertensive effects *in vivo* can be assessed in SHR, which constitute an accepted animal model for studying human essential hypertension (Erdmann *et al.*, 2008). This animal model has been widely used to study the hypotensive effects of ACE inhibitory milk protein-derived peptides in both a short-term and long-term manner (Fitzgerald *et al.*, 2004; Erdmann *et al.*, 2008), and the current literature also includes studies concerning the *in vivo* antihypertensive effects of plant protein-derived peptides in SHR, e.g. pea (Vermeirssen *et al.*, 2005), canola (Alashi *et al.*, 2014) and flaxseed (Udenigwe *et al.*, 2012). The published data concerning the *in vivo* measurement of the antioxidant effects of food protein-derived bioactive peptides is quite limited; however, biomarkers of lipid and protein peroxides as well as DNA damage can be assessed to monitor changes in oxidative stress *in vivo*.

2.4 Production of food protein-derived bioactive peptides

Bioactive peptides can be released by fermentation, food processing and enzyme-catalyzed proteolysis *in vitro* or in the digestive tract after human consumption (Hartmann and Meisel, 2007). Numerous animal and plant protein sources have been exploited as sources of bioactive peptides. Most widely studied protein sources are of animal origin, such as milk proteins and egg, but marine and plant protein sources have attracted increasing attention in recent years. For example chum salmon collagen (Yang *et al.*, 2009), Pacific oysters (Wang *et al.*, 2010) and shrimp cell by-product (Kannan *et al.*, 2011) have been utilized as sources of bioactive peptides with immunostimulating and antitumor

properties. Various plant protein sources have been utilized for bioactive peptide production, of which soy, pulses (lentils, peas, beans, chickpeas), cereals, canola and flaxseed are the typical sources. Based on the current literature, there are two major criteria for the selection of source materials – firstly a pursuit of value-added use of abundant underutilized protein-rich food industry by-products, and secondly utilization of proteins containing specific peptide sequences of particular bioactivity interest. In particular, the combination of these two criteria could lead to the selection of excellent protein sources of bioactive peptides. However, this strategy for selection is restricted due to the fact that the detailed knowledge of structure-function properties of active sequences has been rather limited and hampered thus far.

The specific bioactivity of food protein-derived peptides against different molecular disease targets depends primarily on structural and physicochemical properties such as the molecular size, hydrophobicity and side chain bulkiness of the amino acid residues (Pripp *et al.*, 2005). During the enzymatic production of bioactive peptides several factors affect the resulting peptide composition, and their structural and physicochemical characteristics. These factors include the specificity of the enzyme used for hydrolysis, hydrolysis time, enzyme to substrate ratio, and pretreatment of the protein prior to hydrolysis. For example, thermal treatment of proteins can enhance enzymatic hydrolysis (Inouye *et al.*, 2009).

According to the current literature, enzymatic hydrolysis with single or multiple specific or nonspecific proteases has been the main process for producing ACE inhibitory and antioxidative peptides from food proteins (Pedroche *et al.*, 2002; Wu and Ding, 2002; Yust *et al.*, 2003; Megías *et al.*, 2006; Barbana and Boye, 2010; Udenigwe and Aluko, 2010; Udenigwe *et al.*, 2012). Industrial food-grade proteinases such as Alcalase, Flavorzyme, and Protamex derived from microorganisms, as well as enzymes from plant (e.g. Papain) and animal sources (e.g. Pepsin and trypsin), have been widely used in producing ACE inhibitory and antioxidative peptides. In the case of several plant proteins, the serine-type endoprotease, Alcalase has produced the highest ACE inhibitory activities *in vitro*. A simulated gastrointestinal digestion process could be a cost-effective method for producing ACE inhibitory peptides since extensive processing of the peptide product would not be needed (Vermeirssen *et al.*, 2004a). Several studies have demonstrated the release of potent ACE inhibitory peptides from plant proteins during digestion with either Pepsin alone or in combination with trypsin, chymotrypsin and/or Pancreatin. For example, digestion of red lentils and pea proteins with trypsin, Pepsin and chymotrypsin (Vermeirssen *et al.*, 2004b; Boye *et al.*, 2010; Barbana and Boye, 2011) has resulted in high ACE inhibitory potencies.

On the other hand, a number of studies have shown that antihypertensive peptides are liberated during fermentation. In particular, fermented milk products fermented with lactic acid bacteria strains have been found to possess antihypertensive and antioxidative capacity (Virtanen *et al.*, 2007; Pihlanto *et al.*, 2010). Furthermore, several antioxidative and ACE inhibitory peptide sequences have also been identified from fermented milk (Hernández-Ledesma *et al.*, 2005). Fermentation has also been shown to release ACE inhibitory and antioxidant peptides from plant proteins, such as soybean. For example, the traditional fermented soybean products natto, tempeh and douche contain antioxidative and ACE inhibitory peptides released by fungal proteases. However, enzymatic hydrolysis has been shown to produce higher potent bioactivities than fermentation especially from plant proteins.

2.4.1 Processing methods

A challenge frequently faced in food protein-derived peptide research is how to process the protein hydrolysates further in order to obtain high-yield peptide products with enhanced bioactivity. After protein hydrolysis, the resulting bioactive peptides can be concentrated based on physicochemical and structural characteristics of the peptides of interest, to enhance the bioactivity. The peptide properties that are often focused on include size, net charge and hydrophobicity, depending on the targeted bioactivities. The crude protein hydrolysate can be further processed by being passed through ultrafiltration membranes and size-exclusion chromatography in order to obtain a more uniform product with the desired range of molecular mass, especially to concentrate low molecular-weight peptides that are more resistant to further *in vivo* proteolytic digestion and more potential ACE inhibitors. In addition, reverse-phase chromatography on a hydrophobic column matrix can be used to fractionate peptides according to hydrophobicity, and ion exchange chromatography with selective columns can be utilized to produce peptide fractions of a particular net charge (Pownall *et al.*, 2010; Pownall *et al.*, 2011). These approaches are very advantageous especially when the peptide properties that inhibit the molecular disease targets are known – for example antioxidant activities of peptide fractions have been demonstrated to correlate negatively with cationic property (Pownall *et al.,* 2011). A novel membrane technology known as electrodialysis-ultrafiltration is useful for separating cationic, anionic and neutral peptides with defined molecular size on a large scale (Firdaous *et al.*, 2009). Recently, this method has been utilized to concentrate and separate low molecular-weight bioactive peptides with a net positive or negative charge (Doyen *et al.*, 2011a; Doyen *et al.*, 2011b). Absorbent materials, such as activated carbon, are applicable for enriching particular amino acids in food protein hydrolysates (Udenigwe and Aluko, 2010). This processing method can

result in sufficient yield if the amino acid residues of the target are prevalent in the peptides of interest. In addition, there are extensive bioassay-guided purification methods for the isolation of pure peptides for further analysis, especially for structure-function investigations. However, low peptide yield is still a limiting factor of the feasibility of using food protein-derived bioactive peptides. Therefore, it would be relevant to develop large-scale applicable food-grade processing methods for the production of a high yield of highly active peptide fractions. Understanding the unique structural characteristics of peptides with targeted bioactivity and exploitation of these characteristics in the concentration of the particular peptides is a crucial requirement for this approach.

2.5 Delivery and bioavailability of bioactive peptides

The *in vivo* effects of any suggested health-promoting agent in foods are dependent on their bioavailability. After consumption, they need to appear in the blood to a significant extent to reach the target sites, except for the target sites in the gastrointestinal tract. The bioavailability is determined by several variables, such as gastric digestion, metabolism by the intestinal microflora, absorption and interactions with the intestinal mucus and epithelium, plasma kinetics, the nature of circulating metabolites, cellular uptake and metabolism. A complex enzyme battery is involved in the hydrolysis of proteins and peptides in the human gastrointestinal tract. The enzymes are located in various sites and they generally network in a synergistic manner with other peptidases (Zhang *et al.*, 2011). The gastric digestion in the stomach starts with the action of Pepsin and is followed by the intraluminal digestion in the small intestine where the polypeptides are further cleaved by pancreatic proteases, such as trypsin, chymotrypsin, elastase and carboxypeptidase. The battery of brush border peptidases digests the polypeptides further at the intestinal brush border membrane, which is particularly rich in aminopeptidase activity. Furthermore, the microorganisms of the colon produce large numbers of peptidase enzymes which also participate in protein digestion. The small intestine is the principal site of absorption. After absorption to blood circulation peptides are exposed to serum peptidases which can further hydrolyze the peptide bonds (Zhang *et al.*, 2011). However, peptides that act in the gastrointestinal tract, for example cholesterol-binding and anorectic peptides, do not have to be absorbed to exert their biological properties (Wang *et al.,* 2005).

As human studies are time-consuming, costly and restricted by ethical concerns, the development of *in vitro* models for investigating the effects of digestion on the bioactive peptides has attracted much attention. A plethora of models have been implemented to simulate gastrointestinal digestion; most

typically models simulate digestion in the oral cavity, the stomach and the small intestine. There is substantial variability among the conditions, such as the time of digestion, agitation, enzymes and concentrations of the salts and bile acids used. Moreover, there is great differentiation in the inclusion of various digestion stages and whether the chosen conditions are static with fixed concentrations of enzymes and bile acids etc., or dynamic with varying concentrations of these constituents. For instance, human digestive liquids have been utilized to model digestion *in vitro* (Eriksen *et al.*, 2010; Mäkinen *et al.*, 2012), and meanwhile several reports have concerned the implementation of porcine enzyme mixtures (for example, Vermeirssen *et al.*, 2003; Lo and Li-Chan, 2005; Marambe *et al.*, 2011). The diversity of model conditions has hampered the ability to compare results across the different studies. Thus, a consensus concerning the basic parameters would be relevant in order to harmonize the various *in vitro* digestion models. Recently, *in vitro* models for studying the digestion of secondary plant metabolites (such as polyphenols) were reviewed by Alminger *et al.* (2014). A set of parameters for static *in vitro* models was suggested based on relevant *in vivo* data. Thus far, such a harmonization of parameters based on *in vivo* data has not been reported for modeling the bioavailability of peptides.

In general, small peptides – di-peptides and tri-peptides – have shown *in vivo* bioactivity and resistance to peptidolysis, and can be absorbed intact across the intestine in adequate amounts to reach peripheral target sites (Gardner, 1998; Foltz *et al.*, 2009; Darewicz *et al.*, 2011). Smaller peptides are transported across the enterocytes through intestinal-expressed peptide transporters whereas oligopeptides may be absorbed by passive transport through hydrophobic regions of membrane epithelia or tight junctions (Darewicz *et al*., 2011).

The need for technological tools for the improvement of bioavailability is reinforced, and the focus is on the improvement of limited stability and absorption peptides. Bioactive peptides can be administered in different vehicle matrices, such as beverages and bakery products (Hernández-Ledesma *et al.*, 2011). The current literature includes many strategies for enhancing the bioavailability of bioactive compounds (Nestor, 2009): for example, microencapsulation for controlled release of the active compounds, stabilization of the active molecules to improve the resistance against degradation, and the development of peptide analogues with enhanced stability (Matoba *et al.*, 2001; Yamada *et al.*, 2002; Gomez-Guillen *et al.*, 2011). In addition, amendment of fiber to the meal has been observed to improve the bioavailability of the tri-peptides Val-Pro-Pro, Ile-Pro-Pro and Leu-Pro-Pro (Kies and Van Der Pijl, 2012) and carriers interacting with the peptide molecule have been reported to facilitate intestinal uptake (Shaji and Patole,

2008). However, no general strategy for improving the bioavailability of antihypertensive peptides has been found thus far, and each case needs to be studied separately depending on the characteristics of the peptides of interest.

2.6 Hypotensive effects *in vivo* of plant protein-derived bioactive peptides

The published data demonstrates antihypertensive effects of ACE inhibitory protein hydrolysates and peptides derived from various plant sources. Several studies have utilized the SHR model for studying the potential *in vivo* hypotensive properties after either oral or intravenous administration. The hypotensive effects *in vivo* reported for plant protein-derived peptides and hydrolysates are summarized in Table 5. In addition to the investigations on SHR, published data includes observations of the antihypertensive effects of yam tuber (*Dioscorea alata*) protein in a clinical trial. Lin and co-workers (2006) purified and hydrolyzed yam tuber storage proteins, dioscorins, with Pepsin and demonstrated an antihypertensive effect firstly on SHR. At the same time, the unhydrolyzed dioscorin isolate also showed a significant antihypertensive effect in SHR (Table 5). Subsequently, the antihypertensive effect of the dioscorin isolate was evaluated in a clinical randomized, placebo-controlled crossover study in human subjects (Liu *et al.*, 2009). The dioscorin meal or placebo was administered as a daily drink, and a significant decrease in systolic and diastolic blood pressure was observed after the five weeks of dioscorin drink intervention (Table 5). The dioscorin isolates consisted mostly of protein, and thus the observed antihypertensive effects were related to peptide released from dioscorins during the gastrointestinal digestion. However, the nonprotein compounds may still have some minor effect on the results.

Table 6 The hypotensive effects *in vivo* of plant protein-derived hydrolysates and peptides

Source protein	***Enzyme or other process conditions***	***ACE inhibition IC50 mg/ml***	***Identified peptides***	***ACE inhibition IC_{50} value (μM)***	***Dose (mg prot./kg), in vivo model and administration***	***Change in systolic blood pressure Δ mmHg in comparison to placebo***	***Reference***
Apios Americana Medikus tuber	Water extract (rich in proline)	127			200 mg/kg, SHR, oral	-25 after 1h	Iwai and Matsue, 2007
Rapeseed protein	Pepsin	0.16			500 mg/kg	-7 after 4h	Marczak *et al.*, 2003
	Subtilisin	0.16			500 mg/kg	-16 after 4h	
			VW	1.6	7.5 mg/kg	-11 after 2h	
			VWIS	30	12.5 mg/kg	-13 after 2h	
			IY	3.7	7.5 mg/kg	-10 after 2h	
			RIY	28	7.5 mg/kg SHR, oral	-11 after 4h	
	Alcalase		TF	0.81	30 mg/kg, SHR, oral	-12 after 4h	He *et al.*, 2013b
			LY	0.11		-27 after 2h	
			RALP	0.97		-17 after 2h	
	Alcalase	~80% at 1mg protein/ml			200 mg/kg, SHR, oral	-24 after 8h	He *et al.*, 2013a
	Proteinase K					-5 after 8h	
	Pepsin+Pancreatin					-21 after 24h	
	Thermolysin					-9 after 8h	
	Flavourzyme					-17 after 6h	
	Rapeseed protein isolate					-11 after 6h	
	Pepsin+Pancreatin		GHS	0.52 mg protein/ml	30 mg/kg, SHR, oral	-18 after 6h	He *et al.*, 2013c
	3kDa permeate of Pepsin+Pancreatin hydrolysate	61% at 1mg/protein/ml			100mg/kg, SHR, oral	-21 after 4h	

Table 6 continued

Source protein	*Enzyme or other process conditions*	*ACE inhibition IC50 mg/ml*	*Identified peptides*	*ACE inhibition IC_{50} value (µM)*	*Dose (mg prot./kg), in vivo model and administration*	*Change in systolic blood pressure Δ mmHg in comparison to placebo*	*Reference*
Canola protein	Alcalase Chymotrypsin Pepsin Trypsin Pancreatin	~90% ~90% ~80% ~90% ~80%			200 mg/kg, SHR, oral	-35 after 4h -16 after 6h -24 after 4h no change -15 after 6h	Alashi *et al.*, 2014
Flaxseed protein	Trypsin & Pronase cationic peptide fraction	0.4	QGR RW SVR GQMRQPIQ QQG ASVRT DYLRSC ARDLPGQ RDLPG RGLERA TCRGLERA		200 mg/kg, SHR, oral	-18 after 2h	Udenigwe *et al.*, 2012
Pea protein	*in vitro* gastrointestinal digestion Thermolysin hydrolysate, 3kDa MWCO permeate	0.070 not determined			50 mg/kg, SHR, intravenous 200 mg/kg, SHR, oral 25-30 g/day, Han:SPDR-cy rats, oral Human, consumption 3g/day orally	-44, transient and sharp reduction -19 after 4h -30 at weeks 7 and 8 -6 after 3 weeks	Vermeirssen *et al.*, 2005 Li *et al.* 2011
Mung bean protein	Alcalase 6kDa MWCO permeate	0.64	KDYRL VTPALR KLPAGTLF	26.5 µM 82.4 µM 13.4 µM	600 mg/kg, SHR, oral	-31 after 6h	Li *et al.*, 2006

Table 6 continued

Source protein	*Enzyme or other process conditions*	*ACE inhibition IC50 mg/ml*	*Identified peptides*	*ACE inhibition IC_{50} value (μM)*	*Dose (mg prot./kg), in vivo model and administration*	*Change in systolic blood pressure Δ mmHg in comparison to placebo*	*Reference*
Mung bean sprout	Raw sprout extract Dried sprout extract Pepsin, trypsin and chymotrypsin	ND ND ND			600, SHR, intragastric	-41 after 6h -24 after 3h -29 after 3h	Hsu *et al.*, 2011
Maize, α-zein	Thermolysin		LRP LSP LQP	0.29 1.7 2.0	Peptide (LRP) 30 mg/kg SHR, intravenous	-15 after 2 min	Miyoshi *et al.*, 1991
Wheat bran	autolysis reactions from wheat milling by-products	0.08	LQP IQP LRP VY IY TF	Peptide fraction 0.14 mg/ml	10 mg/ml, oral, SHR	-45 after 2h	Nogata *et al.*,2009 Nogata *et al.*,2011
Sweet potato tuber protein isolate	Thermoase PC 10F , Protease S and Proleather FG-F	0.0182	 ITP IIP GQY STYQT	 19.5 80.8 52.3 300.4	500, SHR, oral	-30 after 8h	Ishiguro *et al.* 2012
Yam tuber	Dioscorin, lyophilized powder Dioscorin, purified by ion exchange chromatography Pepsin hydrolysate				154 mg/kg, SHR, oral 140 mg daily dosage, human, oral 40 mg/kg, SHR, oral	-7 after five weeks -22 after 4h -34 after 8h	Liu *et al.*, 2009; Lin *et al.*, 2006

2.7 Theory synthesis and hypothesis of the study

Cardiovascular diseases are a global health threat and improved nutrition is important to tackle the expected rise in global CVD incidence and societal burden. Hypertension constitutes a high risk factor for the development and causatives of CVD. Nutraceuticals, such as bioactive peptides, may help in the prevention of hypertension and CVD as a part of a healthy diet and lifestyle. ACE is an important agent causing hypertension and ACE inhibitors are widely used in the treatment of hypertension. At the same time, oxidative stress is related to numerous pathological conditions, and in particular, it induces cardiovascular and renal damage with associated with hypertension. Thus, ACE inhition and antioxidant effects are potential molecular targets for bioactive peptides to prevent hypertension and enhance cardiovascular health. Bioactive peptides can be produced from various protein sources and an important aspect in the selection of source materials is the utilization of abundant by-products from the food industry. Huge amounts of protein-rich by-products are formed in the potato starch industry and in the rapeseed oil industry, and due to the high-quality protein content, rapeseed meal and potato fractions are potential source materials for the production of bioactive peptides. Moreover, a vast amount of potato is discarded from the food industry and utilization of the discarded potatoes for the production of bioactive peptides could enhance the value of the entire production chain.

The hypothesis of the present study was that bioactive peptides with antioxidant and ACE inhibitory properties could be produced from rapeseed and potato proteins by developing an effective enzymatic hydrolysis process, and putatively as well by fermentation with proteolytic microbial strains. It was hypothesized that the ACE inhibitory peptides could be concentrated by physicochemical characteristics to enhance the ACE inhibitory capacity and potential bioavailability based on known structural characteristics related to these properties; small molecular size and the presence of hydrophobic amino acid residues are known to correlate with enhanced ACE inhibitory activity and increased bioavailability. It was also hypothesized that preliminary testing of the potential bioavailability of the ACE inhibitory peptides with a proper *in vitro* digestion model would offer valuable information on the potential of the peptides to induce antihypertensive effects *in vivo*. Furthermore, according to the synthezised theory, it was conceivable that studies on animals would be needed to evaluate the *in vivo* antihypertensive effect.

3 AIMS OF THE STUDY

The objective of this thesis was to evaluate the suitability and potential of the industrial potato fruit liquid and rapeseed meal for the production of bioactive peptides with ACE inhibitory and antioxidant properties. Furthermore, the aim was to evaluate the effects of the peptides on blood pressure *in vivo* in animals.

Briefly, the main aims of the present study were:

- To produce bioactive peptides with ACE inhibitory and antioxidant properties from by-products of rapeseed oil industry and potato starch industry by enzymatic hydrolysis and fermentation (I, II, III, IV)
- To concentrate the peptides by physicochemical and structural characteristics in order to enhance the ACE inhibition properties and potential bioavailability (I, III)
- To elucidate the bioavailability of the ACE inhibitory peptides by measuring the resistance of the peptides against peptidolysis in a digestion model *in vitro* using human digestive liquids (III)
- To study the potential of the peptide concentrate to prevent the development of hypertension *in vivo* in a 2K1C rat model (V)

4 MATERIALS AND METHODS

This section is a summary of the materials and methods used in the present study and detailed information is described in the original publications (I - V).

4.1 Sample material for production of potato protein-derived enzymatic hydrolysates with antioxidant and ACE inhibitory properties (I)

Mature potato tubers (*Solanum tuberosum*) of cv. *Lady Christl* and *Asterix*, new-season harvested "immature" tubers of cv. *Timo* and sprouted tubers of cv. *van Gogh* cultivars were purchased from a local supplier. In addition, industrial potato by-product fractions were obtained from companies producing starch and processed potato products. The fractions were: the particulate fraction called potato pulp, and the liquid part obtained after filtration of the pulp obtained from potato starch production, and peel obtained from the manufacture of potato products. The proteins from potato tubers were extracted by the aqueous extraction method described by Ralet and Gueguen (2000).

The proteolytic enzyme preparations used for the hydrolysis of potato proteins were Alcalase (2.4 U/mg, protease from *Bacillus licheniformis*), Esperase (8 U/mg, from *Bacillus sp*.) and Neutrase (0.8 U/mg, from *Bacillus amyloliguefaciens*) from Sigma Chemical (St. Louis, MO).

4.2 Sample material for production of potato protein-derived ACE inhibitory peptides by autolytic process (II)

For the autolysis study the potato cultivar *Asterix* was grown in the seed potato farm of Pohjoisen Kantaperuna Oy (Yppäri, Finland: latitude 64.40° north and longitude 24.09° east) and samples were taken at different stages of potato tubers' life cycle: (i) immature tubers 86 days post planting (dpp) (plants at flowering stage), (ii) tubers at early maturing stage at the time of haulm killing (112 dpp), (iii) mature tubers at harvest time (133 dpp), (iv) dormant tubers after 2.5 months and (v) 5 months in storage, (vi) tubers ready to sprout following 6.5 months in storage, and (vii) tubers stored for 6.5 months and sprouted for 14 days. At each sampling time, 5 kg of tubers was taken and a subsample of 1 kg including tubers of all sizes (diameter 30 - 70 mm) was used for analysis.

Proteins were extracted separately from whole-tuber samples, vascular tissue enriched fraction, pith fraction and peels (Figure 3) by aqueous extraction (Ralet and Gueguen, 2000) and frozen at -70 °C until analysis and autolysis

treatment. Protein contents of the protein extracts were measured by the DC Protein Assay (Bio-Rad Laboratories, USA) and dry matter was measured by drying 500 µl of extract in the oven at 102 °C for 20 h.

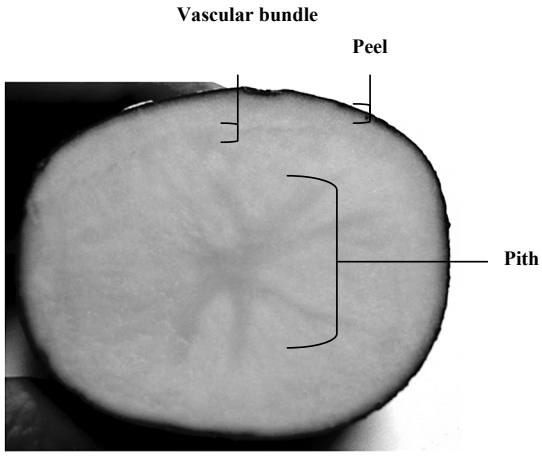

Figure 3 Peel, vascular bundle and pith fractions of the potato tuber used for analysis.

In order to elucidate the capacity of potato protein isolates to hydrolyze endogenous proteins into peptides with ACE inhibitory activity, peptidase activities were measured using p-nitroanilide amino acid substrates as described by Peltoniemi (2004). Prior to the analysis, samples were filtered through a 5 kDa membrane (Millipore) to remove interfering compounds such as salts and sugars.

4.2.1 Autolysis treatments

Firstly, the autolysis was performed at different temperatures, in various pH conditions and with different durations to evaluate the optimal conditions for autolysis. After this, protein isolates from different parts of potato tubers were subjected to autolysis separately and ACE inhibitory activities were measured during the autolysis. Furthermore, the autolysis was performed with the addition of potato proteins as putative sources of ACE inhibitory peptides. The autolysis treatments are presented schematically in Figure 4.

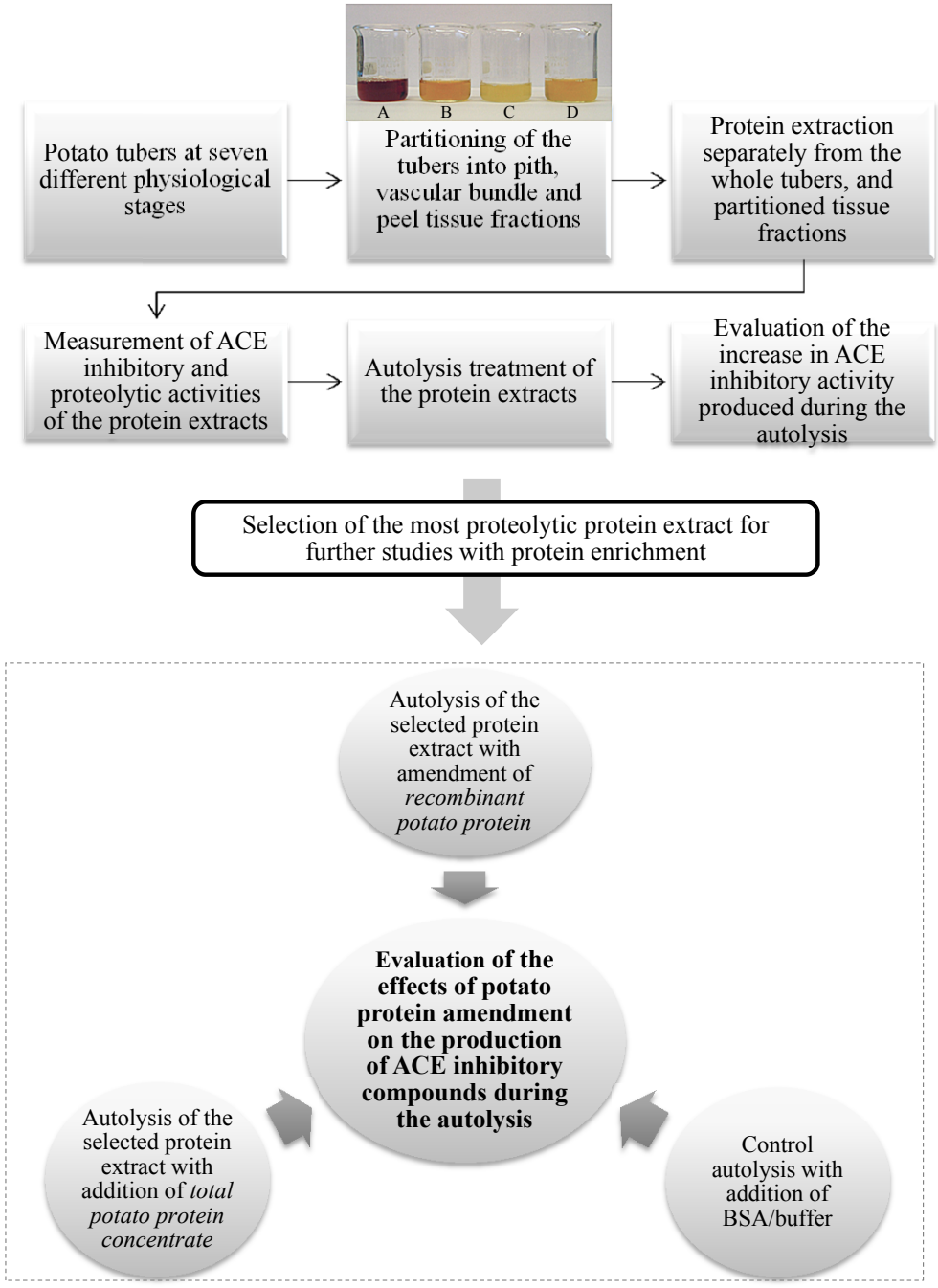

Figure 4 Implementation and different stages of the potato autolysis study. In the upper part, the solutions in the picture are protein extracts from peel (A), vascular bundle (B) and pith (C) fractions, and whole tubers (D).

Two types of protein preparate were used for amendment. The added proteins were purified recombinant potato protein produced in *Escherichia Coli*

predicted to produce six bioactive VPP tripeptides per molecule, and secondly a crude protein concentrate of potato proteins (Figure 4). The protein concentrate was a retentate from ultrafiltration of potato protein extract with a 10 000 Da MWCO membrane.

4.3 Sample material for production of rapeseed protein-derived bioactive peptides by means of enzymatic hydrolysis and fermentation (III, IV)

The rapeseed (*B. napus*) was a by-product from the rapeseed industry: milled rapeseed after oil recovery from Raisio Group Ltd., Finland. Protein isolation from the rapeseed meal was carried out using a method described by Bérot *et al.* (2005). The proteolytic enzyme preparations used for the hydrolysis of rapeseed protein were Alcalase (2.4 U/mg, protease from *Bacillus licheniformis*), trypsin (8.750 U/mg, trypsin from bovine pancreas), Pepsin (2.174 U/mg, Pepsin from porcine stomach mucosa), Subtilisin (11 U/mg, protease Subtilisin Carlsberg) and Thermolysin (42 U/mg, Thermolysin from *Bacillus thermoproteoliticus*) from Sigma Chemical (St. Louis, MO). Fermentation was performed with four proteolytic microbial strains: *Lactobacillus helveticus* CNRZ32, *Lactococcus lactis* ssp. *lactis* 20481, *Kluyveromyces marxianus* ATCC 28244 and *Bacillus subtilis* ATCC 21394, for 48 h at 30 °C. The ACE inhibitory and antioxidant activities were followed during the hydrolysis and fermentation treatments.

4.4 Sample material for producing rapeseed protein-derived peptide fraction for *in vivo* measurement of antihypertensive effects on 2K1C animals (V)

Rapeseed (*B. napus*) meal was obtained after industrial oil recovery from Raisio Group Ltd., Finland. Two different batches of milled rapeseed were used for the *in vivo* studies; the batches originated from two separate harvesting seasons. The protein extraction was performed separately from the two batches according to Bérot *et al.* (2005).

Prior to the *in vivo* studies on animals, rapeseed protein extracts were hydrolyzed with Alcalase and the hydrolysate was subjected to a solid-phase extraction and membrane filtration in order to produce a concentrate of small hydrophobic rapeseed peptides. The solid-phase extraction was performed with Sep-Pak C18 cartridges and the retained compounds were eluted with a stepwise gradient (15% and 60%) of ACN and 0.1% TFA in water. The fraction which was eluted between 15 and 60% ACN, was further subjected to ultrafiltration with a 3 000 Da MWCO membrane. Permeates were pooled,

dried under vacuum and stored at -20 °C until analysis. The peptide concentrates were prepared separately for the *in vivo* animal test phase 1 and phase 2 and were designated as RPHF ph1 (used for animal study phase 1) and RPHF ph2 (respective peptide fraction for animal study phase 2).

4.5 Measurement of selected bioactivities *in vitro* (I, II, III, IV, V)

4.5.1 Determination of ACE inhibitory activity

The method used for determining ACE inhibitory activity was that described by Hyun and Shin (2000). The IC50 value was defined as the concentration of peptide in mg protein/ml required to reduce the height of the hippuric acid peak (50% inhibition of ACE). ACE inhibition mechanisms of selected peptide fractions were estimated by means of enzyme kinetics and the Lineweaver–Burk plot (III). Inhibition types and kinetic parameters were estimated by a nonlinear regression fit using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

4.5.2 Determination of inhibitory activity on lipid peroxidation (III, IV)

The capacity of hydrolysed or fermented rapeseed samples to inhibit lipid peroxidation was tested with soybean phosphatidylcholine liposomes in an Fe-ADP system. Liposomes were prepared according to the method of Ursini *et al.* (1982) and malondialdehyde equivalents (MDAeqv) were measured according to the methods of Tirmenstein *et al.* (1998) and Harper and Hadley (1990) with some modifications. Briefly, liposomes were mixed with sample and oxidative agent (Fe-ADP) in a phosphate buffer solution in various sample concentrations. The concentration of the thiobarbituric acid reactive substances (TBARS) formed during liposome oxidation was determined by a color reaction with thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) (532 nm). The concentration of TBARS was calculated using the malondialdehyde (MDA) standard curve prepared. A control sample was prepared using a corresponding procedure without sample amendment and vitamin E was used as a positive control for inhibition. The oxidation inhibition percentage was defined as follows: $[(MDA_{control}\Delta 48h – MDA_{sample}\Delta 48h)/MDA_{control}\Delta 48h]\times 100\%$.

4.5.3 Scavenging activity of ABTS radical by spectrophotometry and total radical-trapping potential method (TRAP) (I)

The scavenging activity of potato hydrolysates against the ABTS radical [2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] was determined by the decolorization assay of Re *et al*. (1999). The potato hydrolysates with the highest radical scavenging activities were measured by the TRAP method described by Lissi *et al.* (1995).

4.6 Measuring the resistance of ACE inhibitory peptides to gastrointestinal digestion (III)

To mimic digestion in the stomach and duodenum, an *in vitro* digestion assay was carried out as described by Almaas *et al.* (2006). The tested sample was rapeseed protein-derived Alcalase hydrolysate. The digestion was performed by shaking samples with human gastric juice at pH 2 and subsequently with human duodenal juice at pH 8, both steps for 30 min at 37 °C. To analyze the degradation of rapeseed hydrolysate proteins during the digestion, the samples withdrawn during the digestion treatment were subjected to protein electrophoresis. ACE inhibitory activity was followed during the treatment.

4.7 Characterization of the samples (II, III, IV)

4.7.1 Size-exclusion chromatography (III, IV)

The effects of hydrolysis and fermentation treatments on the molecular size distribution of rapeseed samples were determined using size-exclusion chromatography. Molar mass distribution was determined using a calibration curve obtained using b-lactoglobulin (18 400 Da), aprotinin (6 512 Da), bacitracin (1 410 Da), Hip–His–Leu (430 Da) and Leu–Gly (188 Da).

4.7.2 Two-dimensional (2D) gel electrophoresis (II)

The effect of autolysis treatment on the protein compositions of potato protein extracts was measured by 2D gel electrophoresis. Potato protein samples were first subjected to an isoelectric focusing after which the migrated proteins were separated according to molecular size with SDS-PAGE. A mass spectrometry-compatible silver-staining protocol was used to visualize the proteins. In the analysis of tuber pith fraction, the samples were purified by ultrafiltration with a 3 000 Da MWCO membrane before analysis to remove

interfering compounds such as salts and sugars; the retentate was subjected to the analysis.

4.7.3 Analysis of proteins from 2D gels by mass spectrometry (MS) (II)

Selected proteins from 2D gel spots were analyzed at the Turku Centre for Biotechnology (Turku, Finland) in order to identify the proteins degraded during the autolysis treatment as putative sources of ACE inhibitory peptides. Protein spots were picked with a scalpel, washed with NH_4HCO_3/ACN to remove impurities and subjected to an in-gel trypsin digestion after which peptides were desalted by a solid-phase extraction with a C18 membrane (3M). Desalted samples were analyzed with MALDI-TOF/TOF with Matrix Science Mascot Search program (Ohio Supercomputer Center, Columbus, OH). The Swiss-Prot protein knowledgebase 51.7 (http://www.expasy.org/sprot/) was used to search amino acid sequences of known proteins.

4.8 Characterization and identification of ACE inhibitory peptides (I, IV, V)

4.8.1 Concentration of peptides (I, III, IV)

Solid-phase extraction with Sep-Pak C18 cartridges (Waters, Milford, USA) was utilized in order to diminish nonprotein compounds such as salts from the samples prior to the chromatographic purification of peptides. Moreover, solid-phase extraction was utilized for prefractionation of the peptides according to the hydrophobic properties. The retained compounds were eluted with a stepwise gradient (10, 30 and 60 %) of ACN. Ultrafiltration was used for further concentration of the ACE inhibitory and antioxidant peptides. In the case of potato samples, ultrafiltration was performed with 3 000, 5 000 and 10 000 Da MWCO membranes, whereas with rapeseed samples 3 000 Da MWCO was used. Retentates and permeates were subjected to analysis of protein content and bioactivities.

4.8.2 Size-exclusion chromatography (IV)

To elucidate whether the observed bioactivities of the fermented rapeseed samples were due to peptides produced during the fermentation, fractions were collected from the size-exclusion chromatography. Fractions were collected at 2 min intervals using automated fraction collector, concentrated by freeze-drying and analyzed for inhibitory capacities against ACE and lipid peroxidation.

4.8.3 Reversed-phase HPLC (I, V)

In order to analyze the effect of enzymatic hydrolysis on proteins, samples were analyzed by RP-HPLC with a C18 column matrix. Fractions were collected from the RP-HPLC analysis to see whether the observed bioactivities were due to peptides. The fractionated samples were Alcalase hydrolysate of potato proteins, potato fruit liquid, and concentrate of hydrophobic small rapeseed peptides from the Alcalase hydrolysate of rapeseed proteins. To purify the ACE inhibitory peptides, the fractions with the highest ACE inhibitory activities were successively rechromatographed. The purified peaks were subjected to identification with mass spectrometry and the amino acid compositions of the peaks were analyzed by the Pico-Tag method (Millipore Corporation, 1987). The isolated peptide peaks were analyzed by a MALDI-TOF/TOF instrument at the Institute of Biotechnology, University of Helsinki (potato samples) and at the Department of Food Science, Aarhus University, Denmark (rapeseed samples).

4.8.4 Comparison of the peptide compositions between the two rapeseed peptide concentrates (V)

The peptide compositions of RPHF ph1 and RPHF ph2 – the fractions tested on animals – were compared using UPLC-qTOF analysis. The presence of selected previously identified ACE inhibitory peptide sequences in the samples was measured by a targeted peptide search. The peptide sequences Arg-Ile-Tyr, Thr-Phe, Leu-Tyr and Arg-Ala-Leu-Pro were selected for the search according to their hypotensive properties reported in the literature and their prevalence in the primary sequences of the rapeseed proteins napin and cruciferin. The y-type and b-type fragmenting ions of the peptides were used for verification in the fragmenting spectrum.

4.9 Evaluation of the antihypertensive effects *in vivo* (V)

The capacity of rapeseed peptide concentrates (RPHF ph1 and RPHF ph2) to prevent the development of hypertension was studied in 2K1C rats. Rat blood pressure transmitters were used to directly record arterial pressure and heart rate. Blood flow to the left kidney was restricted by the Goldblatt model of hypertension (two kidney one clip, 2K-1C) leading to hypertension due to renal ischemia (Goldblatt, 1964). The experiments were divided into two study phases. In the first phase, the model was established and appropriate dose ranges for oral administration were determined for the RPHF ph1 and a positive control treatment, captopril. Based on the results of the first phase, the second study phase was established. The total observation duration was 28 days.

4.10 Statistical analysis

All samples were tested in triplicate, the analyses were repeated and experiments were carried out at least twice. One-way ANOVA was used to compare the mean ACE inhibitory activities between treatments and samples.

In animal trial phase 2 the normality of the data was tested with the Shapiro-Wilk and Equal Variance tests. The statistical significance of differences in the mean values of hemodynamic parameters was tested using one-way ANOVA followed by the Holm-Sidak Method as a post hoc test. When needed, Kruskal-Wallis one-way ANOVA followed by Dunn's Method as a post hoc test was used.

5 RESULTS AND DISCUSSION

5.1 ACE inhibitory and antioxidant properties of potato protein-derived hydrolysates (I)

5.1.1 ACE inhibition activity

ACE inhibitory activities of the potato tuber protein extracts and industrial by-product fractions before and after hydrolysis are presented in Table 7. Generally, the ACE inhibitory capacities of the hydrolysates were high with IC50 values between 0.018 and 0.086 mg protein/ml. For comparison with plant protein-derived ACE inhibitory IC50 values presented in the current literature, 0.076 mg protein/ml and 0.14 mg protein/ml have been reported for pea and rice protein-derived hydrolysates, respectively (Vermeirssen *et al.*, 2003; Li *et al.*, 2007). In the present study, the highest activities were obtained with Alcalase hydrolysis, although the differences between the tested proteases were minor and not statistically significant ($p > 0.5$). Exogenous enzymes were needed to enhance the ACE inhibitory activity of the protein extracts from mature tubers, but protein extracts from immature tubers were found to be a particular exception as ACE inhibitory activity increased during autolysis without an added enzyme (from 14 to 41%). The result indicated that immature potato tubers contain endogenous proteases that are able to digest potato proteins into ACE inhibitory peptides. Overall, the ACE inhibitory activities of hydrolyzed liquid fraction were significantly higher than hydrolysates of protein extracts from tubers.

However, rather high ACE inhibitory capacity was observed in the liquid fraction already before hydrolysis, meaning that, during processing, compounds such as phenolic acids, alkaloids and amino acids – in addition to proteins – are liberated to the liquid fraction and ACE inhibitory activity might be partly due to nonprotein components.

Thus, the liquid fraction from the potato starch industry was subjected to purification of ACE inhibitory peptides by means of isolating the active peptides and ensuring the role of peptides in the ACE inhibition. The liquid sample was treated with Sep-Pak cartridges prior to fractionation with RP-HPLC in order to diminish the nonprotein compounds and concentrate the sample. The RP-HPLC profile showed rather high complexity of the sample, and ACE inhibition activity was observed throughout the first 30 min of elution, indicating that the sample contained various ACE inhibitory peptides with different hydrophobic properties. The most active peaks were successively re-chromatographed, and as a result, three purified peptide peaks were subjected to MALDI-TOF/TOF analysis, which showed two ions with molecular masses

of 704.2 and 850.5 Da. It could be speculated how the current database contents and increased information on potato proteins could enhance the identification result. The size of ACE inhibitory peptides is known to vary and several ACE inhibitory peptides from other sources with corresponding mass with the detected ions have been identified (Pihlanto and Korhonen, 2003). However, the nonprotein compounds may still have some effect on the results.

In comparison to the peptides which have induced antihypertensive effects in clinical trials, the most extensively studied ACE inhibitory peptides ILE-PRO-PRO and VAL-PRO-PRO from milk have shown respective IC_{50} values of 0.0019 and 0.0066 mg protein/ml indicating around tenfold higher ACE inhibition potential in comparison to potato protein hydrolysates. However, the potato samples are complex mixtures of diverse peptides and thus, the comparison with the ACE inhibitory activity of pure peptides is not straightforward. It also needs to be taken into account, that the *in vitro* ACE inhibitory capacity and antihypertensive effects *in vivo* do not correlate distinctly and thus, the potato peptides need to be investigated *in vivo* to measure the physiological effects on blood pressure.

5.1.2 Antioxidant activity

The radical scavenging activity of the protein extracts as well as by-product fractions before and after hydrolysis treatments are presented in Table 7. In general, enzymatic hydrolysis enhanced the scavenging activity, except for sprouted tubers and there were no statistically significant differences among the tested proteases. The radical scavenging value expressed as IC50 µg dry matter/ml was 15 for the most active hydrolysate, the Alcalase digest of the liquid fraction after 2 h of hydrolysis. For comparison, the IC50 value of positive control, Trolox, was 3 µg/ml. Overall, the liquid fraction produced significantly higher activities than the protein extracts. The results of the ABTS measurements were confirmed by the TRAP method, which gave values corresponding to those obtained by the ABTS method (oxidation time data not shown). The most active sample was the potato liquid Alcalase hydrolysate, which produced an antioxidant capacity of 0.48 g dry matter equivalent to 1 mmol Trolox. Comparison of the present results to those reported in the literature for potato samples is not straightforward due to several different methods being used for testing antioxidant activity. Furthermore, the results are also expressed in a variety of ways, making comparison difficult. The main tuber storage protein, patatin, and its hydrolysates prepared by Amano P and Pancreatin treatments have been found to exhibit antioxidant activity in a series of *in vitro* tests (Kudoh *et al.*, 2003; Liu *et al.*, 2003). Moreover, Wang and Xiong (2005) observed that hydrolysis of potato proteins by Alcalase increased their radical scavenging activity. During enzymatic treatment, the increased

scavenging activity probably correlates with the structural changes of proteins and release of peptides. It has been reported that accessibility to the oxidant-antioxidant test system is greater for small peptides and amino acids than for large peptides and proteins (Moosman and Behl, 2002). However, the role of nonprotein compounds in the observed antioxidant activity cannot be neglected. Previous research has shown that potato contains antioxidative compounds, such as ascorbic acid (Dale *et al.*, 2003) and phenolic compounds, but fairly low antioxidant activity has been obtained by the TOSC assay (Friedman, 1997; Chu *et al.*, 2002). According to current published data, the radical scavenging activity of the peel fraction was most likely due to the phenolic compounds (Nara *et al.*, 2006).

Table 7 ACE inhibitory and ABTS radical scavenging properties of potato protein extracts, by product fractions and hydrolysates

Sample and treatment	***ACE inhibition***			***Radical scavenging***		
	Inhibition[a] %		IC50 mg protein/ml	Scavenging[b] %		IC 50 mg dry matter/ml
	beginning	5 h	5 h	beginning	5 h	5 h
Protein isolates						
Mature, stored tubers (cv. *Lady Christl*)	22.4 ± 2.2	21.4 ± 0.9	nd	5.6 ± 0.7	4.2 ± 0.4	nd
Alcalase		73.7 ± 0.4	0.050		51.0 ± 0.2	27
Esperase		60.2 ± 0.2	0.077		19.9 ± 1.1	nd
Neutrase		53.6 ± 0.6	0.086		16.1 ± 0.6	nd
"Immature tubers" (cv. *Timo*)	13.9 ± 0.2	41.4 ± 0.9	0.079	18.2 ± 2.2	11.5 ± 0.3	nd
Alcalase		49.3 ± 1.9	0.064		39.4 ± 0.3	nd
Esperase		47.9 ± 1.1	0.067		nd	nd
Neutrase		44.5 ± 0.7	0.071		nd	nd
Sprouted tubers (cv. *van Cogh*)	52.5 ± 0.3	60.7 ± 0.8	0.686	37.3 ± 0.2	25.2 ± 0.4	nd
By-products						
Pulp	57.0 ± 0.1	57.4 ± 0.1	0.042	28.3 ± 0.8	30.8[c] ± 0.1	nd
Alcalase		50.6 ± 0.8	0.034		58.9[c] ± 4.0	661
Esperase		86.0 ± 0.6	0.023		54.2[c] ± 1.9	785
Neutrase		74.5 ± 1.8	0.027		49.3[c] ± 1.9	851
Liquid	64.4 ± 0.4	49.5 ± 0.6	0.070	31.0 ± 1.2	45.3 ± 0.2	28
Alacalase		94.2 ± 0.1	0.018		79.3 ± 6.1	15
Esperase		93.2 ± 0.2	0.018		57.5 ± 0.4	23
Neutrase		93.8 ± 0.2	0.025		63.6 ± 2.5	19
Peel fractions (cv. *Asterix*)						
Mature tubers	53.3 ± 0.07	60.0 ± 3.8	nd	41.8 ± 3.2		
From industry	75.0 ± 0.80	70.8 ± 2.1	nd	48.0 ± 3.1		
Ile-Pro-Pro			0.0019			
Val-Pro-Pro			0.0066			
Captopril			0.15 µM			
Trolox						3

Mean values ± standard deviations are shown. [a] Protein concentration 0.07 mg/ml; [b] Dry matter concentration varied between 13.4 and 56.4 µg/ml; [c] Dry matter concentration 824.3 µg/ml

5.1.3 Production of ACE inhibitory peptides from potato protein extracts by autolysis (II)

Potato protein extracts of whole tubers, vascular tissue-enriched fractions, pith fractions and peel fractions of the tubers at different physiological stage were autolyzed and the effect of autolysis treatment on ACE inhibitory activity was measured. Prior to autolysis, the proteolytic activities of the protein extracts were measured with amino acid *p*-NA substrates in order to characterize the peptidase activities of the extracts. The results showed that both physiological age and tuber tissue fraction affect remarkably the ACE inhibition production during the autolysis as well as in the peptidase activities (Figure 5). In general, the highest increase in ACE inhibitory activity was found in the pith fractions in comparison to whole tubers, and vascular tissue fractions. The autolytic activity was at its highest in the dormancy braking stage after five months' storage and prior to sprouting (Figure 5B). After sprouting the autolytic activity was transferred into the vascular tissue fraction (Figure 5C).

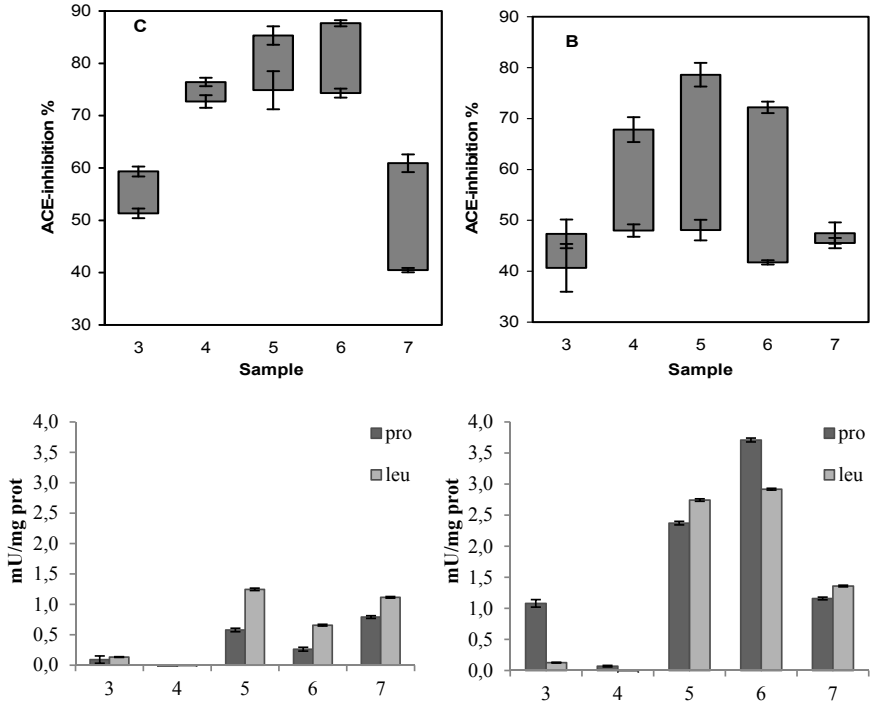

Figure 5 Increase of ACE inhibitory activity following autolysis of protein extracts and the respective peptidase activities from (B) pith fractions and (C) vascular tissue-enriched fractions. The lower edge of the ACE inhibition bar shows the activity prior to autolysis and the upper edge after autolysis. Tubers were sampled at different physiological ages: 3 harvest time; 4 dormancy; 5 dormancy braking; 6 prior to sprouting; 7 sprouted tubers.

Proteolytic activities of the protein extracts were in positive correlation with production of the ACE inhibitory activity. The highest peptidase activities were observed at dormancy braking prior to sprouting, whereas in the dormant stage tubers the proteolytic activity was at its lowest (Figure 5). The peptidase activities were concentrated in the innermost part of the tubers (Figure 5B) although during the dormancy braking and sprouting peptidase activity was also detected in the vascular tissue fractions (Figure 5C). The peptidase activities were highest in proline- and leucine-derived substrates among the tested amino acid derivatives, which is particularly interesting as proline at the C terminus of peptides seems to be especially effective in increasing the ACE inhibitory potential (Murray and FitzGerald, 2007).

Taken together, the data suggested that the bulk of autolytic activity resulting in enhanced ACE inhibition is largely confined to the internal part of the tuber consisting of inner phloem and pith tissue. Thus, peeling potatoes or storing them until the break of dormancy does not lower their value as a source of ACE inhibitory peptides. The current literature shows consistently that the developmental stage and physiological age of potato tubers are associated with significant alterations in the protein composition of potato tubers (Borgmann *et al.*, 1994; Desires *et al.*, 1995a; Desires *et al.*, 1995b; Espen *et al.*, 1999; Kumar *et al.*, 1999; Lehesranta *et al.*, 2006). The results of the present study associate these differences in the production of ACE inhibitory activity.

5.1.3.1 Protein enrichment in the autolysis reaction

The protein extract with the highest proteolytic activity and the greatest increase in the ACE inhibitory activity during the autolysis was amended with a recombinant potato protein predicted to produce six Val-Pro-Pro peptides per molecule. Addition of the recombinant protein resulted in a significant increase ($p < 0.01$) in the ACE inhibition activity during the autolysis (Figure 6). The observed effect was dependent on the concentration of recombinant protein: a 5 % addition produced an increase of 10.4 % in ACE inhibition in comparison to nonamended autolysis whereas a 2 % addition resulted in a 5 % increase (Figure 6 A,B). For comparison, the addition of BSA increased the ACE inhibition only slightly. The effect of total potato protein concentrate addition enhanced the production of ACE inhibitory compounds during the autolysis consistently. The addition of the total protein concentrate resulted in a 10 % ($p < 0.01$) increase in the ACE inhibition (Figure 6 C). The added protein concentrate was a retentate from membrane filtration with 10 000 Da MWCO of the corresponding protein extract of pith fraction.

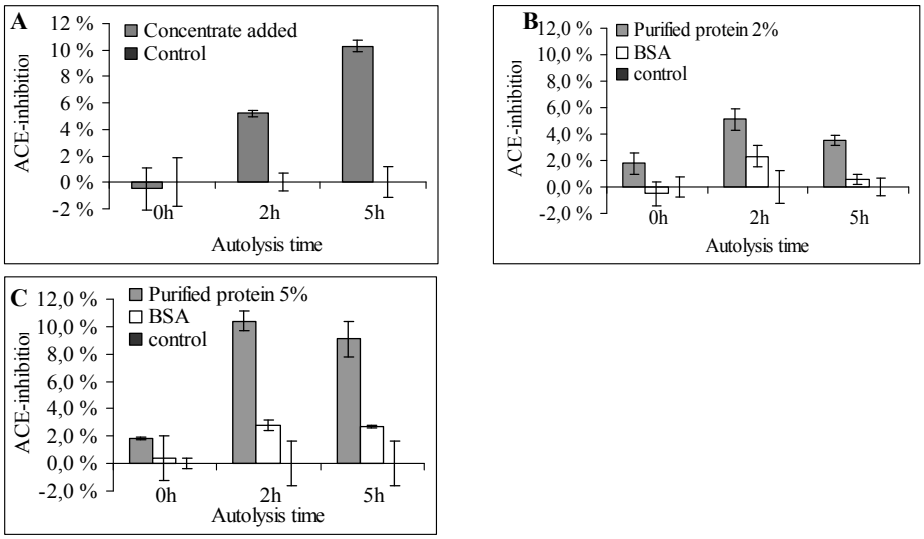

Figure 6 Effects of the addition of purified recombinant protein (A) 5 or (B) 2% (w/v) or tuber pith fraction protein concentrate (C) 10% (w/w) on the production of ACE inhibitory activity during autolysis treatment. Buffer was added as the control, and the relative changes in ACE inhibition activity were compared to this control sample. Thin bars indicate standard error (three autolysis reactions with two replicates; n=6).

Taken together, amendment of putative protein sources for the release of bioactive ACE inhibitory peptides enhanced significantly ACE inhibition production during autolysis. As little as 2 to 5 % of the recombinant protein in the total protein content, caused a significant increase in ACE inhibition. Thus, the results show conceivably that the observed increase of ACE inhibition was due to bioactive peptides released from proteins during autolysis. However, nonprotein compounds such as phenolics or salts in the protein extracts could also play a minor role in the inhibition of ACE.

5.1.3.2 Identification of proteins degraded during autolysis

Proteins of the most proteolytic pith fraction were analyzed by 2D electrophoresis before and after autolysis to detect putative sources of bioactive peptides associated with the observed ACE inhibition. Many protein spots were observed to diminish during the autolysis treatment (Figure 7). A group of proteins that corresponded approximately to 20 kDa and was very pronounced prior to autolysis was hardly detectable after autolysis. Two degrading proteins were identified as potato aspartic protease inhibitors. This indicates that endogenous potato proteases can degrade potato protein inhibitors into ACE inhibitory peptides when released from cellular structures. According to published data,

various protein inhibitors constitute up to 50% of the total tuber proteins (Pouvreau *et al.*, 2001; Lehesrant *et al.*, 2006), which makes them a very potential substrate for the release of ACE inhibitory peptides. The protein inhibitors target different classes of proteases and they have also been reported to degrade during the ageing of potato tubers non-enzymatically (Kumar *et al.*, 1999). Furthermore, we observed a consequent increase in the proteolytic activity during ageing. In summary, there is scope for native endogenous proteases to be active and use protease inhibitors as substrates for the release of bioactive peptides.

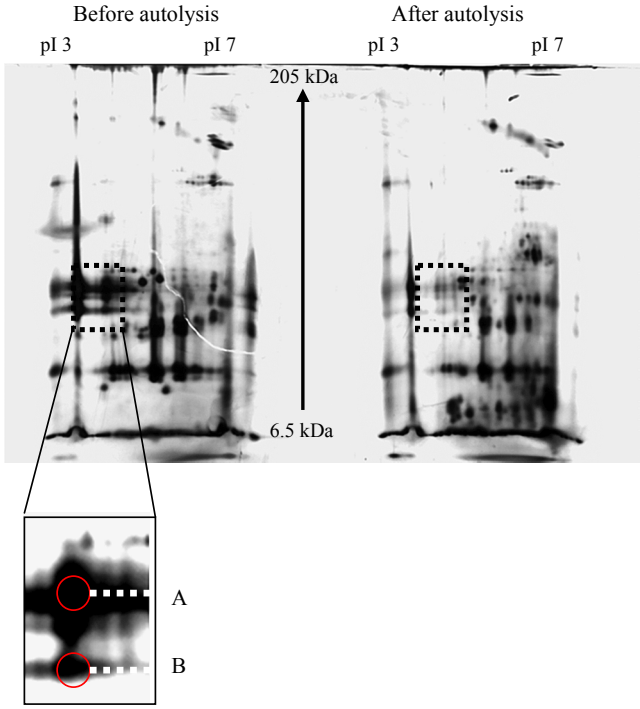

Figure 7 Degradation of proteins during the autolysis of potato tuber pith fraction protein extract. The proteins in the marked area were identified as aspartic proteases. M = protein size marker.

5.2 ACE inhibitory properties of rapeseed protein-derived hydrolysates and fermented samples (III, IV, V)

5.2.1 Chemical characterization of samples (III)

The proximate composition of the rapeseed by-product was 35% dry matter, of which 88% was protein, and 7 µmol/g glucosinolates, and the content of condensated tannins was 174 mg/100 g fresh weight. For comparison, Naczk *et al.* (1994) reported tannin concentrations of a few hundred mg/100 g fresh weight of hulls. Among the seven different glucosinolates identified,

progoitrine (4.5 μmol/g) and gluconapine (1.6 μmol/g) were the dominant ones. Glucosinolates and tannins were diminished during the hydrolysis treatment, as no glucosinolates or condensed tannins were detected after hydrolysis. Small amounts (<100 μg/ml) of sinapic acid derivatives and some unknown flavonoids were present in the hydrolyzed samples.

5.2.2 ACE inhibitory activity of the hydrolyzed and fermented samples (III, IV).

The ACE inhibitory activities *in vitro* of rapeseed samples after hydrolysis and fermentation treatments are presented in Table 8. All enzymatic hydrolysates showed high ACE inhibitory activity of which the Alcalase hydrolysate had the highest ACE inhibitory capacity with an IC50 value of 0.02 mg protein/ml. Differences between the tested enzymes were minor. For comparison to other published data, Marczak *et al.* (2003) reported an IC50 value of 0.16 mg protein/ml for rapeseed Subtilisin hydrolysate and He *et al.* (2013a) reported ACE inhibitory activities around 80% for rapeseed protein-derived hydrolysates at 1 mg protein/ml. The glucosinolates and phenolic compounds were found to diminish during the hydrolysis treatment, but in any case, the effects of other nonprotein compounds such as salts and free sugars in the ACE inhibitory measurement cannot be neglected.

Table 8 ACE inhibitory activities and inhibition of lipid peroxidation by rapeseed protein-derived hydrolysates and fermented samples (n $\geq$ 3)

Sample	***ACE inhibition***	***Inhibition of lipid peroxidation***	
	IC_{50} mg/ml protein	Inhibition %[b]	IER[c] (%/mg/ml)
Hydrolysates			
Pepsin pH 2 40 °C 3 h	0.06	65.0 ± 2.2	139
Subtilisin pH 8 60 °C 3 h	0.07	62.5 ± 4.4	125
Alcalase pH 7 55 °C 3 h	0.02	77.3 ± 0.3	145
Thermolysin pH 8 60 °C 3 h	ND	59.6 ± 5.7	119
Trypsin pH 7 40 °C 3 h	ND	72.8 ± 5.0	151
Control pH 7 40 ˚C 3 h	ND	67.6 ± 0.4	195
Fermented samples			
B.subtilis 48 h	0.58	100 ± 2.1	17.9
K.marxianus 48 h	ND	100 ±12.4	20.8
Lact. helveticus 48 h	0.42	100 ±10.7	20.8
L.lactis 48h	ND	ND	ND
Captopril	1.3×10^{-6}		

[a]The protein concentration in the analysis was 1mg/ml; [b]the protein concentration in the analysis varied between 0.35 and 0.58 mg/ml; [c]IER, inhibitory efficience ration = % inhibition/protein content.

The effects of *L. helveticus*, *B. subtilis*, *L. lactis* and *K. marxianus* fermentation treatments on the ACE inhibition and antioxidant activites are presented in Table 8. Lactic acid bacteria grew well in the medium as the pH decreased substantially from 6.0 to 3.7 during fermentation. The highest increase in ACE inhibition capacities was found with *L. helveticus* and *B. subtilis* with IC50 values of 0.42 mg protein/ml and 0.58 mg protein/ml, respectively. In the published data, *L. helveticus* strains are widely reported for their ability to release ACE inhibitory peptides and for their strong proteolytic effects. The proteolytic effects of the fermentation treatments on the rapeseed proteins are presented in Table 9 on page 70. Characterization of the samples. The importance of strong proteolytic activity has been related to the ability of certain strains to produce bioactivities such as ACE inhibitory peptides. However, it should be noted that higher activities have been found when further enzymatic treatment is combined with fermentation (Vermeirssen *et al.*, 2003; Gibbs *et al.*, 2004), and moreover, enzymatic hydrolysis alone has been shown to produce potent antihypertensive hydrolysates from plant proteins, as was also observed in the present study. Altogether, the results consistently show the high potential of rapeseed meal as a source of ACE inhibitory peptides and the efficacy of enzymatic hydrolysis, especially with Alcalase, for the release of ACE inhibitory peptides.

5.2.3 Concentration of ACE inhibitory peptides (III)

The peptides in the rapeseed protein-derived Alcalase hydrolysate were subjected to concentration with solid-phase extraction and ultrafiltration. ACE inhibitory compounds were slightly concentrated in the hydrophobic fraction eluted between 30% and 60% solvent B (90% ACN, 0.1% TFA), as the IC50 value of this fraction was 0.13 mg protein/ml, whereas the corresponding value for the fraction eluted between 10% and 30% solvent B was 0.27 mg protein/ml. Both fractions were subjected to ultrafiltration with a 3 000 Da MWCO membrane in order to separate small peptides from intact proteins and large polypeptide chains. As a result, the ACE inhibitory peptides were efficiently concentrated into the permeate of the hydrophobic fraction with an IC50 value of 0.08 mg protein/ml. Meanwhile, the IC50 value for the permeate of the less hydrophobic fraction was 0.32 mg/ml. According to current literature, hydrophobic amino acids play an integral role in ACE inhibition and on the other hand, ACE inhibitory peptides are generally short sequences as the active site of ACE cannot accommodate large peptide molecules. The results of the present study support the previously published data, as the ACE inhibitory activity was found to increase in positive correlation with hydrophobicity and small size.

5.2.4 Mechanism of ACE inhibition (III)

All rapeseed samples showed mixed-type inhibition characteristics. The K_i value, which indicates the affinity of peptides to the active site of ACE, was for the concentrate of small hydrophobic rapeseed peptides 0.154 mg protein/ml. For comparison to the reported literature, fermented whey has shown a K_i value of 0.188 mg/ml (Tsai *et al.*, 2008b) and a flaxseed protein hydrolysate of 0.0128 mg/ml (Udenigwe *et al.*, 2009). On the other hand, the hypotensive drug Captopril has shown a Ki value of 0.0067 mg/ ml (Tsai *et al.*, 2008b) indicating a much stronger affinity to the active site of ACE. In general, the most frequently observed type of ACE inhibitory mechanism has been competitive, for example Captopril is known to be a competitive inhibitor. However, noncompetitive and uncompetitive ACE inhibition mechanisms have also been reported (Pedroche *et al.*, 2002; Barbana and Boye, 2010). The mixed-type inhibition of rapeseed samples of the present study was presumably due to the heterogeneity of the samples indicating the presence of various peptides with different inhibition characteristics.

5.2.5 Resistance of ACE inhibitory activity to proteolysis in the digestion model (III)

The rapeseed protein-derived Alcalase hydrolysate was subjected to *in vitro* digestion treatment with human digestive liquids to estimate the potential of rapeseed peptides to avoid structural degradation and loss of ACE inhibitory activity in the gastric and duodenal digestion. The ACE inhibitory activity remained quite stable throughout treatment; the IC50 value changed from 9 to 14 µg protein/ml (Figure 8). The results from microfluid protein electrophoresis revealed that only minor protein fragments in the Alcalase hydrolysate were digested during the treatments, indicating the resistance of ACE inhibitory rapeseed peptides to proteolysis in the gastric and duodenal digestion.

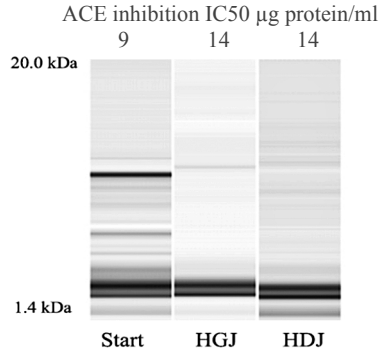

Figure 8 Stability of ACE inhibitory activity of rapeseed hydrolysate sample in simulated digestion treatment, and the corresponding protein patterns from microfluid electrophoresis. Start=before digestion, HGJ=after 30 min treatment with human gastric liquid, HDJ=after 30 min treatment with human duodenal liquid.

In general, the bioavailability of peptides is a major factor for their potential *in vivo* effects. If the protein hydrolysate is orally ingested, it is important to determine the possible loss of ACE inhibitory activity with simulated gastrointestinal digestion *in vitro*. If the activity decreases substantially during digestion, it is unlikely that further *in vivo* evaluations would be made, to prevent unnecessary use of animals. Commercial enzymes have been shown to possess proteolytic properties that differ from human digestive fluids (Eriksen *et al.*, 2010). The proteolytic system in human digestive fluids is highly complex and not thoroughly known as yet, and thus the use of human fluids yields more precise information than commercial enzymes.

Taken together, the results of the present study showed that the most active ACE inhibitory peptides in the Alcalase hydrolysate of rapeseed protein were hydrophobic and rather small, and furthermore, the ACE inhibition was found to be stable in the simulated digestion. In other words, the ACE inhibitory rapeseed peptides can potentially be preserved in the gastrointestinal tract and be absorbed in active form. In any case, measurement of the antihypertensive effects is needed to evaluate the potential *in vivo* antihypertensive effects.

5.2.6 Inhibition of lipid peroxidation (III, IV)

The effects of enzymatic hydrolysis on *in vitro* inhibition of lipid peroxidation of rapeseed isolates are presented in Table 8. All the hydrolysates inhibited lipid peroxidation by percentages above 50 %, indicating rather high antioxidant capacity. Enzymatic hydrolysis enhanced the inhibitory capacity against lipid peroxidation, but there were no significant differences between the tested enzymes. Additionally, we studied the effect of fermentation on *in vitro* lipid peroxidation. All tested fermented samples inhibited lipid peroxidation above 50 % (Table 8), in line with the hydrolyzed samples. Fermentation with *L. helveticus* produced slightly higher potencies when the results were calculated against protein concentration. It is quite difficult to compare results to the previously reported data due to various assay systems being used to measure antioxidant capacities. Most of the studies have used different radical scavenging assays and we assayed the inhibition of lipid peroxidation. The assay was selected due to the importance of oxidative deterioration of polyunsaturated lipids leading to a wide range of cytotoxic products. In any case, antioxidant activities have been reported for rapeseed protein-derived hydrolysates: for example, dose-dependent reducing antioxidant power and hydroxyl radical scavenging activity (Xue *et al.*, 2009; He *et al.,* 2013a).

In general, the amino acid composition of proteins and peptides has been found to affect their antioxidant capacity. In the present study, the increased antioxidant activity due to hydrolysis indicates the role of peptides and amino acids released during hydrolysis, and the effect of fermentation with

L. helveticus for enhancing antioxidant capacity is presumably related to the high proteolytic activity of *L. helveticus* and consequent formation of small molecular-weight degradation products during fermentation. However, nonprotein compounds such as phenols and pigments need to be taken into account when evaluating the antioxidant activities of rapeseed samples. According to our results, the content of tannins and glucosinolates after hydrolysis by proteolytic enzymes was close to zero, but the presence of some phenolic compounds such as sinapic acid derivatives was observed. Thus, further fractionation studies are necessary to identify the antioxidant peptides present in rapeseed hydrolysates and fermented samples.

5.2.7 ACE inhibitory properties of rapeseed protein-derived peptide fractions subjected to *in vivo* studies (V)

Based on the previously described results, the concentrate of hydrophobic small rapeseed peptides was prepared for *in vivo* studies by means of solid-phase extraction and membrane filtration. At this time, concentration was performed in larger volumes than the previously reported concentration and the hydrophobic fraction was eluted between 15 and 60 % of ACN. The ACE inhibition IC50 value obtained after the solid-phase extraction was 0.531 mg protein/ml for the fraction eluted between 15 and 60 % ACN and ultrafiltration with a 3 000 Da MWCO membrane concentrated the ACE inhibitory peptides into the permeate; the ACE inhibitory IC50 value of the permeate, designated as RPHF ph1, was 0.375 mg protein/ml. This fraction was subjected to the first phase of *in vivo* study on animals. Subsequently, the hydrolysis and concentration procedure was repeated in order to generate an adequate amount of rapeseed peptide concentrate for the animal test phase 2. The procedure was identical to that conducted previously, but the milled rapeseed used as raw material was obtained from another industrial batch. The ACE inhibitory activity of the fraction was slightly weaker this time; the IC50 value of the hydrophobic small peptide fraction, designated as RPHF ph2, was 0.470 mg protein/ml. For comparison to published data, He *et al*. (2013a) reported consistent ACE inhibitory activity with RPHF ph1 and RPHF ph2 for small molecular-weight ultrafiltration fractions of rapeseed protein-derived Alcalase (ACE inhibition of 80 – 90 % with 1 mg protein/ml).

5.3 Characterization of the samples

5.3.1 Size-exclusion chromatography (III, IV)

Size-exclusion chromatography was utilized to determine the degradation of proteins during the hydrolysis and fermentation treatments. Enzymatic hydrolysis degraded >10 kDa proteins effectively into <5 kDa degradation products, although Thermolysin was less proteolytic than Alcalase, trypsin and Subtilisin (Table 9). During fermentation, proteins were hydrolyzed by the extracellular proteinases of the microbes, resulting in an increase of small molecular-weight compounds. The effects differed among the microbial strains: *K. marxianus* and *L. lactis* were not proteolytically active as no significant degradation of proteins was observed with either of the strains, whereas *B. subtilis* and *L. helveticus* degraded efficiently the high molecular-weight proteins (Table 9). In particular, *L. helveticus* produced a high amount of degradation products with molecular mass below 1 kDa. The results showed that the ACE inhibitory activity is distinctly correlated with the efficacy of proteolytic treatment and proportion of small degradation products.

Table 9 Proteolytic effects of microbial fermentations on rapeseed proteins

Treatment	***Molecular weight distribution (kDa)%***			
	< 1	1.01 - 5.0	5.01 - 10.0	>10
Rapeseed 0 h	13.8	23.4	21.1	30.0
B.subtilis 48 h	16.1	29.6	53.1	0
K.marxianus 48 h	15.3	27.2	22.4	29.2
Lact. helveticus 48 h	22.2	32.2	41.9	2.9
L.lactis 48 h	13.7	24.3	22.5	37.8

To see that the observed increase in bioactivities is due to peptides produced during fermentation with *L. helveticus*, fractions were collected from gel filtration chromatography. The highest ACE inhibitory potencies were found in small molecular-weight 300 – 500 Da fractions, whereas the inhibition of lipid oxidation was found mostly in 1 200 – 1 300 Da fractions (see Figure 9).

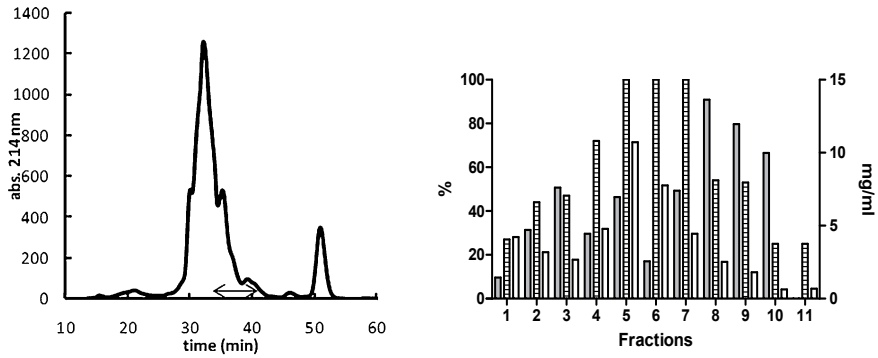

Figure 9 The molecular size profile of rapeseed meal fermented by *L. helveticus* for 48 h at 37 °C. The lines correspond to fractions with ACE inhibitory or inhibition of lipid peroxidation. This profile is representative of several runs that were carried out. The columns show ACE inhibition%, inhibition of lipid peroxidation%, and protein concentration mg/mL in collected fractions.

The results indicate that the most potent peptides inhibiting lipid peroxidation have a molecular mass of 1 200 – 1 300 Da and the most effective ACE inhibitory peptides are of 300 - 500 Da in molecular size. Several ACE inhibitory peptides with corresponding molecular mass have been reported in the current literature, for example rapeseed protein-derived peptide Arg-Ile-Tyr (450.54 Da, Marczak *et al.*, 2003). For comparison to antioxidative peptides in the current literature, peptides with molecular masses consistent with the present results have been identified for example, from whey of 1 200 Da (Hernandez-Ledesma *et al.*, 2005), giant squid muscle of 747 and 1 307 Da (Rajapakase *et al.*, 2005), and chicken essence of 1 400 Da (Wu *et al.*, 2005). However, the amino acid content should be analyzed to see whether the activities are due to specific amino acid residues.

5.3.2 Reversed-phase chromatography (V)

The rapeseed peptide concentrate RPHF ph1 was fractionated by RP-HPLC chromatography in order to isolate the ACE inhibitory peptides. ACE inhibitory activity was widely observed in all of the fractions, indicating the presence of several ACE inhibitory peptides with a wide range of hydrophobicity. Four fractions possessed the highest ACE inhibition, 86 to 89 %, and these fractions were successively re-chromatographed several times. Purified peptide peaks were collected and subjected to MALDI-TOF/TOF analysis for identification. As a result, the peaks of the MS/MS spectra with a positive charge ion at m/z 1139.64 and 1008.69 were identified as Ser-Ser-Val-Ser-Pro-Ser-Ala-Ala-Ala-Ala-Ala-Ala-Pro-Gly-Gly-Ser and Lys-

Lys-Arg-Ser-Lys-Lys-Lys-Ser-Phe-Gly, respectively, with respective molecular weights of 1139 Da and 1008 Da. In general, the RP-HPLC and MALDI-TOF/TOF analysis showed that the ACE inhibitory potency of RPHF ph1 was due to several ACE inhibitory peptides with a wide range of hydrophobicity. The identified peptides are presumably not very effective ACE inhibitors as such due to their large size. However, they contain primarily hydrophobic amino acid residues and may degrade into more efficient ACE inhibitors during gastrointestinal digestion.

5.4 Preventive effects against hypertension of rapeseed peptide fractions in 2K1C rats (V)

In order to measure the antihypertensive effects, rapeseed peptide concentrates (RPHF ph1 and RPHF ph2) were evaluated *in vivo* using oral administration to 2K1C rats. The mean arterial pressure (MAP) of the rats was recorded by using a radiotelemetry method (Tonkiss *et al.*, 1998).

5.4.1 First phase of the study

The RPHF ph1-treated (600 mg/kg body weight) rats showed lower MAP values than vehicle-treated rats and it was observed that the RPHF ph1 prevented the elevation of MAP in the 2K1C model (Figure 10). The maximum reduction in the systolic blood pressure (SBP) of the RPHF ph1, -50 mmHg, was observed after ten days of dosage and the preventive effect of RPHF ph1 on the elevation of MAP could be seen throughout the 28 days of the study. The respective antihypertensive effect of the positive control, Captopril, after ten days of dosage was -70 mmHg. The number of animals per group used in this study phase (n=4) was too small to perform formal statistical analysis and the observations are based on differences between the means ± SEM.

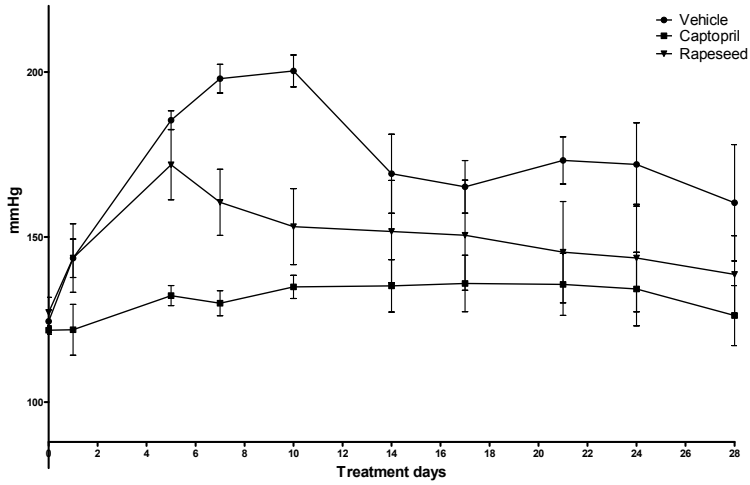

Figure 10 Effects of RPHF ph1 on the blood pressure of 2K1C animals (n=4), values expressed as MAP±SEM.

5.4.2 Second phase of the animal study

Based on the results of phase 1 of the study, a further study with a larger number of animals per group (n=6) was established. The results are presented in the Figure 11. Overall, the results from phase 2 of the animal study were partly incoherent with the results of phase 1 with the RPHF ph1. The RPHF ph2 was observed to prevent blood pressure elevation in 2K1C rats to some extent, but the effect was weaker than with the RPHF ph1 in the first study phase and not statistically significant.

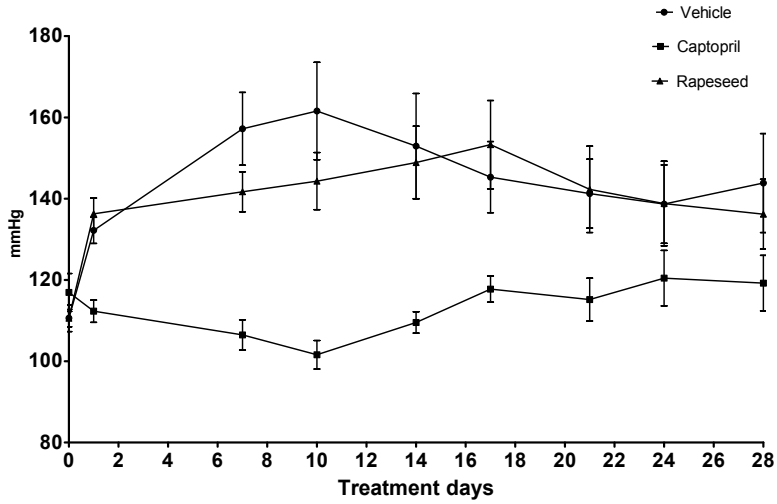

Figure 11 Effect of RPHF ph2 on blood pressure in 2K1C animals (n=6), values expressed as MAP±SEM.

The current literature includes information on the hypotensive effects of plant protein-derived hydrolysates and peptides, rapeseed peptides among them, but the previously reported hypotensive effects have been measured on the SHR model, and thus the comparison with the present study is not straightforward. For example, the increase of vascular mass in SHR may have a role when comparing the results to 2K1C model rats (Fouda *et al.*, 1987). However, some correlation with the previously demonstrated hypotensive effects of plant protein-derived hydrolysates and peptides can be found. For example, corresponding SBP reductions with the RPHF ph1 have been demonstrated for rapeseed protein-derived Alcalase hydrolysate (100 mg/kg bw) with a decrease in SBP of -24 mmHg at 8 h after oral administration to SHR (He *et al.*, 2013a) and for 3 000 Da membrane ultrafiltration permeate of a rapeseed protein-derived Pepsin+Pancreatin digest with an SBP reduction of about -21 mmHg after 4 h of oral administration (100 mg/kg bw) to SHR (He *et al.*, 2013a). For comparison to the antihypertensive effects of plant protein-derived hydrolysates of other origins, an SBP reduction of around -20 mmHg has been reported after oral administration (100 mg/kg bw) of sweet potato protein hydrolysate to SHR (Ishiguro *et al.*, 2012). The SBP reductions observed with the RPHF ph1 were more pronounced than the previously reported antihypertensive effects, but with a relatively higher dose of 600 mg/kg bw. However, it needs to be emphasized that comparison of the results is not straightforward due to the different model used. The premise of the current study was preventive and the effects of the rapeseed peptide fraction on blood pressure were detected for a longer period in comparison to the previously reported studies on SHR. Overall, the present results indicate preventive potential against hypertension for rapeseed protein-derived peptides.

5.4.3 Comparison of the peptide compositions of the two rapeseed peptide concentrates

Due to the incoherent results from the *in vivo* studies, the peptide compositions of RPHF ph1 and ph2 were analyzed by UPLC-qTOF/TOF. The results showed that the samples were composed of a highly complex mixture of peptides and demonstrated also that the compositions between the two samples were similar in the majority. However, a targeted search for the previously identified rapeseed peptides Arg-Ile-Tyr, Thr-Phe, Leu-Tyr and Arg-Ala-Leu-Pro revealed differences between the RPHF ph1 and RPHF ph2. Peptide Arg-Ile-Tyr was detected in both of the samples as well as Leu-Tyr, but Thr-Phe was found only in the RPHF ph1 and not in the RPHF ph2, indicating that the peptide compositions of RPHF ph1 and PFH ph2 were not identical.

Taking the results from animal studies and targeted peptide analysis together, it can be considered that the reason for the incoherent *in vivo* results from study

phase 1 and phase 2 may lie at least partly in the peptide composition of the samples. The ACE inhibitory IC50 values of the RPHF ph1 and RPHF ph2 used for animal study phase 1 and phase 2, respectively, were not equal although they were rather close. The predominant peptide compositions of the two RPHF were found to be correspondent, but a specific difference was observed concerning peptide THR-PHE with an IC50 value of 3.061 mM. This dipeptide has been previously reported to possess antihypertensive properties *in vivo* in SHR. The observed specific difference in the peptide composition is presumably at least partly responsible for the weaker antihypertensive effects of the RPHF ph2, but there may also be other specific differences between the two samples. Further extensive analysis of peptide compositions would be needed to measure the differences throroughly. The peptide compositions of RPHF ph1 and RPHF ph2 may differ basically for two reasons: the sample preparation process and the milled rapeseed used as raw material. The RPHF ph1 and RPHF ph2 were prepared separately in different volumes – although both at the laboratory scale – and this may result in some differences in the hydrolysis and fractionation of peptides. However, an essential factor for the peptide composition is presumably the milled rapeseed used as raw material. The milled rapeseed was manufactured in different years for the RPHF ph1 and RPHF ph2 and this may cause differences in the extensive protein composition of the raw material used for the hydrolysis. To summarize, the results indicate that the final hypotensive capacity of rapeseed peptide concentrates is potentially a result of synergistic actions of several peptides, and that the raw material quality has a particular effect on the final peptide composition.

5.5 Some aspects concerning practical applications of plant protein-derived antihypertensive peptides

Plant-derived bioactive peptides possess a widely documented antihypertensive potential *in vitro*, but thus far the data concerning the antihypertensive effects *in vivo* is limited to rather few studies on animals. In general, few commercial product applications have been established with antihypertensive milk peptides. The most studied milk peptides Val-Pro-Pro and Ile-Pro-Pro have shown antihypertensive effects in several studies on animals and human subjects, but the effects have not been unambiguous. The extent of the antihypertensive effects has been suggested to depend on the nature of delivery system, dose, study duration, genetic background of the subjects, stages of hypertension (reviewed by Udenigwe and Aluko, 2012).

Much emphasis has been put on the legal regulation of the health claims attached to the products. Systematic approaches for the review and assessment of scientific data have been developed by authorities around the world. In the

EU, the European Regulation on nutrition and health claims was established in January 2007 and the regulations are governed by EFSA. Recently, the aspects concerning the scientific information needed for the use of a health claim in the functional food product labeling and marketing were summarized as follows (reviewed by Pihlanto and Mäkinen, 2013):

- The scientific evidence on the beneficial effects of the product needs to be sufficiently detailed, extensive and conclusive:
- It is necessary to identify and quantify the active sequences in the product. It is mandatory to monitor the hydrolytic or fermentative industrial production process as the antihypertensive peptides are only minor constituents in highly complex food matrices.
- The antihypertensive effect in humans as well as the minimal dose needed to show the effect have to be proven in extensive investigations to fulfill the requirements of the legislation concerning functional foods.

In general, the feasibility of the food protein-derived bioactive peptides depends on the bioavailability in intact forms in target tissues, which in turn depends on the structure of the peptides. Information on the important structure-function parameters of peptides is increasing constantly, which can greatly enhance the production and processing of peptides. To enable sufficient scientific evidence on the antihypertensive effects of the plant protein-derived peptides, there is a crucial need for further research on the elucidation of the *in vivo* molecular mechanisms of action, safety at various doses, and pharmacological activity in the prevention of abnormal health conditions in human subjects. Recent advances in specific analytical techniques enable these measurements as they have made it possible to follow small amounts of the peptides and derivatives in biological fluids and complex matrices. At the same time, emphasis has been put on developing technological tools to enhance the bioavailability of these peptides. A great technological challenge is also to develop food industry-applicable large-scale processing methods for concentration of the active peptides according to the structure-function parameters, leading to high-yield peptide products. These findings and challenges open up an interesting field aimed at the revaluation of the protein-rich by-products formed in the food industry.

6 SUMMARY AND CONCLUSION

Hydrolysis of defatted rapeseed meal from the rapeseed oil industry by proteolytic enzymes, Alcalase being the most effective among them, produced high *in vitro* ACE inhibitory activity as well as activity against lipid oxidation. Protein extraction and hydrolysis treatments effectively removed anti-nutritional compounds such as glucosinolates from rapeseed meal. Fermentation of rapeseed meal with *L. helveticus* and *B. subtilis* strains also produced peptides with ACE inhibitory activity and inhibition capacity against lipid peroxidation, but the efficacy of fermentation was minor in comparison to enzymatic hydrolysis. ACE inhibition was mainly due to low molecular-weight peptides (300 - 500 Da) whereas inhibition of lipid peroxidation was due to higher molecular-weight peptides (1 200 – 1 300 Da).

The ACE inhibitory activity of rapeseed protein-derived Alcalase hydrolysate remained stable in treatment with human gastric and duodenal fluids, and ACE inhibition was concentrated in the fraction of small hydrophobic peptides. This indicated the potential bioavailability of the produced peptide concentrate, and thus the potential antihypertensive effects of the peptide fractions were tested *in vivo* on animals. The results indicated that the concentrate of small hydrophobic rapeseed peptides may possess preventive effects against the elevation of blood pressure after oral administration to 2K1C rats. However, the results demonstrated, in particular, the essential effect of the quality of milled rapeseed used as a raw material on the peptide composition obtained after hydrolysis. Two peptides, which may be precursors of ACE inhibitory peptides, were identified from the hydrophobic, small molecular-weight rapeseed peptide concentrate.

Hydrolysis of industrial by-products from potato starch production and potato tuber protein extracts with commercial proteolytic enzymes yielded effective ACE inhibition capacity and radical scavenging activity. Among the tested enzymes, Alcalase produced the highest ACE inhibitory activities with corresponding values to rapeseed protein-derived Alcalase hydrolysate. The highest antioxidant activities were also observed in samples hydrolyzed with Alcalase. Overall, potato fruit liquid from the potato starch industry yielded higher ACE inhibitory and antioxidant activities than the other potato fractions. The ACE inhibitory activity was most likely related to peptides released during the enzymatic hydrolysis, but phenolic compounds were at least partly responsible for the antioxidant activity of potato by-product fractions.

In addition to enzymatic treatments, autolysis was found to enhance ACE inhibition of potato tuber extracts. Protein extracts from inner tuber tissues of potato showed particular autolytic capacity. The physiological age of the tuber affected the production of ACE inhibition production during the autolysis; the

highest autolytic activity was observed in stored tubers at dormancy braking. The production of ACE inhibitory activity was positively correlated with protease activity in tuber tissues. Amendment of the autolysis reaction with protein substrates from which bioactive ACE inhibitory peptides may be released enhanced ACE inhibition. Many tuber proteins including aspartic protease inhibitors were observed to degrade during autolysis. The results showed that the observed increase in ACE inhibition was conceivably due to bioactive peptides released from proteins during autolysis.

Altogether, the results indicate that rapeseed and potato proteins are rich sources for the production of bioactive peptides with ACE inhibitory and antioxidant properties. Moreover, food industry by-products have the additional advantage of representing a value-added outlet for inexpensive starting material. Further studies are still required to identify the active peptides and to investigate the *in vivo* antihypertensive effects of the peptides. An economic analysis of the whole process from the collection of by-products to the production of the powered peptide mixture would provide valuable information for the food industry.

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APPENDIX: ORIGINAL PUBLICATIONS

I. Reprinted from *Food Chemistry* 2008, 109, 104-112, with permission from Elsevier Ltd.

II. Reprinted from *Journal of Agricultural and Food Chemistry* 2008, 56, 9875-9883, with permission from American Chemical Society.

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V. Submitted manuscript

Doctoral thesis in Food Sciences at the University of Turku

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic Herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols—approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: Isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: Food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: Estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: Analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: Influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole—compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: Studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **YANG BAORU (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria × ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota—a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: Effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: Effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
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41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: Studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
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45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and Medicines as Legal Categories in the EU and China. Functional Foods as a Borderline Case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: Basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: Contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants—analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
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