Antioxidant Activities of Hydrolysates and Peptides Generated from High Hydrostatic Pressure-Treated Soy Protein Isolates

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MASTERS OF SCIENCE

Your file Votre référence
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This document is dedicated to my grandparents Bor-Nan Chen and Mai-Yu Chen-Wong and my parents Red Chang and Dorothy Chen.

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ABSTRACT

Native and pressure-treated (600 MPa) soy protein isolates (SPI) were subjected to *in vitro* digestion to study the effect of pressure processing on the digestibility of SPI and the antioxidant activity of the hydrolysates and isolated low molecular weight (<1 kDa) peptides. The digestibility of SPI increased significantly ($P < 0.05$) with pressurization following 10 min of pepsin digestion. The total peptide content in the pepsin-pancreatin hydrolysates was unaffected by pressurization; however, the peptide profiles were altered. Peptides from hydrolysates of pressurized SPI showed higher antioxidant activity than peptides from native SPI hydrolysates as measured by the FRAP assay. In contrast, peptides from native SPI hydrolysates exerted higher antioxidant activity than peptides of hydrolysates of pressurized SPI as assessed by the DPPH assay. These results indicate that peptides from hydrolysates of native and pressurized SPI produce differential *in vitro* antioxidant activities that might impact their *in vivo* antioxidative effects.

ABRÉGÉ

Les isolats de protéine soja (IPS), natifs et traités par la pression (600 MPa), étaient soumis à la digestion *in vitro* pour étudier l'effet du traitement par la pression sur la digestibilité des IPS, ainsi que sur l'activité anti-oxydante du produit de l'hydrolyse et des peptides isolés de poids moléculaire bas (<1 kDa). Avec la pressurisation, la digestibilité des IPS était augmenté significativement ($p<0,05$) après 10 minutes de digestion en présence de pepsine. Dans le produit pepsine-pancréatine de l'hydrolyse, le contenu total de peptides n'était pas affecté par la pressurisation ; néanmoins, le profil des peptides était modifié. Les peptides produits par l'hydrolyse des IPS traités par la pression ont démontrés une plus haute activité anti-oxydante que les peptides produits par l'hydrolyse des IPS natifs quand celle-ci était mesurée par l'analyse FRAP. Par contre, l'inverse était trouvé par l'analyse DPPH, avec les peptides produits par l'hydrolyse des IPS natifs possédants plus d'activité anti-oxydante que les peptides produits par l'hydrolyse des IPS traités par la pression. Ces résultats indiquent que les peptides produits par l'hydrolyse des IPS natifs et les peptides produits par l'hydrolyse des IPS traités par la pression fournis de différents activités anti-oxydante *in vitro* qui pourraient peut-être influencer leurs effets anti-oxydante *in vivo*.

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Carole Chang (candidate) - Assisted in the experimental design of the study, ordered the equipment, performed all experimental assays, organized the data, analyzed the data, and drew conclusions from the analyzed data. The candidate wrote the manuscript under the guidance of the co-authors and made modifications to it in response to their comments.

Dr. Stan Kubow (Supervisor, Associate Professor at McGill) - Advice and guidance on all aspects of the study including study design, assay techniques, interpretation and statistical analysis of data, solving equipment problems, and editing of the manuscript.

Dr. Larry Lands (Co-Supervisor, MD at Montreal Children's Hospital) - Provided advice on study design and guidance throughout the study. Provided experimental materials and editing of the manuscript.

Dr. Hosahalli S. Ramaswamy (Committee Member, Professor at McGill University) - Advice on hydrostatic pressure treatment of the soy protein isolates. Guidance on the selection of different pressure levels. Provided facility and equipment for the pressuziation process. Editing of the manuscript.

Dr. Laurie Chan (Committee Member, Professor at University of Northern British Columbia) - Provided guidance throughout the study. Provided facility and equipment for CZE analysis.

Dr. Yasuo Konishi (Research Scientist, Biotechnology Research Institute) - Advice on HPLC and MS results and interpretation. Provided facility and equipment for HPLC and MS analysis.

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ABBREVIATIONS

ABTS	2, 2'-azino-bis (3-ethylbensothiazoline-6-sulfonic acid)
Arg	Arginine
ß-lg	ß-lactoglobulin
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CFTR	Cystic fibrosis transmembrane conductance regulator
CZE	Capillary zone electrophoresis
DPPH	1, 1-diphenyl-2-picrylhydrazyl
ESI-MS	Electrospray ionization mass spectroscopy
ESR	Electron spin resonance spectroscopy
FRAP	Ferric reducing ability power
FRBR	Fenton reaction based radical
GMP	Glycomacropeptide
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S-transferase
His	Histidine
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IgE	Immunoglobulin E
IgG	Immunoglobulin G
kDa	Kilodaltons
LDL	Low density lipoprotein
Leu	Leucine
Lys	Lysine
MES	Morpholinoethanesulfonic acid
MPa	Mega-pascal
MWCO	Molecular weight cut-off
OD	Optical density
ORAC	Oxygen radical absorbance capacity

PCA-ORAC	Oxygen radical absorbance capacity using perchloric acid
Phe-Gly	Phenylalanine - glycine
PQ	Paraquat
Pro	Proline
RONS	Reactive oxygen and nitrogen species
RP-HPLC	Reversed phase high-performance liquid chromatography
-SH	Sulfhydryl group
SPI	Soy protein isolates
S-S	Disulfide
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoroacetic acid
TOF	Time of flight
TPTZ	2, 4, 6-tripyridyl-s-triazine
TRAP	Total radical trapping antioxidant parameter
Trolox	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
VCEAC	Vitamin C equivalent antioxidant capacity
WPI	Whey protein isolates

CHAPTER 1

INTRODUCTION

Soybeans are considered to be the world's most inexpensive source of protein and provide high quality edible oil (Messina, 1995). In addition, proteins and oils derived from soybeans are utilized in numerous foods, feeds and industrial applications. Consumption of soy protein is associated with a lower risk of cardiovascular disease in man and reduced atherosclerosis in a variety of experimental animals (Clair and Anthony, 2005). Other properties of soy include antioxidant, antiinflammatory and potentially antithrombogenic effects. Consequently, research concerning the health properties of soybean products is an important topical research area. Although there is evidence that soy protein can exert antioxidant effects *in vivo*, mechanisms of action have yet to be determined. It has been suggested that one or more of the components present in soy protein isolates, including isoflavones as well as various protein components especially peptides, could be responsible for its antioxidant effects (Chen et al., 1998). Many studies have been conducted to investigate the antioxidant effects of soy isoflavones with inconsistent results. There is relatively little research regarding the antioxidant potential of soy protein components, particularly for the digestive protein end-products in the form of peptides. Additionally, high pressure processing has recently been shown to increase the *in vitro* digestibility of whey proteins and improve the release of the bioactive peptides that appear to increase the cellular synthesis of the antioxidant glutathione (Vilela et al., 2006). Hence, the research topic of this thesis has focused on the antioxidant capacities of the hydrolysates of native and pressurized soy protein isolates (SPI) and the low molecular weight peptides resulting from the *in vitro* digestion of SPI using a dual enzyme system involving pepsin and pancreatin.

Rationale

Soy protein derived peptides or amino acids have been found to exert antioxidative effects in two recent *in vitro* studies (Chen et al., 1998; Saito et al., 2003). Additionally, lowered tissue lipid peroxidation and increased cellular glutathione levels were seen in rats fed a soy protein diet (Madani et al., 2000; Aoki et al., 2002). Several clinical trials have also observed antioxidant activity in their experimental subjects exerted by soy protein intake (Ashton et al., 2000; Swain et al., 2002; Vega-Lopez et al., 2005; Heneman et al., 2006). Moreover, soy protein intake showed higher antioxidant activity when compared to the potent antioxidant action of whey protein isolates in the prevention of training-induced decreases in antioxidant capacities in male athletes (Brown et al., 2004).

Hyperbaric pressure treatment of whey proteins can induce major structural changes with an increase in freely exposed sulfhydryl residues for digestion that were not available before the pressurization (Funtenberger et al., 1997). When subjected to single cycle pressurization of 550 MPa, whey proteins showed greater accessibility of disulfide bonds and increased exposure of amino acid sequences that led to a more rapid proteolysis resulting in peptide profile changes and the greater release of small bioactive peptides (Vilela et al., 2006). Furthermore, conformational changes induced by high pressure treatment (i.e., 200, 400 and 600 MPa) induced unfolding of 7s and 11s components of soy protein as well as the dissociation of glycinin (Puppo et al., 2004). Hence, pressurization of soy protein could lead to the greater accessibility of the protein to digestion, which would allow for a more rapid proteolysis resulting in increased release of bioactive antioxidant peptides. In that regard, it would be of particular interest to evaluate the production of low molecular weight peptides less than 1 kilodaltons (kDa), which are primarily absorbed and the most bioactive *in vivo* (Qiao et al., 2004). Hence, the focus of this thesis are *in vitro* studies to investigate whether low molecular weight peptides liberated from the hydrolysis of pressurized soy proteins differ structurally and in their antioxidant properties from those released from the digestion of native soy proteins.

1.2 Project Objectives

There are three specific objectives for this thesis:

a) To assess whether pressure processing of SPI affects its *in vitro* digestibility.

b) To study whether pressure treatment of SPI affects the concentration or profile of low molecular weight (< 1 kDa) peptides generated from its *in vitro* digestion.

c) To assess whether low molecular weight (< 1 kDa) peptides isolated from the hydrolysates of pressure-processed SPI have an increased antioxidant capacity as compared to peptides isolated from the hydrolysates of native SPI.

1.3 Hypotheses

1) Pressurized SPI will have a higher *in vitro* digestibility than native SPI leading to an increase in the low molecular weight (< 1 kDa) peptide content and a change in the peptide profile as assessed by mass spectrometry (MS), reversed phase high performance chromatography (RP-HPLC) and capillary zone electrophoresis (CZE).

2) There will be an improved antioxidant activity obtained from soy peptides generated enzymatically from pressurized SPI versus peptides obtained enzymatically from native SPI.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Soybeans

The utilization of soybeans in the Asian population has dated back to more than 5000 years ago (Rhee, 1994). The soybean is an important food seed that contains many good quality proteins and oils. Depending on the variety of the soybeans, the natural seeds of soybean contain 35-40% protein, 15-20% lipid and 20-25% carbohydrates (Messina, 1995).

Soybeans are an important food source in terms of their nutritional value. From a nutritional perspective, soy products may hold many advantages over animal products; for instance, soybean oil is low in saturated fats and is therefore less likely to cause heart disease (Bakhit et al., 1994). In addition, soybeans contain a number of minor constituents such as trypsin inhibitors, phytate, saponins, and isoflavones. Several of these dietary compounds are believed to exert health benefits such as the lowering blood cholesterol or the prevention of cancer (Anderson and Wolf, 1995). In 1999, the US Food and Drug Administration (FDA) approved a health claim for soy protein stating that daily intake of 25 g of soy protein could help lower blood cholesterol and reduce coronary heart disease risk, which further emphasized the dietary importance of soy protein.

Among the compounds present in association with soy protein-based products, soy isoflavones are of particular interest as these compounds have been accredited to support the health benefits of soy intake for maintenance of a healthy heart, strong bones and alleviating post-menopausal symptoms (Brouns, 2002). Isoflavones may also support the proper functioning of the immune system and may have the potential to reduce risk factors involved in the etiology of certain cancers (Brouns, 2002). Furthermore, soy isoflavones have been shown to exert possible beneficial effects on cell proliferation, cell growth and cell maturation and may function as important regulators of organ function

maintenance (Messina, 1995). For health benefits, recommended intakes have ranged from 60 – 100 mg aglycones/day (100-160 mg glycones) (Brouns, 2002). In Asian countries, soy isoflavone intake is estimated to range from 20-100 mg/day (Murphy, 1997).

Another important observation has been the suggestion that isoflavones may have antioxidant properties (Khan and Sultana, 2004). As antioxidants, genistein and daidzein may thus protect cells from the damaging effects of free radicals that are known to promote aging and inflammatory diseases. The antioxidant importance of isoflavones; however, has been put into doubt by a recent study which found that consumption of soy protein, with or without isoflavones, only resulted in a very modest increase in postprandial antioxidant capacity (Heneman et al., 2006). Moreover, treatment with soy protein without isoflavones exerted statistically higher increases in the plasma antioxidant capacity compared to the intake of soy protein with isoflavones. Therefore, this latter research appears to indicate that isoflavones might not be the principal component responsible for the antioxidant activity of soy proteins.

2.2 Soy Isoflavones

Soy isoflavones are a group of biologically active compounds that have several functions in the soybean: they contribute color to the soybean, protect the plant against bacterial and fungal infections and serve a hormone-like role (as a phytoestrogen) in plant cell regulation. Isoflavones occur predominantly as glycosides in soybeans and consequently are highly polar (water soluble) compounds (Setchell and Cassidy, 1999). Most isoflavones are bound to carbohydrate called glycones and referred to as daidzin, genistin and glycetin and only a small fraction appears to be unbound isoflavones called aglycones referred to as daidzein, genistein and glycetein.

Isoflavones aglycones are released from their glycosides in the digestive tract by the hydrolytic action of microflora. It is therefore likely that hydrolysis enhances the efficiency of intestinal absorption of dietary isoflavonoids (Murota et al., 2002). The

aglycones may be absorbed directly in the intestine by passive diffusion or may first undergo further biotransformation to a range of metabolites. These aglycones are either absorbed by the enterocytes or in the case of daidzein, partly converted into equol and/or methlangelolensin. However, not all persons seem to absorb isoflavones to the same extent. This may be due to individual differences in the status of the flora of the large intestine (Brouns, 2002). Setchell and Cassidy (1999) reported that a high carbohydrate diet causes increased intestinal fermentation and results in more extensive biotransformation of phytoestrogens, with greatly increased formation of equol (metabolite of daidzein).

Soy isoflavones have been indicated to have the capability to behave as antioxidants, inhibit enzyme systems and influence transport proteins and cell signalling pathways by their effects on tyrosine kinase and growth factors (Setchell and Cassidy, 1999). Hence, soy isoflavones may play a role in reducing lipid peroxidation, improving arterial reactivity, reducing blood pressure and decreasing pro-inflammatory cytokines. Soy isoflavones were also shown by Khan and Sultana (2004) to be effective chemopreventive agents against ferric nitrilotriacetate (Fe-NTA)-mediated renal oxidative stress, toxicity and cell proliferation in rats. Furthermore, soy isoflavones are structurally similar to estrogen and therefore may have the ability to interact with cellular receptors for estrogens. Such an interaction may exert weak estrogenic effects to help the maintenance of strong bones and alleviate post menopausal symptoms (Brouns, 2002).

2.3 Soy Proteins

Soy protein is a mixed or heterogeneous protein made up of several different individual proteins as indicated by the listing of the major protein factions in Table1. The classifications of soy protein are based on sedimentation characteristics. The conventional nomenclature for soy protein fractions is the 2s, 7s, 11s and 15s fractions (Yamauchi et al., 1991); the major fractions are the 7s fraction (7s globulin or β-conglycinin) and 11s fraction (11s globulin or glycinin). These two fractions account for about 70% of the protein in the seed and play important roles in several food systems because of their high

nutritional value and functional properties (Puppo et al., 1995). Some soy bean proteins, as trypsin inhibitors, contribute to the nutritional quality of soybeans by virtue of their relatively high cysteine content (Tan-Wilson and Wilson, 1986). The functional properties of soy proteins are affected by size, amino acid composition, net charge, sequence of amino acids, method of extraction, isolation of pH, temperature and ionic strength (Zayaz, 1997).

Table 1: Components of ultracentrifuge fractions of water extractable soybean proteins. Source: Wolf (1970)

Fractions	% of Total	Components	Molecular Weight (Da)
2s	22	Trypsin inhibitors	8000- 21500
		Cytochrome C	12000
7s	37	Hemagglutinin	110000
		Lipoxygenase	102000
		β-Amylase	61700
		7-s Globulins	180000-210000
11s	31	11-s Globulins	350000
15s	11		≈600000

2.4 Soy Protein Isolates

Soy protein isolates (SPI) are the most refined form of soybean proteins that contain 90% or more protein. Glycinin (11s) and β-conglycinin (7s) globulins are the major components of SPI. According to Wolf (1970), SPI are prepared by removing the water-insoluble polysaccharides, as well as the oligosaccharides and other low molecular weight components. First, the defatted soybean flakes or flour are treated with minimal moist heat. They are then extracted with water and alkali at a pH of 7 to 8.5. After the

extraction, the insoluble residue, which contains the water-insoluble polysaccharides and residual protein, is then separated from the clarified extract. This clarified extract contains the bulk of the proteins and carbohydrates. In the next step, the pH of this extract is adjusted to about pH 4.5. This treatment precipitates the proteins and the precipitations are then removed by centrifugation or filtration. The precipitated proteins are then washed and dried to give the isoelectric protein which is insoluble in water. However, most of the protein is neutralized before drying and this procedure yields the proteinate form, which has the advantage of being water dispersible, unlike the isoelectric protein. According to Setchell and Cassidy (1999), SPI prepared by an ethanol wash process generally do not carry significant amounts of isoflavones unless they are fortified with isoflavone extracts. SPI that are generated via isoelectric precipitation and the acid wash process do retain a large proportion of the isoflavones in association with the protein. SPI produced by different methods can then be stored in the refrigerator or freezer for future use. Pinto et al. (2005) found that SPI stored at – 18°C and 42°C for one year presented similar digestibility values as assessed by pepsin-mediated *in vitro* digestibility studies, indicating that the nutritional value of the protein in terms of digestibility may not be affected greatly by storage conditions. However, prolonged storage at higher temperatures may cause protein denaturation and as a consequence, protein insolubilization, which can limit product utilization (Pinto et al., 2005).

One of the major components of SPI is the 7s protein, which is composed of six similar sized subunits held together mainly by hydrophobic bonds. The 7s protein can also dimerize when ionic strength is increased. The other major component of SPI is the 11s molecule, which is composed of six subunits, each of which contains two disulfide-linked protein molecules. Both 7s and 11s globulin protein have the ability to form disulfide-linked polymers, which contribute to insolubility of SPI (Wagner et al., 1996). Furthermore, these disulfide polymers also cause turbidity and increase viscosity of soybean protein solutions. However, depolymerization occurs readily when these proteins are treated with mercaptoethanol, sodium sulfite or cysteine.

According to Molina and Wagner (2002), the native soy protein 7s and 11s globulins show high solubility (> 90%) at alkaline conditions. Acidic treatment (pH 2-3) in combination with a thermal treatment is useful to increase the solubility through partial deamination and mild hydrolysis of proteins. Thermal denaturation reduces water solubility of soy proteins, especially at high concentrations and the mechanical strength of the gel also depends upon protein concentration, since it is related to the number of crosslinks formed per protein chain. According to Catsimpoolas and Meyer (1970), the minimum protein concentration required for soy isolates to form a heat-induced gel is 8%. Mori et al. (1982) also reported that high concentrations of glycinin led to gel formation, while low concentrations of glycinin favored disaggregation. However, ß-conglycinin dissociated and then recombined into soluble aggregates at high protein concentrations and ionic strength (Iwabuchi and Yamauchi, 1984). Both protein solubility and foaming properties of SPI and hydrolysates were affected at pH 4.5 because this is the isoelectric point of the storage proteins in soybean seeds (Molina and Wagner, 2002).

Both 7s and 11s globulins are sensitive to their ionic environment. They undergo association-dissociation reactions with changes in ionic strength. The most clear-cut demonstration of these reactions is observed with the 7s globulin. At pH 7.6 and 0.5 ionic strength, the 7s protein exists as a monomer with a molecular weight of 180 – 210 kDa, whereas at 0.1 ionic strength the 9s protein has a molecular weight of 370 kDa, and is obviously a dimer of the unit observed at the higher ionic strength. The 11s protein is also converted into a faster sedimenting form when ionic strength is lowered from 0.5 to 0.1, but the extent of association is low. Both proteins undergo these reactions reversibly (Molina and Wagner, 2002).

2.4.1 β- Conglycinin (7s globulin)

The 7s globulin is one of the major storage proteins in soybean. It can be separated into three individual proteins designated β-conglycinin, γ-conglycinin and basic 7s globulin (Yamauchi et al., 1991). Basic 7s globulin is composed of four subunits each consisting of a high molecular weight polypeptide (26 kDa) and a low molecular weight

polypeptide (16 kDa) linked by a disulfide bond (Utsumi et al., 1997). The 7s globulin contains nine amino-terminal residues and, presumably, these nine subunits undergo a number of reactions. In acid solutions at low salt concentrations, the 7s protein forms two species that sediment with coefficients of 2s and 5s. Conversion into the 2s and 5s species in acid is inhibited by salts, and is reversed by dialyzing the protein to pH 7.6, 0.5 ionic strength. In 0.01N sodium hydroxide, the 7s globulin is converted to a form with a sedimentation coefficient of only 0.4%. Conversion of the 7s protein into the slow-sedimenting forms suggests disruption of a subunit structure (Wagner et al., 1996).

β-Conglycinin is composed of three subunits designated as α′, α and β. These subunits are glycoproteins with one oligosaccharide unit attached to the aspartic acid residue at the N-terminal end of the molecule. There is no difference in carbohydrate content between the α′ and α subunit, although they contain twice as much carbohydrate as the β subunit. β-Conglycinin is considered to be a compactly folded protein with high degree of unordered regions. The secondary structure of β-conglycinin is composed of 5% of α-helices, 35% of β-sheets and 60% of random coils (Lewis and Chen, 1979).

2.4.2 Glycinin (11s globulins)

Glycinin represents ~ 30% of total protein in soybeans. It is composed of an acidic (~38 kDa) and a basic polypeptide (~20 kDa) linked by a single disulfide bridge. The 11s protein contains eight glycine, two phenylalanine, and two leucine (isoleucine) amino terminal residues per mole suggesting a minimum of 12 polypeptide chains and 12 subunits per molecule (assuming absence of disulfide cross linkages between polypeptide chains). A dimer structure of two identical monomers each containing six subunits was proposed for the 11s molecule (Wagner et al., 1996). The quaternary structure of the 11s molecule is disrupted by high and low pH, by high concentrations of urea, detergents, phenolacetic acid-mercaptoethanol-urea mixtures and by temperatures above 80° C (Wagner et al., 1996).

Heat denaturation is often a prerequisite for gel formation. Glycinin has a denaturation temperature of 90°C at neutral pH and an inonic strength of 0.25M; reducing the ionic strength lowers the denaturation temperature. The gel characteristics of pure glycinin are affected by ionic strength, heating temperature and pH during heating. Heat treatment induces denaturation and aggregation of soy protein molecules; at high soy protein concentrations greater than 7% (w/w), the aggregated formed produces a self-supporting gel (Van Kleef, 1986).

2.5 Soy Peptides

Peptides are breakdown products produced by the digestion of ingested protein. Most peptides and proteins are large hydrophilic compounds with significant instability in the gastrointestinal environment. The molecular weight is likely a major determinant of peptide and protein absorption. Peptides > 1 kDa are poorly absorbed probably due to the relatively poor diffusibility of peptides through aqueous pores of the tight junction such that only relatively small peptides can readily transverse this aqueous pathway (Fix, 1996). This is confirmed by the study of Debroas et al. (1998) which found that the growth of *Prevotella ruminicola* seems to depend on the availability of small peptides (< 1 kDa) derived from hydrolysis of higher molecular weight peptides. They also found that small peptides and amino acids were converted to volatile fatty acids more rapidly than high molecular weight peptides (> 2 kDa).

Proteolytic enzyme treatment of soy proteins could also introduce undesirable attributes to the products such as bitterness. Soluble peptides fractionated from commercial soy protein hydrolysates into various molecular mass ranges between 0.4 and 10 kDa have different degrees of bitterness and differ in their amino acid composition from their parent proteins and from each other (Cho et al., 2004). The bitterness intensity of the fractionated peptides depends on the degree of hydrolysis of their parent proteins. The bitterness of the soy peptides is predominantly associated with the medium molecular mass range peptides at 0.4 and 10 kDa. The small peptide fractions below 1 kDa are much less bitter than the higher molecular weight fractions (Cho et al., 2004).

2.6 Antioxidant Assays

A biological antioxidant is defined as any substance that when present at low concentrations compared to those of an oxidizable substrate, can significantly delay or prevent oxidation of that substrate (Benzie and Strain, 1996). Antioxidant properties, especially free radical scavenging activity, are important due to the deleterious role of free radicals in foods and in biological systems. Free radicals are atoms or molecules with an unpaired electron in the outer orbit. The unpaired electron may also take an electron from another molecule, join with another molecule, or completely disengage itself and reattach itself to another molecule, thus producing more free radicals. When the generation of free radicals exceeds the antioxidant defences in the body, oxidative stress can occur and tissues often respond by generating additional antioxidant protection. Free radicals have also been associated with the aging process and age-related diseases (Lee et al., 2004). Ultimately, severe oxidative stress can cause cellular injury and death (Hu et al., 2004).

Several methods are used to measure the antioxidant activity of a biological material. In all cases, the simplest method to measure antioxidant activity is to: 1) dissolve the radical chromogen in the appropriate medium; 2) add antioxidant; and then 3) measure the loss of the radical chromogen photometrically by observing the decrease in absorbance at a fixed time. Lastly, in the final step, the decrease in absorbance can be correlated in a dose-response curve with a standard antioxidant (e.g., Trolox, ascorbic acid) expressing the antioxidant activity as equivalents of standard antioxidant (Arnao, 2000).

The methods for evaluation of antioxidative action should be based on the identification of different antioxidative mechanisms under various conditions (Becker et al., 2004). There are different assays for quantification of radical scavenging activity. These assays primarily operate by direct measurement of hydrogen atom donation or electron transfer from the potential antioxidant to free radical molecules in simple lipid free systems. However, these assays also lack oxidation substrates and may not

necessarily reflect the situation in an oxidized food or *in vivo* situation involving oxidative stress. The assays can be classified as: 1) classical assays for detection of antioxidant activity; 2) assays for detection of scavenging of stable radicals and 3) assays for detection of scavenging of short-lived radicals (Becker et al., 2004).

Classical assays for detection of antioxidant activity includes the Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) and the ferric reducing ability power (FRAP) assay. The TEAC method relies on the reduction of the colored cation ABTS (2, 2'-azino-bis (3-ethylbensothiazoline-6-sulfonic acid)), which is an artificial radical that is not found in biological systems and therefore is not truly representative of an *in vivo* situation. The FRAP assay measures antioxidant capacity by the reduction of the ferric tripyridyltriazine complex to the ferrous complex. Non-enzymatic antioxidants such as ascorbic acid can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions in which one reactive species is reduced at the expense of the oxidation of another. In this context, antioxidant power may be referred to analogously as reducing ability, which is the ferric reducing ability power. Thus, at low pH, when a ferric tripyridyltriazine complex is reduced to the ferrous form, an intense blue color with an absorption maximum at 593 nm develops (Benzie and Strain, 1996). One limitation to this assay; however, is that the reducing capacity measured does not necessarily correlate with actual *in vivo* antioxidant capacity (Becker et al., 2004).

Assays for detection of scavenging of stable radicals include the DPPH (1, 1-diphenyl-2-picrylhydrazyl) and electron spin resonance (ESR) spectroscopy. DPPH is a very stable radical chromogen that presents an absorbance peak at 517 nm in methanolic media. When DPPH reacts with an antioxidant compound that can donate hydrogen, it is reduced. The changes in color from deep violet to light yellow are measured on a (ultraviolet) UV/visible light spectrophotometer. ESR spectroscopy directly measures free radical concentrations by applying stable galvinoxyl or Fremy's salt radicals and the antioxidant capacity to scavenging these radicals is quantified by the decrease of the ESR signal intensity relative to an assay without addition of antioxidant (Becker et al., 2004).

Assays for detection of scavenging of short-lived radicals include oxygen radical absorbance capacity (ORAC) assay, spin trapping and Fenton reaction based radical (FRBR) assay. ORAC assay is based on quenching of fluorescence from the protein β-phycoerythrin by radicals. However, β-phycoerythrin is not photostable and interacts with polyphenols. Spin-trapping agents are specially designed molecules, which react readily with highly reactive free radicals forming relatively stable spin adducts. The FRBR assay is based on coupled reactions as hydroxyl radical scavenging by antioxidants competes with the trapping of radicals by the added spin trap (Becker et al., 2004).

A variety of chemical assays can be used that include the total radical trapping antioxidant parameter (TRAP) assay and the ORAC method, which are typically used to express the total antioxidant capacity of foods. These assays can express antioxidant activity as Trolox equivalent antioxidant capacity (TEAC) or IC_{50} values based on molar units. Trolox, a water soluble analogue of vitamin E having potent antioxidant activity, is not a natural compound found in foods but is typically used as a standard on a comparison basis in the above chemical antioxidant assays. In that regard, there is also typically use of naturally occurring vitamin C as an antioxidant standard. Kim et al. (2002) have suggested that the antioxidant capacity of foods should be measured by a simple, reliable method and be reported using more familiar terms such as vitamin C equivalent antioxidant capacity (VCEAC) as opposed to the use of Trolox equivalents. The VCEAC can be calculated on a weight basis (mg/100 g or mg/100 ml) to show the total antioxidant capacity of a food.

Due to their ease, speed and sensitivity, the most commonly used antioxidant assays are those involving chromogen compounds of radical nature to simulate reactive oxygen and nitrogen species (Arnao, 2000). The methods using ABTS or DPPH scavenging are among the most popular spectrophotometric methods for determining the antioxidant capacity of foods and chemical compounds (Kim et al., 2002). The blue-green ABTS and the violet DPPH radical anions are easy to use, have a high level of sensitivity, and allow for analysis of a large number of samples in a timely fashion. The DPPH assay; however, has been found to underestimate antioxidant capacity by about 34% compared to the

ABTS assay (Arnao, 2000). This underestimation of VCEAC by DPPH radicals may be due to the interference of other absorbing compounds at 517 nm, whereas the blue-green ABTS radical chromogens are detected specifically at 734 nm, a wavelength far from the visible region. One of the limitations regarding the use of chromogens to estimate the antioxidant activity of biological samples is that interference at specific wavelengths may occur due to interfering compounds in the analyte. Hence, the more color that might reside within a sample, the smaller the absorbance decrease and the less antioxidant activity measured. The interferences arise because of the presence of colored compounds (anthocianins, carotenoids, etc.) in the samples or due to the production of secondary reaction products between the chromogen and the samples being analyzed. Therefore, it is crucial to choose a wavelength far from the visible region to avoid interferences (Arnao, 2000). In the case of the ABTS test, the problem is less serious since the chromogen presents absorbance peaks at 734 and 842 nm. However, the DPPH assay measures wavelength at 517 nm, which is near the visible region causing interference, resulting in underestimation of antioxidant activity. Furthermore, DPPH is a free radical that is acquired directly without preparation while ABTS must be generated by enzymatic or chemical reactions. The ABTS assay; however, can measure the total antioxidant activity of any food sample in which the compounds are either lipophilic or hydrophilic since ABTS radical chromogens can be dissolved not only in aqueous phase but also in organic phases, whereas DPPH radical chromogens can be solubilized only in organic media (Kim et al., 2002).

The solvent used in the assay system can also affect the efficiency of antioxidants as the scavenging rate strongly depends on the nature of the solvent (Becker et al., 2004). In non-polar solvents, a hydrogen bonded complex between the antioxidant and the peroxyl radical is formed and a direct hydrogen transfer is facilitated. In an aqueous environment such as the water/lipid interface in emulsion, electron transfer is facilitated through stabilization of the ion pair, which results in the acceleration of the overall process of hydrogen transfer for the chain breaking antioxidant. Polar solvents may also hinder formation of the complex between the phenol and the peroxyl radical due to a preferential

formation of a hydrogen bonded complex between the phenol (hydrogen bond donor) and the solvent molecule (hydrogen bond acceptor).

2.7 Antioxidant Activity of Soy Proteins and Soy Peptides

Soy protein derived peptides or amino acids are suggested to exert antioxidative effects. A variety of *in vitro* tests of antioxidant activity have been used for direct comparison of antioxidant activities of soybean extracts. Chen et al. (1998) reported that histidine (His)-containing peptides from soy protein act as metal-ion chelators, active oxygen quenchers and hydroxyl radical scavengers to produce the *in vitro* antioxidative activity as assessed by 1, 1-diphenyl-2-picrylhydrazyl radical scavenging (DPPH) assay. In this study, 22 synthetic His-containing peptides were designed on the basis of the antioxidative peptide (leucine (Leu)-Leu- proline (Pro) -His-His) derived from proteolytic digests of a soybean protein. However, only a marginal DPPH scavenger activity was observed from these peptides.

Saito et al. (2003) constructed two series of combinatorial tripeptide libraries to explore antioxidative properties of soy derived peptides; one was composed of 108 peptides containing either two His or tyrosine (Tyr) residues in the peptides and the other was composed of 114 peptides structurally related to Pro-His-His. Antioxidant activities of the tripeptide library were examined against the peroxidation of linoleic acid and it was found that the peptides containing two Tyr residues had higher activities than the corresponding peptides containing two His residues at the concentration of 40 μM. Among the tested peptides, Tyr-(His, lysine (Lys), arginine (Arg))-Tyr showed the highest antioxidant activity. Saito et al. (2003) also found that the tripeptides containing (tryptophan) Trp or Tyr residue at the C-terminus showed high radical scavenging activities using ferric reducing power ability (FRAP) assays due to the special capability of phenolic and indolic groups serving as hydrogen donors.

β-Conglycinin hydrolysates were reported by Chen et al. (1995) to have antioxidative activities according to the antioxidant activities of six peptides isolated from the hydrolysates in their study. In this study, a 3% β-conglycinin solution was treated with five different proteases with distinct specificities. Upon enzymatic hydrolysis, antioxidative effects of β-conglycinin increased with protease S; however, the antioxidant activity as measured by the ferric thiocyanate method did not increase with increasing degree of hydrolysis. The peptides from the hydrolysates were then separated by size exclusion chromatography and isolated by reversed-phase HPLC using a 0.1% TFA-acetonitrile system and then a 10 mM ammonium acetate-acetonitrile system. Chen et al. (1995) also found that the antioxidant activity of the hydrolysates is inherent to the characteristic amino acid sequences of the peptides, which depended upon the protease specificities. From the six peptides isolated, four antioxidant peptides contained His residues in the sequence and the two remaining peptides contained Tyr residues, which are potent hydrogen donors. All the antioxidant peptides isolated from β-conglycinin contained a Pro residue in the sequence. The antioxidant activity of His-containing peptides could be attributed to their chelating ability and the lipid radical trapping ability of the imidazole ring (Uchida and Kawakishi, 1992). It also appears that the characteristic amino acid sequences of peptides are required to express the antioxidant effects since no antioxidant activity was observed when the constituent amino acids were mixed at the same concentration as the peptides (Chen et al., 1995).

Soybean extracts were found to possess free radical scavenging activities, which were influenced by genetic and environmental differences. Processed soy products such as tofu had approximately 50% of free radical scavenging activity as assessed by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) free radical and photochemiluminescence (PCL) methods as compared to that of raw soybean, which indicates that some processing methods can affect the free radical scavenging activity in soybeans. The highest and the lowest free radical scavenging activity were 12.18 and 7.51μmol butylated hydroxytoluene (BHT) equivalent/g soy (Lee et al., 2004).

In animal studies, Madani et al. (2000) revealed that rats fed a 20% soy protein diet were found to have lower concentration of plasma thiobarbituric acid-reactive substances (TBARS) than rats fed a 20% casein diet, indicating lowered lipid peroxidation. In a study by Aoki et al. (2002), rats were fed casein or soy protein isolate based diets and exposed to paraquat (PQ), a widely used herbicide, which causes severe injury to the lungs and other organs. PQ produces oxidative stress by redox cycling with a variety of cellular diaphorases and oxygen, generating superoxide radicals. Intake of SPI inhibited the PQ-induced increase in the hepatic TBARS/total lipid ratio, indicating an inhibition of PQ-induced lipid peroxidation. Intake of SPI was also found to increase hepatic glutathione (GSH) concentrations. PQ intake elevated hepatic glutathione disulfide (GSSG) concentration in rats fed the casein diet and these effects were attenuated in rats fed SPI suggesting that intake of SPI might increase the antioxidative capacity of the liver. Furthermore, the hepatic glutathione S-transferase (GST) activity of rats fed PQ was significantly increased by SPI. This latter finding suggested that the radical scavenging activities and enhancement of the activity of glutathione-related enzymes induced by SPI intake are factors that prevented the oxidation of the reduced form of GSH by PQ. Aoki et al. (2002) suggested that the antioxidative activity of soy protein might be explained by either its amino acid composition or via an effect induced by soy-derived peptides. For example, L- Arg, which reportedly has antioxidative activity (Wallner et al., 2001), is abundant in soy protein. Takenaka et al. (2003) found that dietary soy peptides as well as SPI reduced the PQ-induced oxidative stress in rats as shown by lower tissue concentrations of TBARS and higher hepatic total GSH concentrations. On the other hand, the feeding of an amino acid mixture simulating the amino acid composition of soy protein did not have any antioxidative effect. Moreover, the SPI and soy peptide treatment groups showed a lower hepatic GSSG/GSH ratio relative to the casein and amino acid fed groups. This latter study thus suggests that the peptides rather than the amino acids released from the digestion of soy protein exert the antioxidant effects attributed to soy intake.

The antioxidant activity of soy protein has been consistently noted in human feeding studies. Ashton et al. (2000) conducted a 30-day study supplementing 45 healthy female

volunteers with either 150 g meat or 290 g tofu (containing 119.8mg isoflavones) and found a decrease in susceptibility to *in vitro* oxidation of low density lipoprotein (LDL) isolated from subjects fed the tofu diet as compared to meat-fed group. Swain et al. (2002) showed that postmenopausal women supplemented with either 40 g SPI with or without isoflavones or 40 g whey protein over 24 weeks had a higher plasma total antioxidant capacity as assessed by ABTS assay when provided with the soy protein supplements than whey protein supplemented group, regardless of the isoflavone content. Likewise, Vega-Lopez et al. (2005) showed that both hypercholesterolemic males and post menopausal women had higher plasma antioxidant capacities as measured by high performance liquid chromatography (HPLC) with subjects supplemented with 25 g SPI either with or without isoflavones as compared to subjects receiving 25 g animal protein supplements. In the latter study, the antioxidant effects of SPI were unaffected by the presence of isoflavones. Similar findings were observed in a recent study conducted by Heneman et al. (2006), who studied the antioxidant capacity of serum following intake of 25 g of either milk protein shakes or soy protein shakes with or without added isoflavones. The antioxidant capacity was assessed at baseline as well as 4, 6 and 8 h after the protein shake consumption. Surprisingly, only subjects fed soy protein shakes without isoflavones showed a significant increase in the plasma antioxidant capacity whereas no antioxidant effect was noted with the groups fed the soy protein shakes containing isoflavones or the milk protein supplemented group. The isoflavonoids may not impact *in vivo* antioxidant since over 75% of genistein and diadzein exist in conjugated form as glucuronide or sulfate in the plasma and urine of humans (Heneman et al., 2006). Conjugation with sulfate or glucuronide occurs on the site of the only hydroxyl group available on the isoflavone molecule, which thus decreases the antioxidant capacity of the isoflavonoid metabolite. Recently, Brown et al. (2004) who compared the intake of soy protein versus whey protein in 27 male athletes found that 33 g of soy protein per day for nine weeks prevented training-induced drops in antioxidant capacities to a significantly greater degree when compared to whey protein intake. In summary, although intake of soy protein appears to exert *in vivo* antioxidant effects, there appears to be little contribution of isoflavones in this regard.

2.8 Principle of High Pressure Processing

High pressure food processing generally relies on the application of isostatic hydraulic pressures in the range of 100 to 1000 mega-pascal (MPa). The high pressure process is usually carried out with water as a hydraulic fluid to facilitate the operation and compatibility with food materials. The process temperature during pressure treatment ranges from below 0°C to greater than 100°C with exposure times ranging from a few sec to over 20 min. The isostatic principle indicates that pressure is transmitted in a uniform and quasi-instantaneous manner throughout the biological sample independent of size, shape and food composition (Tabilo-Munizaga and Barbosa-Canovas, 2004). The pressurization process time is therefore independent of sample volume, in contrast to thermal processing. Keeping the sample for extended period of time does not require any additional energy. Raising the pressure up to 400 MPa necessitates approximately the same amount of energy as heating at 30°C. The low energy levels involved in pressure processing may explain why covalent bonds of food constituents are usually less affected than weak interactions (Cheftel and Culioli, 1997). The application of pressures less than 800 MPa leads to the disruption of the native structures of most proteins in solution because the volume of the system (i.e., protein plus solvent) becomes smaller when the protein adopts an unfolded conformation (Tabilo-Munizaga and Barbosa-Canovas, 2004).

High pressure induces modification of macromolecules that include proteins, via disruption of hydrophobic and electrostatic interactions. Pressure treatment can cause a variety of structural effects on proteins, inducing reversible or irreversible structural modifications leading to protein denaturation, aggregation or gel formation. Proteins can thus be denaturated not only by high temperatures but also by high pressures and low temperatures. Depending on the pressures used, the protein conformational changes can induce a variety of modifications of functional properties of the food (Chapeau and Lamballerie-Anton, 2003). Conformational changes in proteins resulting from pressure application often imply small volume changes, i.e., below 1% (v/v). Such conformational changes affect inter-atomic distances in weak intra- and intermolecular interactions, including protein-bound water. Covalent bonds and the primary structure of proteins;

however, are unaffected. High pressure treatment does not have sufficient energy to cause a breakdown of the disulfide (bond energy 213.1 kJ/ mol), carbon–carbon (83.1 kJ/mol), carbon–oxygen (84.0 kJ/mol), or carbon–nitrogen (69.7 kJ/mol) bonds. Even at 10,000 MPa, the energy provided is less than 8.37 kJ/mol. Since covalent bonds are unaffected by high pressure, the primary structure of proteins remains intact during pressure treatment. Consequently, high pressure treatment can only break down relatively weak hydrogen bonds and van der Waals forces and the effect may also be temporary (Hayakawa et al., 1996). The resistance of covalent bonds to pressure means that low molecular weight food components responsible for nutritional and sensory characteristics remain intact during pressure treatment, whereas high molecular weight components whose tertiary structure is important for functionality determination are sensitive to pressure (Tewari et al., 1999).

In general, high pressure treatment induces the breakdown of: (1) salt bonds, due to electrostriction; (2) hydrophobic interactions (i.e., alignment and volume reduction of water molecules close to hydrophobic groups); and (3) apparent re-enforcement of hydrogen bonds (Ramaswamy and Riahi, 2003). The re-enforcement of hydrogen bonds is attributed to a decrease in the O-H inter-atomic distance resulting in smaller molecular volumes. As hydrogen bonds are affected by pressurization, the secondary structure of proteins (α-helix and ß-sheets), in contrast to the tertiary or quaternary structure, is affected under pressure. Various protein structural studies have indicated that pressures above 100 – 200 MPa often cause: (1) the dissociation of oligomeric structure into their subunits; (2) partial unfolding and denaturation of monomeric structures (in most cases irreversibly); (3) protein aggregation (probably as a consequence of unfolding); (4) protein gelation whenever the pressures and protein concentrations are high enough (Heremans et al., 1997). These structural changes depend on the nature and concentration of the protein, as well as on the applied pressure, temperature, treatment time and characteristics of the surrounding environment, such as pH and ionic strength (Tabilo-Munizaga and Barbosa-Canovas, 2004).

The extent of protein denaturation depends on the hydrophobicity or hydrophilicity of molecules. Chapeau et al. (2003) reported that high pressure treatment induces modification of surface hydrophobicity and sulfhydryl interactions. Pressure denaturated proteins, unlike heat denatured proteins, retain a compact structure with water molecules penetrating their core. Also, pressure-induced denaturation of protein in solution has been related to the cleavage of hydrogen bonds between the surrounding and/or boundary water on the surface of globular protein molecules (Hayakawa et al., 1996). In general, pressures in the range of 100 – 300 MPa can lead to reversible denaturation, while pressure greater than 300 MPa lead to irreversible denaturation. The denatured protein tends to produce a gel stabilized by an intermolecular network, preventing refolding to the native state (Hayakawa et al., 1996).

2.9 High Pressure Processing of Food Proteins

High pressure processing can induce changes to the conformational structure of the proteins indicated by the increase in the exposure of hydrophobic groups, which can then alters the functional properties of the protein (foaming, emulsifying, gelling and water binding capacities). Johnston et al. (1992) showed that pressure processing of skim milk can increase the number of exposed hydrophobic groups on the surface of the protein with increasing severity and duration of high pressure, which indicates irreversible protein unfolding. Gaucheron et al. (1997) subjected milk to high pressures ranging from 250, 450 and 600 MPa for 30 min at temperatures of 4°C, 20°C and 40°C. All pressure treatments except 250 MPa led to an increase in protein hydrophobicity, a decrease in average diameter of particle and slight solubilization of calcium and phosphorous from the colloidal to the aqueous phase of the milk. At 40°C and 250 MPa, the pressure effects were different because protein hydrophobicity remained unchanged.

High pressure processing can also increase the formation of intra to intermolecular disulfide bonds. Tanaka et al. (1996) showed that the reactivity of the sulfhydryl (-SH) group of ß-lactoglobulin B (ß-lg B), which is buried within the protein, increased with increasing pressure as a result of exposure of the -SH group to the protein surface. The

intermolecular and intramolecular reactions of the -SH group with increasing pressure was indicated to be the main cause for irreversible pressure-induced denaturation of ß-lg B. Likewise, Funtenberger et al. (1997) showed that the formation of intermolecular disulfide bonds through SH/disulfide (S-S) interchange reactions appears to be enhanced under pressure. Formation of high pressure induced S-S bonds was also observed by Nabhan et al. (2004) when investigating the effect of high hydrostatic pressures applied at different temperatures on the structural changes of milk proteins. Raw bovine milk was submitted to high pressure (300 to 600 MPa) and a range of temperatures (4 to 70°C) for either 2 or 5 min. The combined effects of pressure and temperature on milk protein-induced structural changes and polymer and copolymer formation was characterized by anion-exchange and size-exclusion fast protein liquid chromatography and electrophoretic techniques. Approximately one-half of the ß-lg B formed polymers and the other half of the molecules formed large copolymers, mainly with κ-casein, α-lactalbumin via intermolecular S-S bond exchange, and αs1-casein via physicochemical interactions. Other whey proteins (i.e., serum albumin, immunoglobulins, lactoferrin) also participated in the formation of the copolymers but to a lesser extent (Nabhan et al., 2004).

Pressure processing can also induce secondary changes to the protein. Hosseini-nia et al. (2003) studied the effect of structural changes in physical and chemical conditions on the secondary structure of whey proteins with an increasing hydrostatic pressure (up to 12.0 kbar). These workers found that high pressure caused both reversible and irreversible changes in the conformation of ß-lg proteins including ß-lg A, ß-lg B and the combination of ß-lg A + ß-lg B. Most of the secondary structural changes of ß-lg A+B induced under high pressure were reversible and were attributed to differences in hydrophobic interactions and/or S-S bonds in the secondary structures induced to ß-lgB as opposed to ß-lg A. Hence it is likely that the functional or biological attributes of ß-lg, which is typically composed of a combination of ß-lg A + ß-lg B may be differentially affected by a single application of hydrostatic pressure depending upon the genetic variant of ß-lg.

Pressure processing produces little energy and does not provide sufficient high energy required to break the S-S bonds. Ahmed and Ramaswamy (2003) studied rheological

changes of glycomacropeptide (GMP) upon exposure to high pressures from 100 to 400 MPa for 30 min and temperature from 20 to 80°C for 15 min. GMP is a nutritionally important minor protein present in whey along with lactoperoxidase, lactoferrin and protease peptones. Ahmed and Ramaswamy (2003) found relatively insignificant changes in the magnitude of rheological parameters during pressurization. Coagulation of the protein was observed at the pressure level of 400MPa. The slight decrease noted in rheological parameters at 400 MPa might be attributed by slight deformation of κ-CN bonds and change of orientation. The energy generation from high pressure is only 9.6KJ/g/mol per 10,000 MPa and so could not disrupt completely κ-CN bonds.

3.0 Pressurized Soy Proteins: a New Perspective

High pressure processing has been applied to soy proteins. Puppo et al. (2004) subjected soy protein to the high pressure (200 – 600MPa) and similar observations in protein conformational changes as previously observed with other food proteins was also found with soy protein. Puppo et al. (2004) further tested the high pressure effect in combination to acidic and alkaline pH environment. The solubility of pressure treated soy protein in an acidic environment was found to increase with 200 MPa but no effects were found with 400 and 600 MPa. The surface hydrophobicity of pressure-treated SPI increased in both acidic and alkaline environments, indicating that pressure treatment produced a molecular unfolding of the protein with the exposure of the hydrophobic groups to the medium. However, the increases in surface hydrophobicity were more pronounced at alkaline pH. As previously described for other proteins, protein unfolding of soy proteins was accompanied by the formation of S-S bonds. In addition, Puppo et al. (2004) showed that with increasing pressure, the -SH groups of the SPI decreased. They also observed that the content of -SH groups was higher at acidic pH due to the protonation. Puppo et al. (2004) suggested that high pressure treatment at 200, 400 and 600 MPa produced unfolding of 7s and 11s of soy protein as well as the dissociation of glycinin.

In the research directed by Dr. Kubow at McGill University, whey protein solutions of different concentrations were studied under five different modes of pressure of 400 MPa at 25°C which included: (a) one pulse cycle; (b) three pulse cycles; (c) a combination of two pulse cycles + 10 min holding time followed an additional one pulse cycle, (d) 5 pulse cycles, and (e) a combination of two pulse cycles + 5 min holding time followed by another three pulse cycles (Hosseini-nia et al., 2003). A greater or lesser number of S-S bonds were detected following pressurization depending upon the pressure treatment used. This research demonstrated that hyperbaric pressure treatment of whey proteins leads to the changes in the accessibility of S-S bonds to solvents. Hence, the surface polarity of the molecule that exposes the hydrophobic groups to the polar environment can be altered by pressurization.

Recent studies have indicated that pressure treatment could cause conformational changes in whey proteins that might expose its amino acids sequences for digestion that were not available before the pressurization (Vilela et al., 2006). This greater accessibility of pressurized whey proteins to digestion would allow for a more rapid proteolysis of the proteins resulting in the liberation of small bioactive peptides. This was confirmed by the analysis by mass spectroscopy (MS). After 30 min of pepsin digestion, MS studies demonstrated that pressure treated whey proteins showed the release of different peptide profiles, presumably due to increased access of digestive enzymes to different regions of the whey proteins (Vilela et al., 2006). Additionally, single cycle pressurization of 550 MPa of whey proteins was similarly effective in enhancing *in vitro* proteolysis as repeated three cycle pressurization (Vilela et al., 2006). Dr. Kubow and collaborators have produced a series of animal studies testing the bioactivity of whey proteins as affected by pressure processing (Kubow et al., 2005). In the initial studies, the rats were fed semi-purified diets containing either pressurized (three cycle pressurization involving 400 MPa) whey protein isolates (WPI) or native WPI at a concentration of 20 wt% for either 17 or 35 days. The animals fed pressurized WPI were found to have decreased serum IgG and IgE, decreased levels of plasma TBARS, increased hepatic GSH concentrations, greater weight gain and feed efficiency ratios when compared with the controls fed native WPI (Hosseini-nia et al., 2003) It was suggested that the enhanced

weight gain and tissue GSH concentrations could be due to an increase in the digestibility of the pressurized WPI; however, effect of pressurization on protein digestibility was not tested.

In another study where the male Sprague Dawley rats were fed a diet containing native WPI, 1-cycle (550 MPa) or 3-cycle (400 MPa) pressurized WPI for 40 days (Jing, 2005). The 1-cycle pressure treatment group showed significantly greater growth than the group fed native WPI suggesting that 1-cycle pressure treatment could be used to increase feed efficiency. No differences; however, were observed among the dietary groups in terms of tissue lipid peroxidation indices, liver peroxides, or tissue GSH concentrations, which conflicts with the results of Hosseini-nia (2003). Many variables associated with commercial WPI production could have led to the differential impact of pressurization in these two animal feeding trials including differences in the type of WPI used as different processing techniques were used to manufacture the WPI used in the two different studies.

To our knowledge, only one animal study has been conducted regarding the study of the nutritional benefits from the consumption of soy proteins exposed to pressure processing. Rats were fed for 28 days with either standard laboratory diet, tofu-containing diet or diet with tofu treated with high pressure of 400 MPa at 20°C for 30 min (Prestamo and Arroyo, 2000). They found that the soy isoflavones such as genistein and daizein were unaffected with high pressure treatment. It was postulated that high pressure processing can destroy the phytates, which normally act to inhibit the action of a number of digestive enzymes, including pepsin, trypsin and alpha amylase. By removing the phytate inhibition of amylase, high pressure processing may have contributed to the increased in blood glucose levels observed in rats fed with pressurized tofu. Also, the tofu was more dispersed and thus more tofu components could become accessible to digestive enzymes with the high pressure treatment. Moreover, liver weight and serum calcium content were significantly lower in rats fed the high pressure-treated tofu as compared to rats fed conventional diet (Prestamo and Arroyo, 2000).

In conclusion, more studies are still needed to confirm the antioxidant potential of peptides derived enzymatically from SPI. In addition, no studies have been carried out to investigate the antioxidant potential of peptides or hydrolysates produced from the enzymatic digestion of pressurized soy protein. In that regard, there are potential applications for the development of new functional foods based on pressure treatment of SPI, which could potentially produce products with more potent nutritional and nutraceutical properties including improved antioxidant action. It is conceivable that pressurized SPI would be more accessible to digestive enzymes, which would allow for a more rapid proteolysis of the proteins and an increased or altered release of small bioactive antioxidant peptides. The present thesis has thus focused on the possible effects of pressure treatment of SPI on the digestibility and antioxidant activity of the resulting peptides and hydrolysates.

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CHAPTER 3

MANUSCRIPT

Antioxidant Activities of Hydrolysates and Peptides Generated from Hydrostatic Pressure-Treated Soy Protein Isolates

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ABSTRACT

Background: Soy protein intakes as well as peptides derived from soy have been indicated to exert potent antioxidant effects; however, the antioxidant capacities of hydrolysates or low molecular weight peptides generated from digestive enzyme action have been sparsely studied. Hydrostatic pressure has been indicated to enhance the digestibility and antioxidant capabilities of whey protein isolates but no studies have been performed with soy protein isolates (SPI) in this regard. The purpose of the present study was to investigate the *in vitro* antioxidant effects of hydrolysates and low molecular peptides generated from the hydrolysis of native and pressure-treated SPI. **Materials and methods:** SPI were exposed to hyperbaric pressure treatment (600 MPa) and hydrolysates were generated via *in vitro* dual enzyme pepsin-pancreatin digestion. Low molecular weight (< 1 kilodaltons) peptides from the SPI hydrolysates were obtained using membrane ultrafiltration and characterized by capillary zone electrophoresis (CZE), reversed phase high performance liquid chromatography (RP)-HPLC and electrospray ionization-mass spectroscopy (ESI-MS). *In vitro* antioxidant activities of hydrolysates and low molecular weight peptides isolated from native and pressure-treated SPI were assessed by the ferric reducing antioxidant power (FRAP) and 1, 1- diphenyl-2-picrylhydrazyl (DPPH) assays. **Results:** Hydrostatic pressure treatment increased significantly ($P < 0.05$) SPI digestibility following 10 min of pepsin digestion. Differential peptide profiles were observed following pepsin and pancreatin digestion of pressurized SPI in comparison to native SPI as evaluated by ESI-MS, RP-HPLC and CZE. The low molecular weight peptides obtained from pressurized SPI exerted significantly ($P < 0.05$) greater antioxidant activity as assessed by FRAP whereas the hydrolysates of native SPI showed higher FRAP antioxidant activity. Both the low molecular weight peptides and hydrolysates obtained from native SPI exerted significantly ($P < 0.05$) greater antioxidant activity as assessed by the DPPH radical scavenging assay than the peptides and hydrolyates derived from pressure-treated SPI. **Conclusion:** These results indicate that pressure treatment alters hydrolysis of SPI leading to hydrolysates and peptides that have differential antioxidant capacities.

***Key words*:** soy protein isolates, peptides, antioxidant, hyperbaric pressure

3.1 INTRODUCTION

Soy proteins and isoflavones have gained considerable attention for their potential health benefits which include the lowering of blood cholesterol and blood pressure levels, prevention of heart disease and the reduction of the risks for certain forms of cancer (Messina et al., 2002). Soy products may be beneficial to cardiovascular and overall health because of their relatively high content of polyunsaturated fats, fiber, vitamins, minerals and their low content of saturated fat (Sacks et al., 2006). In addition, some of the health benefits of soy protein have been attributed to its antioxidant properties. Human and animal studies have consistently shown that soy protein intake is associated with decreased oxidative stress as demonstrated by diminished *in vivo* tissue lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) levels, lowered susceptibility to *in vitro* low density lipoprotein (LDL) oxidation and enhanced plasma antioxidant capacities as assessed by 2, 2'-azino-bis (3-ethylbensothiazoline-6-sulfonic acid) (ABTS) assay (Madani et al., 2000; Aoki et al., 2002; Swain et al., 2002). Moreover, clinical trials have also found that the antioxidant effects of soy protein supplements are not enhanced by the inclusion of isoflavones thereby indicating that peptides might be the *in vivo* bioactive components (Heneman et al., 2006).

A limited number of *in vitro* studies have demonstrated that soy protein derived peptides can exert antioxidant effects (Chen et al., 1998; Saito et al., 2003). Chen et al. (1998) reported that the *in vitro* antioxidant activity of histidie (His) -containing peptides derived from soy protein can act as metal-ion chelators, active oxygen quenchers and hydroxyl radical scavengers. Saito et al. (2003) constructed two series of combinatorial tripeptide libraries to explore antioxidant properties of soy-derived peptides against the peroxidation of linoleic acid. One library was composed of 108 peptides containing either two His or tyrosine (Tyr) residues whereas the other library was composed of 114 peptides structurally related to proline (Pro)-His-His. Peptides containing two Tyr residues were shown to have relatively higher antioxidant activities than corresponding peptides containing two His residues. Saito et al. (2003) also found that the tripeptides containing tryptophan (Trp) or Tyr residue at the C-terminus showed high radical

scavenging activities due to the special capability of phenolic and indolic groups serving as hydrogen donors.

Hydrostatic pressure processing has been used in the food industry to decrease the microbial population, reduce secondary contamination and to retain good taste (Basak et al., 2003). Additionally, high pressure processing can induce protein conformational changes that lead to an increased exposure of hydrophobic amino acids groups to solvent and conversion of intramolecular to intermolecular disulfide bonds (Tanaka et al., 1996; Funtenberger et al., 1997). Soy proteins subjected to high pressure processing (i.e., 200, 400 and 600 mega-pascal (MPa) of pressure) demonstrated conformational changes such as unfolding of 7s and 11s and the dissociation of glycinin (Puppo et al., 2004). Other studies have demonstrated that pressure processing of food proteins can improve functional properties within foods such as gelation (Montero et al., 2002). The physiological consequences of pressure processing of soy proteins; however, have been relatively unexplored. Prestamo and Arroyo (2000) demonstrated that tofu became more dispersed and more tofu components became more bioavailable with high pressure treatment (400 MPa). Pressure processed tofu was also associated with an increase in blood glucose levels. This latter finding was suggested to be due to the destruction of phytates within tofu, which inhibit the action of digestive enzymes, including alpha amylase, leading to a more rapid rise in blood glucose level (Prestamo and Arroyo, 2000). Prestamo and Arroyo (2000) also demonstrated that the soy isoflavones within tofu such as genistein and daizein were unaffected with high pressure treatment. We have recently demonstrated that treatment of whey protein isolates (WPI) with single cycle pressurization of 550 MPa resulted in a more rapid *in vitro* proteolysis resulting in whey peptide profile changes (Vilela et al., 2006). The treatment of cultured mutant CFTR (cystic fibrosis transmembrane conductance regulator) cells with the low molecular weight peptides (< 1 kDa) isolated from the hydrolysates of pressure-treated WPI resulted in enhanced glutathione status as compared to cells treated with peptides obtained from native whey protein hydrolysates (Vilela et al., 2006).

Despite several studies indicating the *in vivo* antioxidant potency of soy proteins, the antioxidant capabilities of peptides generated from soy protein hydrolysis remains relatively unexplored. Moreover, the impact of pressure processing on soy protein digestion and the functional consequences of the released peptides in terms of antioxidant effects is unclear. The present study thus examined whether pressure processing of soy protein isolates (SPI) alters: (1) the hydrolysis of SPI and resulting peptide profiles; and (2) the antioxidant capabilities of the hydrolysates and low molecular weight peptides isolated from SPI digestion. The antioxidant capabilities of peptides with molecular weights < 1 kDa was studied since these peptides are primarily absorbed *in vivo* (Qiao et al., 2004).

3.2 MATERIALS AND METHODS:

Materials

Soy protein isolates (SPI) were kindly provided as a gift by ADM Protein Technologies Inc. (Decatur, Illinois). The soy protein isolate PRO-FAM 974 (catalogue # 066-974) contains approximately 90% protein and 4% fat. This SPI is alcohol washed and contains minimal concentrations of isoflavones (0.4 mg of total isoflavones/ g protein). The pH of the isolate is 7.0-7.4 when 1:10 (w/v) dispersed in water. All samples were stored and sealed in plastic containers and froze at - 80°C. Pepsin (catalogue # P6887) and pancreatin (catalogue #P1625) were obtained from Sigma-Aldrich Co (Oakville, Ontario). DPPH (1, 1-Diphenyl-2-picrylhydrazyl) ~97 %, (catalogue #D0909) was obtained from TCI America (Portland, U.S.A.). TPTZ (2, 4, 6-tripyridyl-s-triazine) (catalogue # 3682357) was obtained from Acros (Pittsburgh, USA). Bovine serum albumin (BSA), Fraction V (catalogue # A2153) was obtained from Sigma-Aldrich Co.

Methods

High Pressure Treatment of SPI

The SPI was dissolved in doubly distilled water to make a 12.5% solution (w/v) and packaged in low-density polyethylene bags (Whirl-Pak®, USA). Entrapped air was removed by hand pressing and heat sealed. Samples were pressure treated at 500-600 MPa for selected time in a 5 L semi-continuous Hyperbar System (ACIP 6500/5/12VB; ACB Pressure Systems, Nantes, France) equipped with temperature controller and pressure regulator. The pressurization rate was about 4.4 MPa/s and released at 26 MPa/s. Tests were done at room temperature (20°C) which increased to 25-30°C due to the adiabatic effect during pressurization; however, temperature during the hold time was maintained around 25°C because of the temperature controlled water circulation in the jacket. Various pressure treatments and holding times were tested (i.e. 500MPa, 550MPa, 600MPa + 0/ 5 min holding time) and 600MPa + 5 min holding time was selected for its optimal protein digestibility (data not shown). The native and pressurized 12.5% (w/v) SPI solutions were lyophilized at -80°C and 90 MT vacuum in a Flexi-Dry MP lyophilizer (FTS Systems, Stone Ridge, NY) in preparation for future *in vitro* digestibility studies. Approximately 48 to 72 h was needed to freeze-dry the samples depending on the volume used. Some lyophilized proteins were flushed with N_2 and stored at - 20°C in sealed plastic containers with desiccants for subsequent *in vitro* digestibility studies and were used within 2 months. The remaining lyophilized proteins were placed into aliquots in Cryovials (5ml) and stored in a deep freezer at - 80°C to avoid repeated freeze thaw cycles.

In Vitro Enzymatic Digestion

The *in vitro* enzymatic digestion method to simulate the *in vivo* gastrointestinal digestion of milk proteins was performed as previously described by Vilela et al. (2006). The freeze-dried native and pressurized soy proteins were diluted in double distilled water to a concentration of 3 mg protein/ml and the pH of the solution was adjusted to 1.5 with HCl by drop-wise addition of 10 N HCl. Digestibility assays were performed in five replicates

and repeated in six separate experiments. The 0.3% (w/v) SPI solution was filtered by 45 μm filter (Millex GV; Millipore, Bedford, Massachusetts) and placed into five (10 ml) plastic tubes, which were added into a shaking water bath at 37°C. In order to digest the protein, 650 μl of pepsin solution (5 mg/ml in 0.01 N HCl) was added to the protein solutions to start the reaction. Spectrophotometric (Beckman, DU 640, Fullerton, CA) readings were taken at 10, 20 and 30 min. At the end of the 30 min, 2 drops of 10 N NaOH were added to terminate the enzymatic reaction. The peptidic digests were placed into a water bath at 40°C and the pH was adjusted to 7.8 for the optimal reaction of the pancreatin enzyme. Freshly prepared pancreatin stock solution (5 mg/ml in sodium phosphate buffer pH 7.0) was added to the digests, which were incubated in a water bath for 60 min with gentle shaking. Aliquots of the digestion solution were taken every 15 min for spectrophotometric readings. At the end of the 60 min digestion, 70 μl of 150 mM Na_2CO_3 solution was added to the pancreatic digests to interrupt the enzymatic digestion and the tubes were placed in an ice bath to rapidly decrease the temperature of the solution of the pancreatic hydrolysates.

Peptide Isolation

After the pepsin-pancreatin digestion, the resulting hydrolysates from native and single cycle pressure treated SPI were subjected to ultrafiltration to remove large proteins and the proteolytic enzymes. The ultrafiltration consisted of addition of the hydrolysates to cellulose membranes with a molecular weight cut-off (MWCO) of 1 kDa (Millipore) in a stirred ultrafiltration membrane reactor (Micon Millipore™ system, Model 8050, Millipore, Nepean, Ontario) under nitrogen gas pressure of 40 psi. The isolated peptides in the permeate were freeze-dried overnight in a vacuum concentrator (Flexi-Dry MP Lyophilizer, FTS Systems Inc., Stone Ridge, NY) at - 80°C and 90 MT vacuum and stored at - 20°C in sealed tubes for later use in capillary zone electrophoresis (CZE), reversed phase high-performance liquid chromatography (RP-HPLC), electrospray ionization mass spectroscopy (ESI-MS) and *in vitro* antioxidant assays (i.e. FRAP, DPPH). The freeze-dried peptides were stored in sealed tubes flushed with N_2 in the - 80°C freezer.

Protein and peptide content

The protein and peptide content of the SPI solutions were determined before and after the *in vitro* enzymatic digestions. The protein content of the SPI solutions during pepsin digestion was measured at 0, 10, 20 and 30 min using the Bio-Rad Protein Assay kit (catalogue #500-0006, Bio-Rad; Hercules, CA) in accordance with the procedure of Bradford (1976). The method of Church et al. (1983) was used to quantify peptide concentrations, which involves the measurement of α-amino groups released by hydrolysis via reaction with *o*-phthaldialdehyde (OPA) and β-mercaptoethanol that form an adduct that absorbs strongly at 340 nm. Briefly, 50 ml of OPA solution was freshly prepared as follows: 25 ml of 100 mM sodium tetraborate solution in water; 2.5 ml of 20% (wt/wt) SDS; 40 mg of OPA (dissolved in 1 ml of ethanol); 100 μl of β-mercaptoethanol, and water to complete the volume. Aliquots of each sample were collected before and after digestion with pepsin and pancreatin and added to 1 ml of OPA solution and incubated for precisely 2 min. As the α-amino group content increases with digestion time, the volume of the aliquot to be taken was determined at time 0 and was based on the minimum linear absorbance obtained from the standard curve using phenylalanine - glycine (Phe-Gly) standard with the concentration ranging from 25 to 150 μM. The optical density (OD) was measured 2 min later at 340 nm wavelength since OPA absorption is stable only after 20 min (Panasiuk et al., 1998). Since the absorbance is sensitive to pH, the efficiency of the digestion was determined by measuring the OD at 0 and 30 min at pH 1.5 for the pepsin digestion and at 0 and 60 min at pH 7.8 for the pancreatin digestion. The OD was also determined after ultrafiltration to detect the peptides with molecular weight less than 1 kDa. The efficiency of the digestion was determined by taking into consideration the net α-amino groups detected (i.e. OD before filtration less OD after filtration) after digestion with pepsin and after digestion with pancreatin. The results were expressed as μM of Phe-Gly.

Mass spectrometry (ESI-MS)

Electrospray ionization mass spectroscopy (ESI-MS) was used to analyze the lyophilized MWCO fractions of less than 1 kDa from pancreatin hydrolysates obtained from native and pressure-treated (600 MPa + 5 min holding time) SPI using a hybrid mass spectrometer (Waters Micromass QTOF Ultima Global, Micromass, Manchester, UK) equipped with a nanoflow electrospray source, operated in positive ionization mode (+ESI) at 3.80 kV. The source temperature was 80°C and the desolvation temperature was 150 °C. A 10 mg/ml solution was prepared using the lyophilized (< 1 kDa) pancreatin hydrolysates of both native and pressure-treated SPI for the mass spectrometry. The time of flight (TOF) was monitored at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, and a collision energy of 10 eV (for MS survey). The MS survey mass range, m/z, was 300-1999, which was scanned continuously over the direct infusion run. The mass spectrometer was tuned and calibrated with [Glu]-Fibrinopeptide B (Sigma Chemicals; St. Louis MO). Instrumental control and data analysis were manipulated using software-MassLynx V4.0 (Waters, Massachusetts, USA).

Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis (CZE) was performed to assess peptide profiles of the pancreatin digests using a P/ACE ^{TM} 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to an IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated fused silica capillary column (57 cm × 50 μm, length 50 cm) was assembled in the P/ACE cartridge (Polymicro Technologies, Phoenix, Arizona USA) for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time. Prior to CZE analysis, the peptide solutions were filtered through a 0.2 μm low binding cellulose acetate membrane (Nalgene-nalge®, Nunc International Corporation, Rochester, NY). The running sodium phosphate buffer (0.1 M, pH 2.5) was obtained from Sigma-Aldrich. The sample stock peptide solutions

were prepared by dissolving 50 mg of the freeze-dried filtered hydrolysates in 1.0 ml of the running buffer that was diluted (1:10) with Nanopure water. The solutions were either kept on ice for immediate CZE analysis or stored at –80°C for later CZE analyses. The separation buffer solution was degassed before use. The CZE analyses were performed for a total duration of 30 min at a constant voltage (27 kV) and temperature (30°C) using a UV-detection at 214 nm. The capillary column was pre-rinsed with nanopure water for 2 min and separation buffer for an additional 2 min or until the baseline line was stable before the first sample application. Between sample runs, the capillary was flushed with NaOH 1M for 2 min, and HCL 1N for an additional 2 min followed by the pre-rinse. A mixture of peptide standards (peptide standard for CZE, catalogue # P2693; Sigma-Aldrich Co, Oakville, Ontario) was used to verify the precision and accuracy of the method. The best peak resolution was achieved by injecting 40 µl of samples containing 2 mg/ml of lyophilized peptide extracts obtained following the dual enzyme digestion of pepsin and pancreatin digestion described above. The migration time of the individual peptides were compared relative to the migration time of Bovine serum albumin (BSA) and results were expressed as percent of the internal standard (BSA; 400 µg/ml).

Reversed Phase (RP)-HPLC Peptide

Native and pressure treated lyophilized MWCO fractions of less than 1 kDa SPI (50 mg) were dissolved in Nanopure water (1:10), filtered through 0.22 µm filters (Millex GV; Millipore, Bedford, Massachusetts) and loaded on an analytical C18-reverse phase (RP) column (5 µm; 250 mm × 50 mm) (Vydac Series-218TP54, Vydac Company, Herperia, CA) and separated with a Waters HPLC system. Mobile phase A was prepared by adding 1.0 ml trifluoroacetic acid (TFA) to 1000 ml HPLC grade water. Mobile phase B was prepared by adding 1.0 ml TFA to 1000 ml acetonitrile. A binary gradient of 0.1% TFA in water (phase A) and 0.1% TFA in acetonitrile (phase B) on a gradient from 5% phase B at 5 min to 60% phase B at 60 min at a flow rate of 1.0 ml/min and column temperature of 25 °C was used to separate different peptide components. The column was washed for 5 min with 100% phase B and re-equilibrated for 15 min between injections with the injection volume of 100 µl. The UV data were recorded from 210 nm to 285 nm.

FRAP (Ferric Reducing-Antioxidant Power) assay

The FRAP (ferric reducing-antioxidant power) assay was conducted according to the method of Benzie and Strain (1996). The principle of the assay is based on the reduction of the colorless Fe^{3+}–2, 4, 6-tripyridyl-*S*-triazine complex to the ferrous form (Fe^{2+}), which absorbs light at 593 nm. At low pH, when a ferric-tripyridyltriazine complex is reduced by electron-donating antioxidants to the ferrous form, an intense blue color with an absorption maximum at 593 nm develops. A 0.3 M acetate buffer of pH 3.6 was mixed with 10 mM 2, 4, 6-tripyridyl-*S*-triazine (TPTZ) solution and 20 mM ferric chloride solution in the ratio of 10:1:1, respectively, to form the working FRAP reagent. The absorbance change was converted into a FRAP value (in μM) by relating the change of absorbance at 593 nm of test samples to that of a standard solution of bovine serum albumin (BSA) that has a known FRAP value. A standard curve of BSA was included in each assay to determine linearity at a concentration range from 100 – 1200 μM. The obtained curve was linear over the range of 100 – 1200 μM, a concentration that included all the obtained values. In addition, a concentration range from 100 to 1000 μM of ascorbic acid was tested with the FRAP assay to obtain an indication of the antioxidant potency of the tested peptides in terms of ascorbic acid equivalence. A 10 mg/ml SPI solution was made from lyophilized (1 kDa) pepsin-pancreatin hydrolysates of both native and pressure-treated (600 MPa + 5 min holding time) and centrifuged (Beckman NVT 65.2, Mississauga, Ontario) at 11,070 x g for 5 min at 4°C. Only the clear portion of the centrifuged solution was used. Briefly, 50 μl of either filtered or unfiltered SPI solutions was added to 1.5 ml of the working FRAP reagent. The absorbance was recorded at time 0.5, 1.0, 2.0, 3.0, 4.0 and 21 h to assess the relative stability of the antioxidant activity of the samples. Absorbance at 593 nm was determined relative to the reagent blank solution that contained only the working FRAP reagent. The difference in absorbance between the tested sample and the blank reading was calculated and the data was expressed as μM. Each experiment was performed with three replicates and repeated six separate times.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay

The DPPH assay was adapted from Lijun et al. (2003). The DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a stable radical chromogen that presents peak absorbance at 520. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color from deep violet to light yellow correlate with the loss of the radical chromogen. A concentration range from 0.5 to 1.0 mM of ascorbic acid was tested with the DPPH assay to produce a range of 91 to 98% of DPPH radical scavenging ability. This latter approach was used as a reference for the antioxidant potency of the tested peptides in order to express their values in terms of ascorbic acid equivalents. Solutions were made that contained 10 mg/ml of lyophilized (< 1 kDa) pancreatin hydrolysates from either native or pressure-treated (600 MPa + 5 min holding time) SPI. The hydrolysates were centrifuged (Beckman NVT 65.2, Mississauga, Ontario) at 11,070 x g for 5 min at 4 °C. Following centrifugation, one-half of the upper layer was filtered with a 0.5 μm filter to produce a filtered SPI solution. The other half of the upper layer was kept as the unfiltered SPI solution. Both of the filtered and unfiltered SPI solutions (0.3 ml) were combined with 0.3 ml of 0.2 M morpholinoethanesulfonic acid (MES) buffer (pH = 6.0) and 0.6 ml of ethanol to which was added 2 ml solution of 0.4 mM DPPH. The absorbance of the mixture was measured spectrophotometrically at 520 nm (Beckman, DU 640, U.S.A.) at 0.5, 1.0, 2.0, 3.0, 6.0, 9.0, 12.0 and 24 h. The control contained only ethanol. The result was expressed as % inhibition which was calculated as (OD control minus OD sample)/OD control x 100. Each experiment was performed with three replicates and repeated six separate times.

Statistical Analysis

Data is presented as mean ± SD. To test the protein and peptide content of pressurized SPI fractions following hydrolysis, repeated measures analysis of variance (ANOVA) was used to test the significant differences. Independent sample Students' t-test and multiple comparison tests using Tukey's *post hoc* test were used to compare differences among treatments when significant differences were established via ANOVA. Repeated measures analysis was also used to differentiate significances among different time

points. A p value < 0.05 was considered statistically significant. Statistical analyses were computed with SPSS-11.5 (SPSS Inc., Chicago, Illinois).

3.3 RESULTS

The protein content following 30 min pepsin digestion of native SPI and SPI subjected to isostatic high pressure of either 500 MPa with 0 min holding time or 600 MPa with 5 min holding time is shown in Figure 1. Analysis of variance showed significant ($P < 0.05$) main effects of both length of time of enzyme treatment and type of protein treatment. There were significant differences ($P < 0.05$) detected at 20 min between the native protein and the protein exposed to the 500 MPa + 0 min treatment. There were also significant differences ($P < 0.05$) detected at 10 and 20 min between the native SPI and the 600 MPa + 5 min pressurized SPI. No significant differences were observed between the two types of pressure treated SPI (i.e., 500 MPa + 0 min and 600 MPa + 5 min). The 600 MPa + 5 min holding pressure condition was chosen for this study since this pressure treatment exhibited significant differences at two time points (10, 20 min) whereas the 500MPa + 0 min treatment was only significant at one time point (20 min) when compared to the native SPI samples.

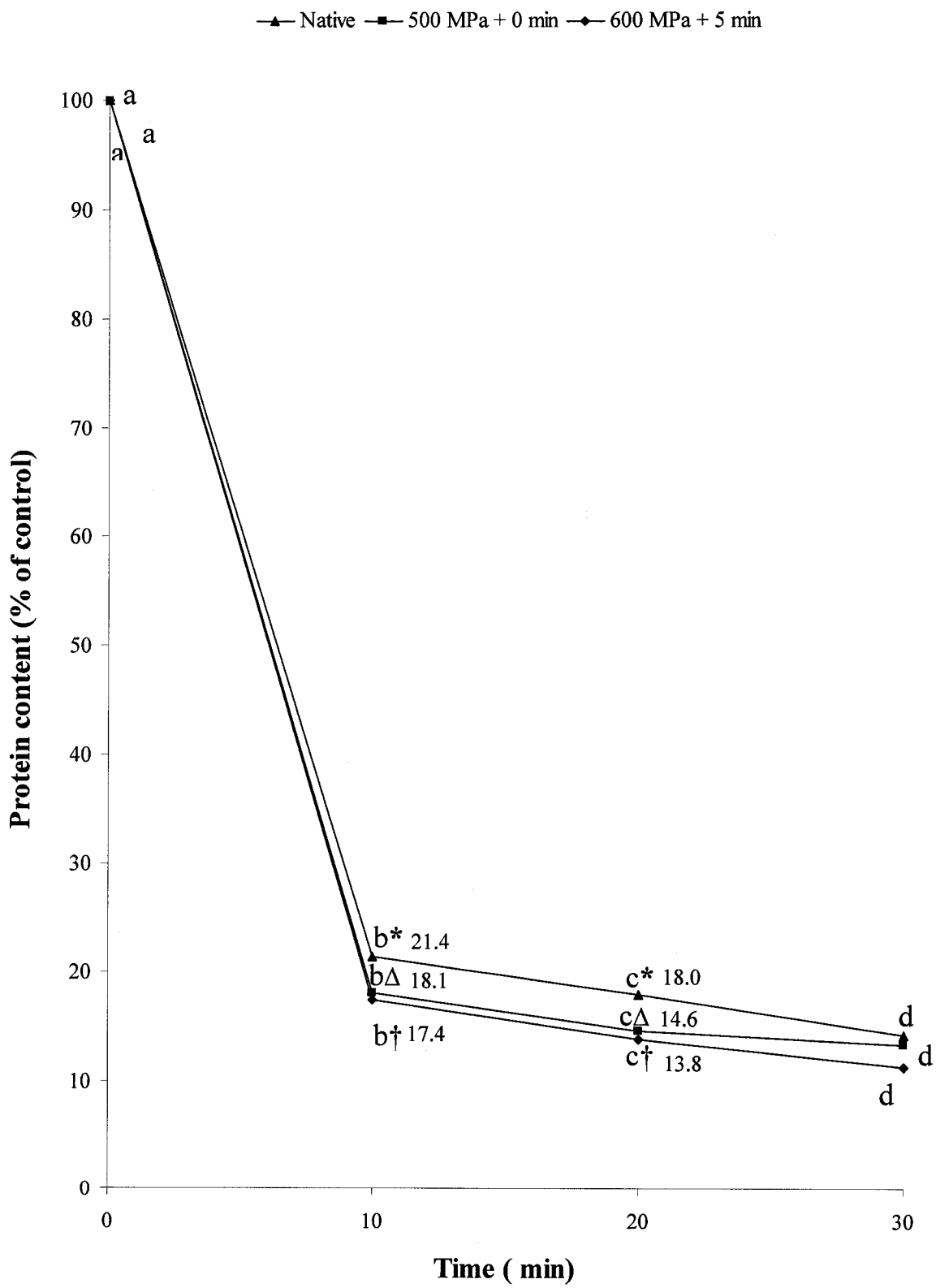

Native
500 MPa + 0 min
600 MPa + 5 min
Protein content (% of control)
Time (min)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
a
a
a
b* 21.4
bΔ 18.1
b† 17.4
c* 18.0
cΔ 14.6
c† 13.8
d
d
d

Figure 1. Effect of hydrostatic pressure treatment on *in vitro* digestion of SPI. SPI were exposed to either 500 MPa + 0 min or 600 MPa + 5 min and the solutions were lyophilized. Native represents lyophilized SPI solution that did not undergo pressure treatment. Native SPI and pressure-treated lyophilized SPI samples were prepared at a concentration of 3% (w/v) and digested with pepsin during 30 min at 37°C. Aliquots were taken every 10 min and the protein content was determined at 590 nm (n = 6). The Y-axis refers to the amount of protein remaining in the peptidic solutions compared to the undigested control. The numbers along the curves represent the percentage of proteins detected at 10 and 20 min. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. Treatments not sharing common symbols (*, Δ, †) indicate significant differences ($P < 0.05$, ANOVA) between groups at each time point by Tukey's *post hoc* comparison.

Peptide Content

No differences were observed in terms of the total peptide content of the native versus pressurized (600 MPa + 5 min) SPI (Fig. 2). Likewise, the content of < 1 kDa peptides did not differ between native and pressurized SPI after 30 min of pepsin digestion or after an additional 60 min of pancreatin digestion (Fig. 3).

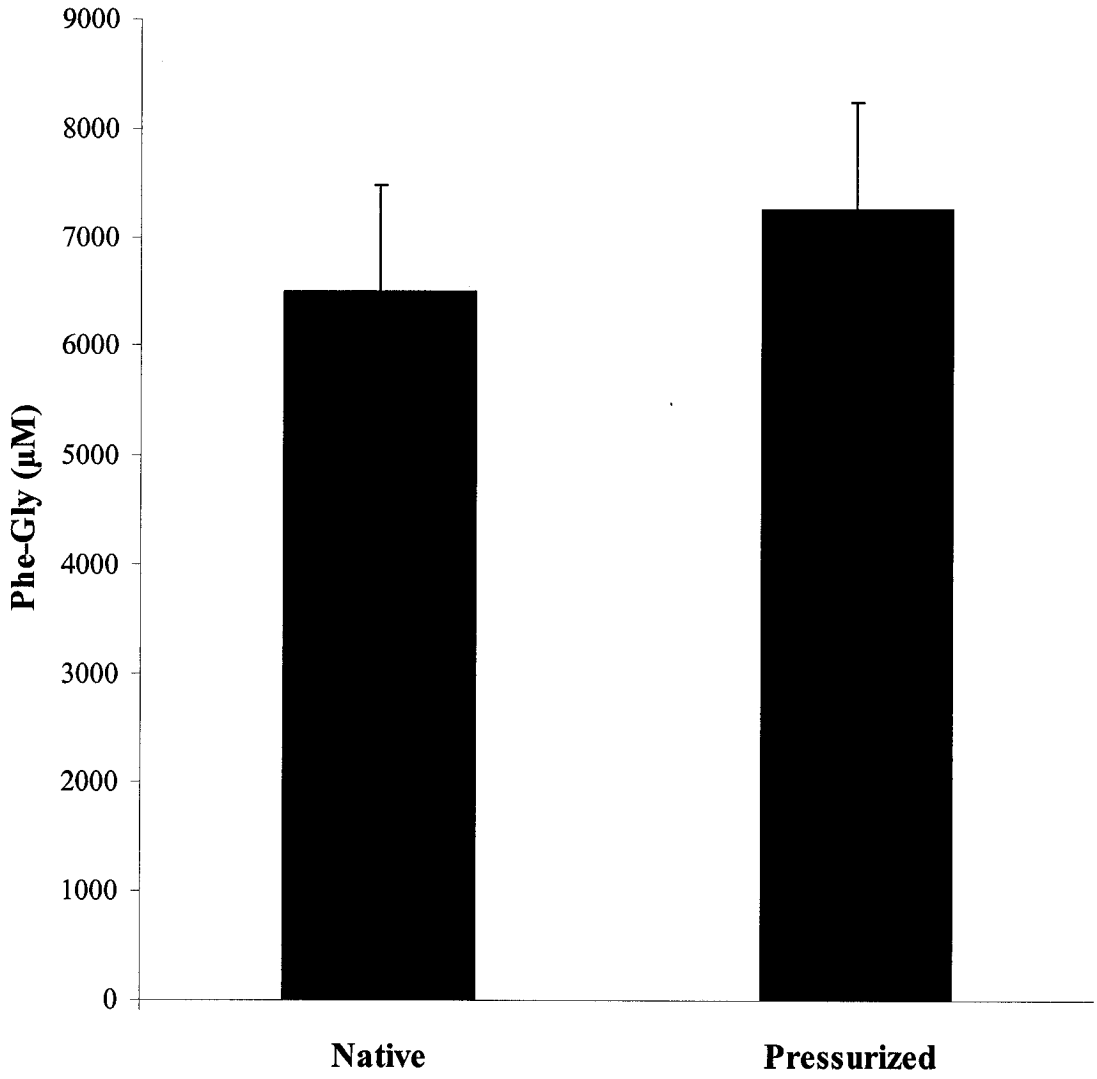


Figure 2. Effect of hydrostatic pressure treatment on *in vitro* digestion of SPI. Using either lyophilized native SPI or SPI pressurized at 600 MPa + 5 min, 3% solutions (w/v) were prepared and digested with pepsin for 30 min followed by pancreatin digestion for an additional 60 min. The enzyme/substrate ratio used was 1/100 for pepsin and 1/30 for pancreatin. Ultrafiltration was used to separate peptides with molecular weight cut-off MWCO < 1 kDa. The peptide content of the filtrate was determined at 340 nm (n = 6). Error bars show 95% CI of mean.

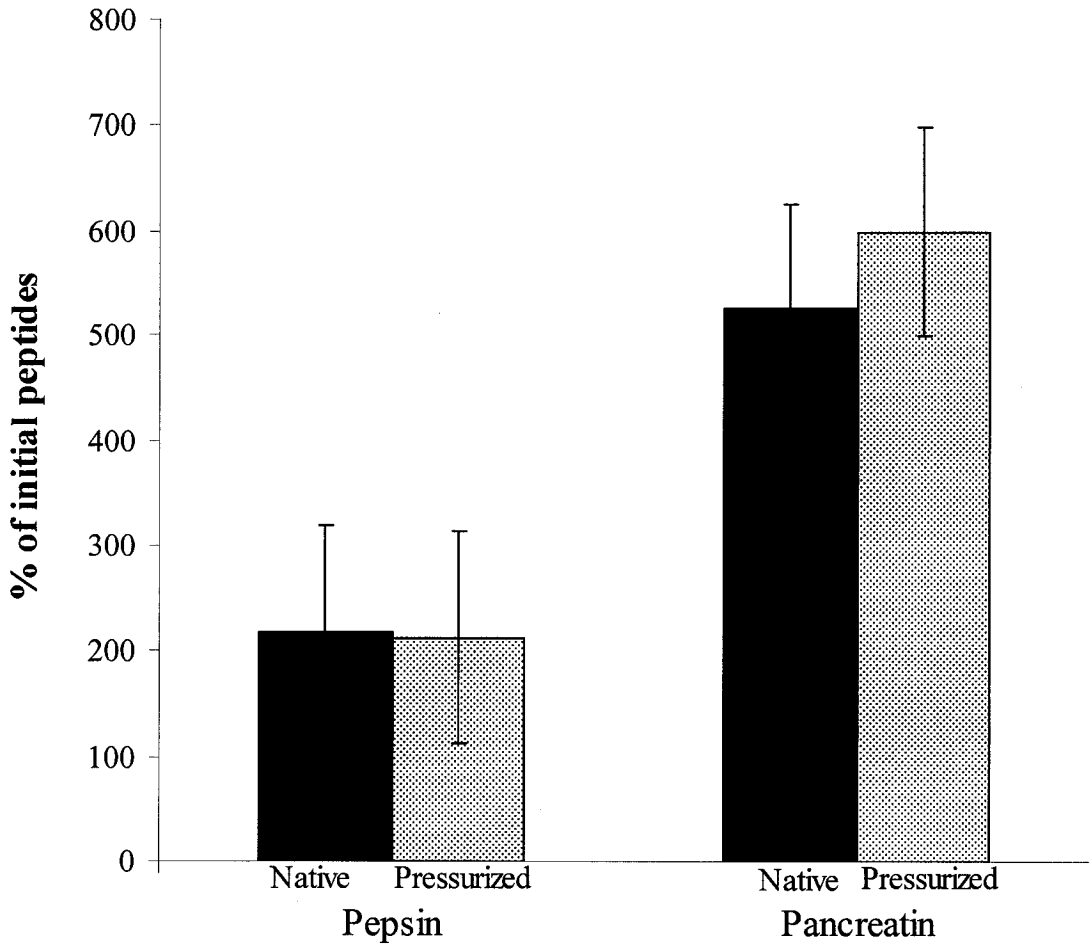


Figure 3. The < 1 kDa peptide content of native and pressure treated (600 MPa + 5 min) SPI after *in vitro* enzymatic digestion by pepsin alone, and by pepsin followed by pancreatin treatment. The enzyme/substrate ratio used was 1/100 for pepsin and 1/30 for pancreatin. The amount of peptides < 1 kDa released at the end of the digestion with pepsin (on the left) and pancreatin (on the right) was collected by ultrafiltration and determined at 340 nm (n = 6). Error bars show 95% CI of mean.

Mass spectrometry

The electrospray ionization mass spectrometer (ESI-MS) profile of MWCO fractions < 1 kDa isolated from native and pressurized (600 MPa) SPI hydrolysates following pepsin digestion is shown in Figure 4. The results from the mass spectrometry showed that the hydrolysates of native and pressurized SPI have similar profiles; however, there were clearly differences in the relative amounts of peptides between the samples. Thus, relatively more peptides at molecular weight of 520.27 were observed in native SPI whereas the pressurized SPI sample had greater amounts of peptides with molecular weight of 494.25. Also, there were higher amount of peptides of the molecular weight of 643.32 in native SPI and the pressurized SPI contained relatively higher amounts of peptides with the molecular weight of 601.79.

Capillary zone electrophoresis (CZE)

The electropherogram profiles of peptides less than 1 kDa isolated from pepsin-pancreatin hydrolysates of native and pressurized (600 MPa + 5 min) SPI is shown in Figure 5. The number of peaks and the peak areas relative to the internal BSA standard were different between the pepsin-pancreatin hydrolysates of native (Figs. 5A and 5B) and pressurized SPI (Figs. 5C and 5D). The pressurized (600 MPa + 5 min) SPI generated more peptide fragments than the native SPI as well as a different migration pattern and peak areas relative to BSA. Hence, the electropherograms of peptides resulting from the pepsin-pancreatin digestion of pressurized SPI showed two additional peaks eluting later than BSA around 29.3 min (Fig. 5D), which was not observed with the native soy protein hydrolysates (Fig. 5B). The electropherogram of peptides resulting from the pepsin-pancreatin digestion of native SPI also showed an additional peak that eluted soon after BSA at 8.7 min (Fig. 5B), which were not seen with the pressurized SPI hydrolysates (Fig. 5D).

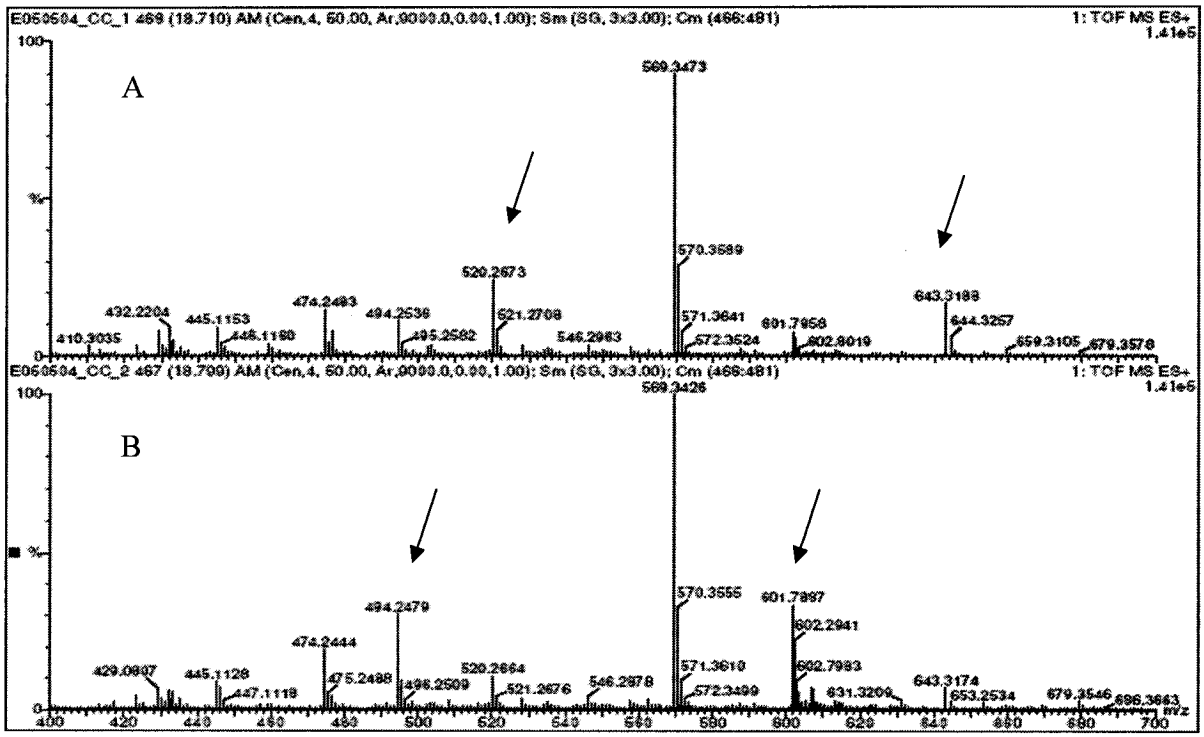
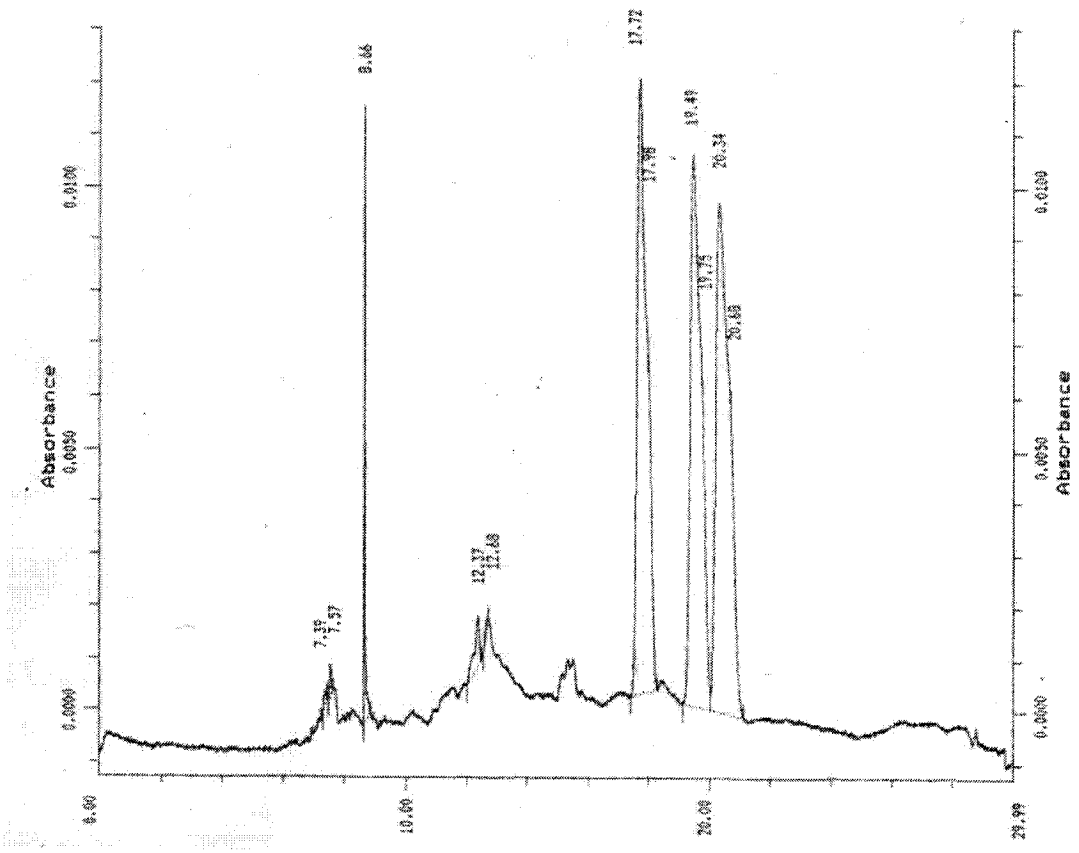
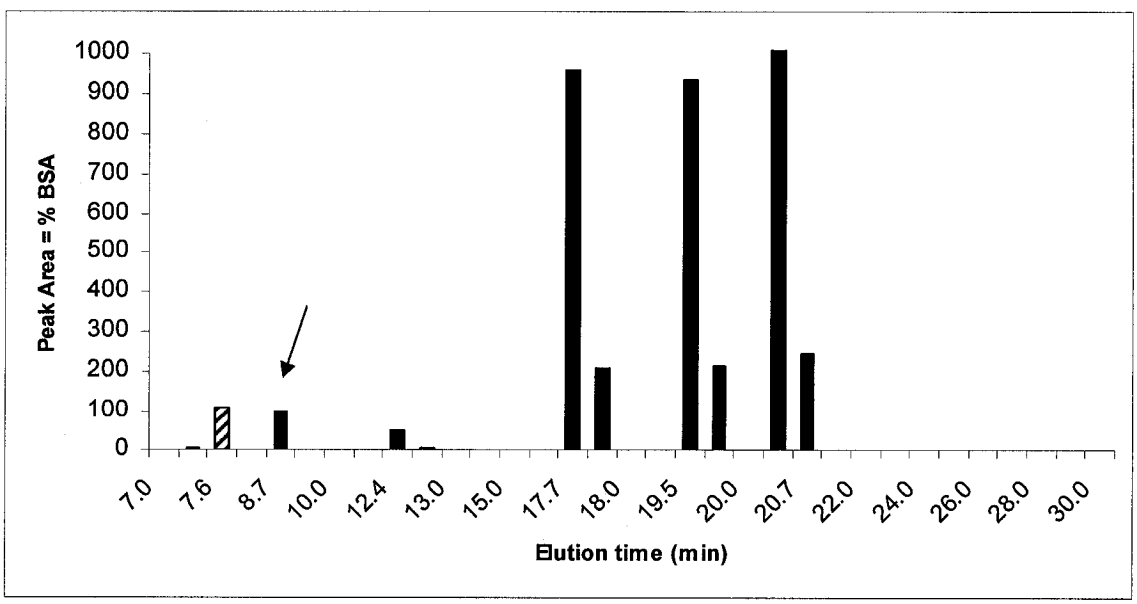


Figure 4. ESI-MS profiles of peptide extracts with molecular weight < 1 kDa obtained from *in vitro* pepsin digestion and ultrafiltration of (**A**) native SPI; (**B**) pressurized SPI (600 MPa + 5 min). The arrow indicates where differences exist in relative amounts of peptides between the native and pressure-treated SPI.

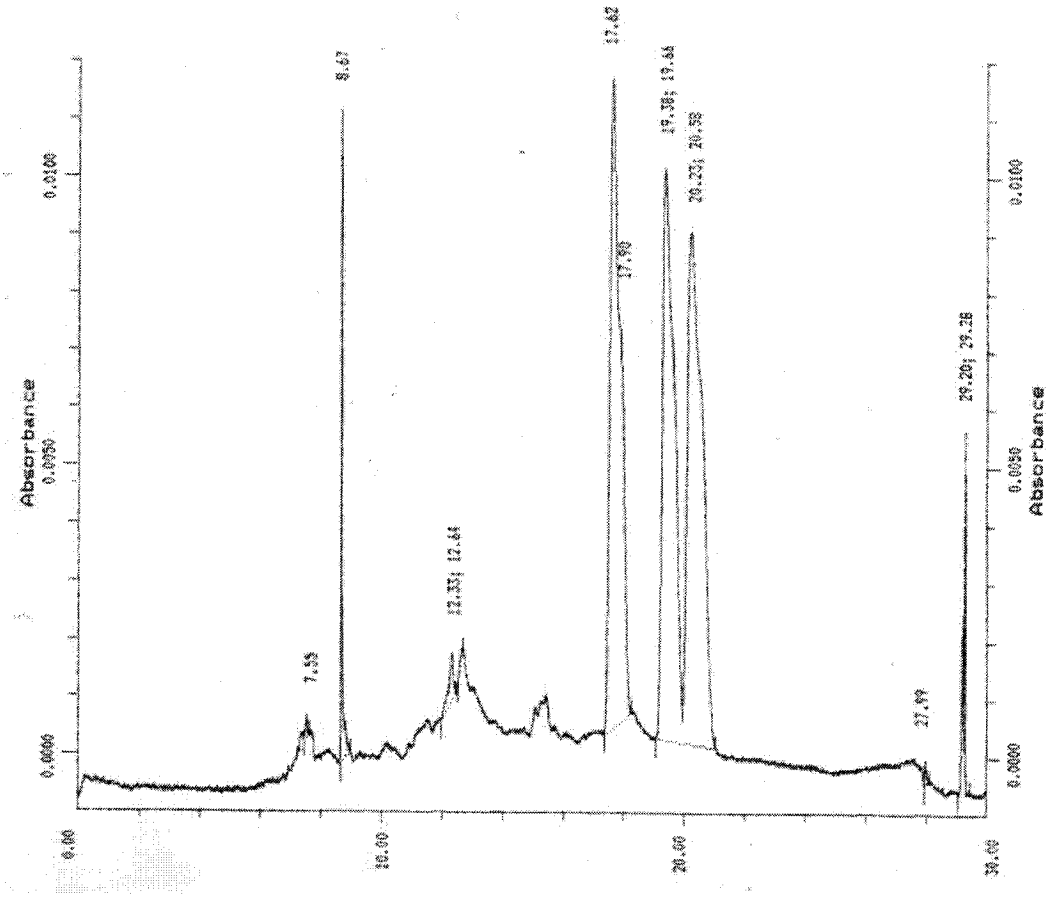
A


Absorbance
0.0100
0.0050
0.0000
8.66
17.72
19.49
20.34
12.17
12.68
7.57
10.00
20.00
29.99

B


Peak Area = % BSA
1000
900
800
700
600
500
400
300
200
100
0
7.0
7.6
8.7
10.0
12.4
13.0
15.0
17.7
18.0
19.5
20.0
20.7
22.0
24.0
26.0
28.0
30.0
Elution time (min)

C


Absorbance
Absorbance

D

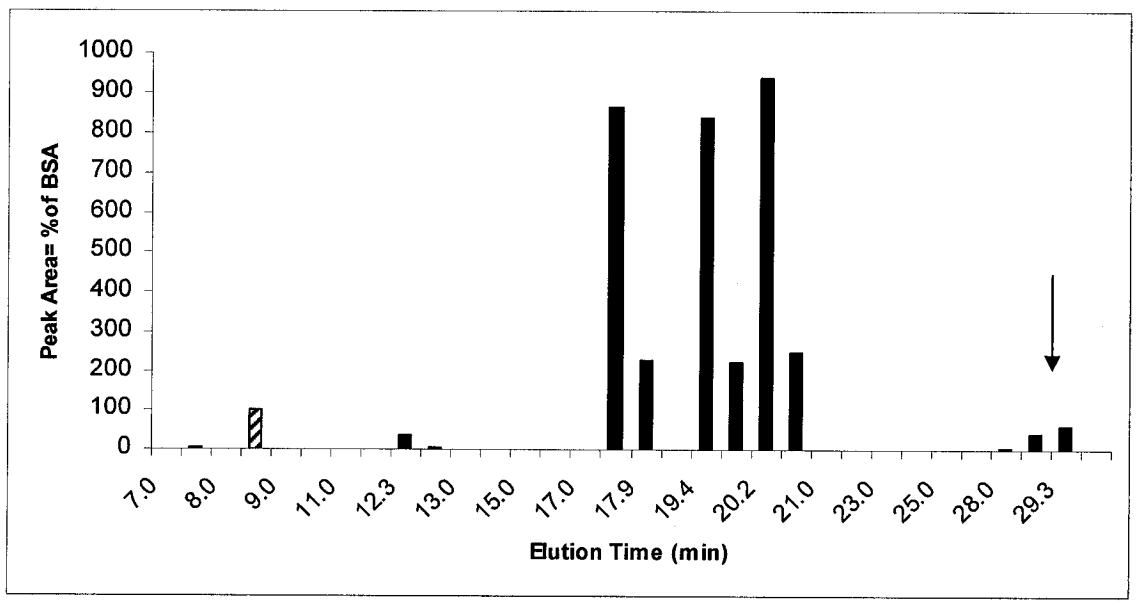

Peak Area= %of BSA
1000
900
800
700
600
500
400
300
200
100
0
7.0
8.0
9.0
11.0
12.3
13.0
15.0
17.0
17.9
19.4
20.2
21.0
23.0
25.0
28.0
29.3
Elution Time (min)

Figure 5. Electropherogram profiles of peptide extracts with molecular weight < 1 kDa. Capillary zone electrophoresis was used to generate the electropherograms of the separated peptides, which were detected at 214 nm. CZE conditions: 40 µl of the sample solution at a concentration of 40 mg/ml was injected and the peptides migrated from the positive to the negative pole at 27 kV and 30°C using 0.1 M phosphate buffer (pH 2.5). BSA (400 µg/ml) was used as internal standard and represents 100%.
(A) Electropherogram of peptides obtained following *in vitro* pepsin-pancreatin digestion and ultrafiltration of native SPI; (B) Graph represents the relative amounts of peptides obtained following *in vitro* pepsin-pancreatin digestion and ultrafiltration of native SPI as compared to the internal standard bovine serum albumin (small grid background) as calculated from the peak areas (100% corresponds to the albumin peak area) of native SPI; (C) Electropherogram of peptides obtained following *in vitro* pepsin-pancreatin digestion and ultrafiltration of pressure-treated SPI (600 MPa + 5 min); and (D) Graph represents the relative amounts of peptides obtained following *in vitro* digestion and ultrafiltration of pressure-treated SPI (600 MPa + 5 min) as compared to the internal standard bovine serum albumin (small grid background) as calculated from the peak areas (100% corresponds to the albumin peak area). Arrows indicate the peaks where differences exist.

Reversed Phase (RP)-HPLC peptide profiles

The topographic image of UV absorbances at multiple wavelengths ranging from 210 to 285 nm of < 1 kDa peptides isolated from hydrolysates of native and pressurized (600 MPa + 5 min) SPI following *in vitro* pepsin-pancreatin digestion is shown in Figure 6. The blue lines signify the lowest intensity while the red lines represent higher intensity. There are differences in intensity between treatments as indicated by the presence of peptides from pressurized sample absorbing at 200 – 210 nm, 240 – 270 nm and 265 – 285 nm (Fig. 6a), which was not seen in the chromatograms of the native SPI samples. In addition, peptides were present in the native SPI that absorbed at 210 – 220 nm, 250 – 280 nm (Fig. 6b) which were not observed in the HPLC chromatograms from the peptides of the pressurized SPI samples.

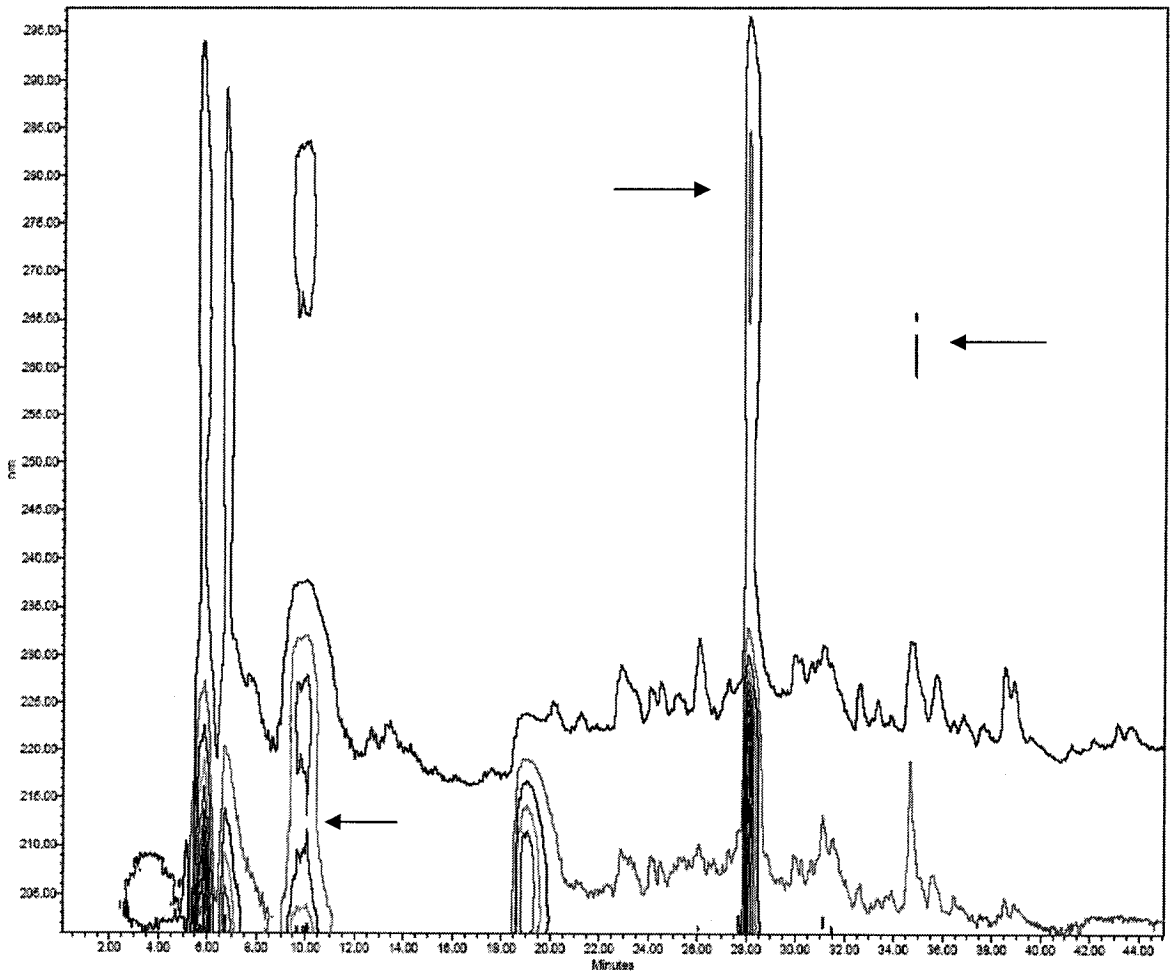


Figure 6a. Reversed-phase HPLC chromatogram obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm of peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of pressurized (600 MPa) SPI. Samples were loaded on analytical C18-reverse phase column (5 μm; 250 mm × 4.6 mm) (Vydac Series-218TP54, Vydac Company, Herperia, CA) and separated with a Waters HPLC system (Waters, Milford, MA). Conditions for RP-HPLC separation are as described in methods section. Arrows indicate area where differences exist between the native and the pressure-treated SPI.

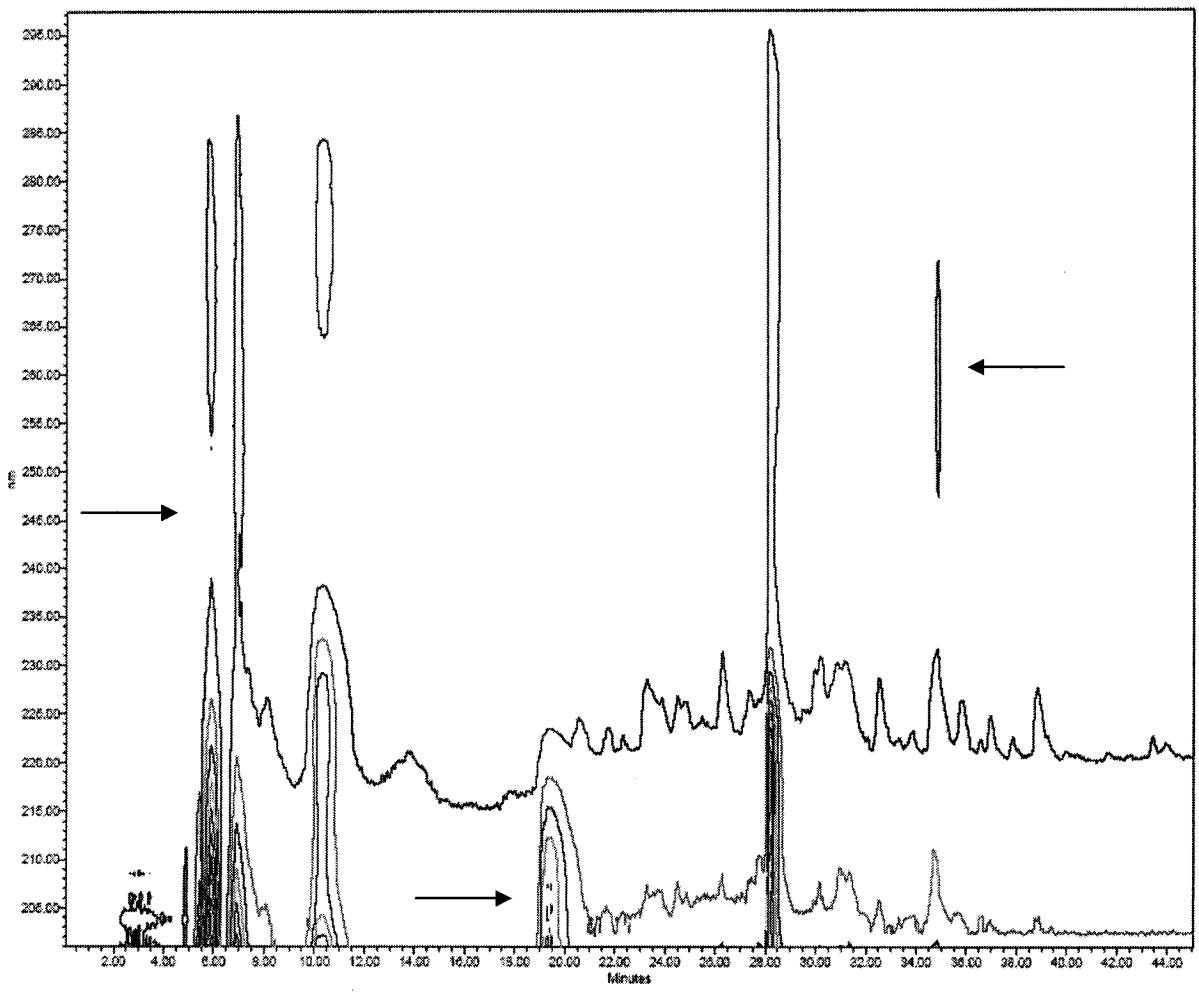


Figure 6b. Reversed-phase HPLC chromatogram obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm of peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of native SPI. Arrows indicate area where differences exist between the native and the pressure-treated SPI.

RP-HPLC chromatogram obtained via detection at 214 nm

Since most peptides absorb at lower wavelengths, further analysis of peptides using RP-HPLC at 214 nm was done to assess differences in percent peak areas. The typical chromatograms of RP-HPLC analysis of < 1 kDa peptides isolated from hydrolysates of native and pressurized (600 MPa) SPI following *in vitro* pepsin-pancreatin digestion are presented in Figure 7. Most peaks eluted at the 4 to 12 min and 15 to 40 min intervals.

Although the RP-HPLC peptide profile of pressure-treated SPI did not differ markedly from peptide profiles of native SPI, peptide peaks were present at 31.1 min, 38.5 min and 38.9 min in the hydrolysates of pressurized SPI, which were not observed with the native soy protein hydrolysates (Fig. 7a). Additionally, peptide peaks were observed at 31.2 min, 38.8 min in the native soy hydrolysates group, which were not observed in peptide profiles obtained from pressurized soy hydrolysates (Fig. 7b).

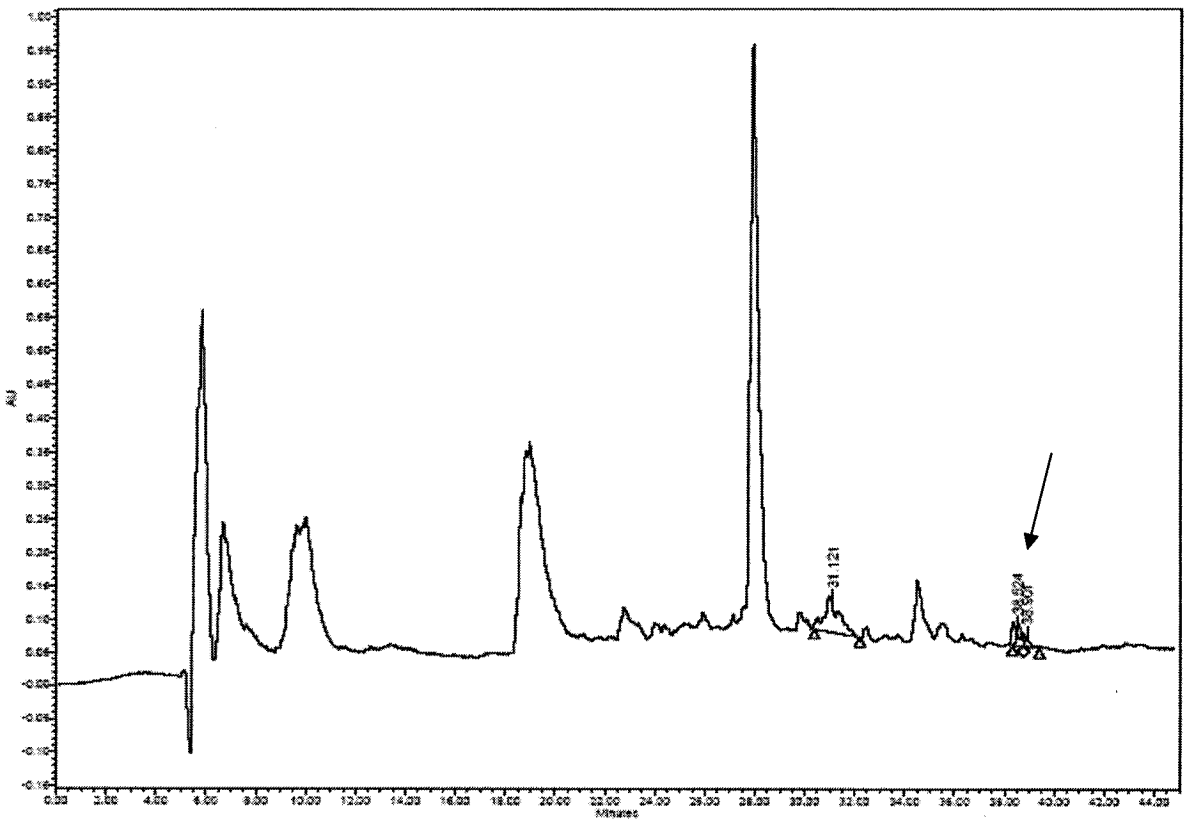


Figure 7 a. Reversed-phase HPLC chromatogram obtained via detection at 214 nm of peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of pressurized (600 MPa) SPI. The arrow indicates area where differences exist between the native and the treatment group.

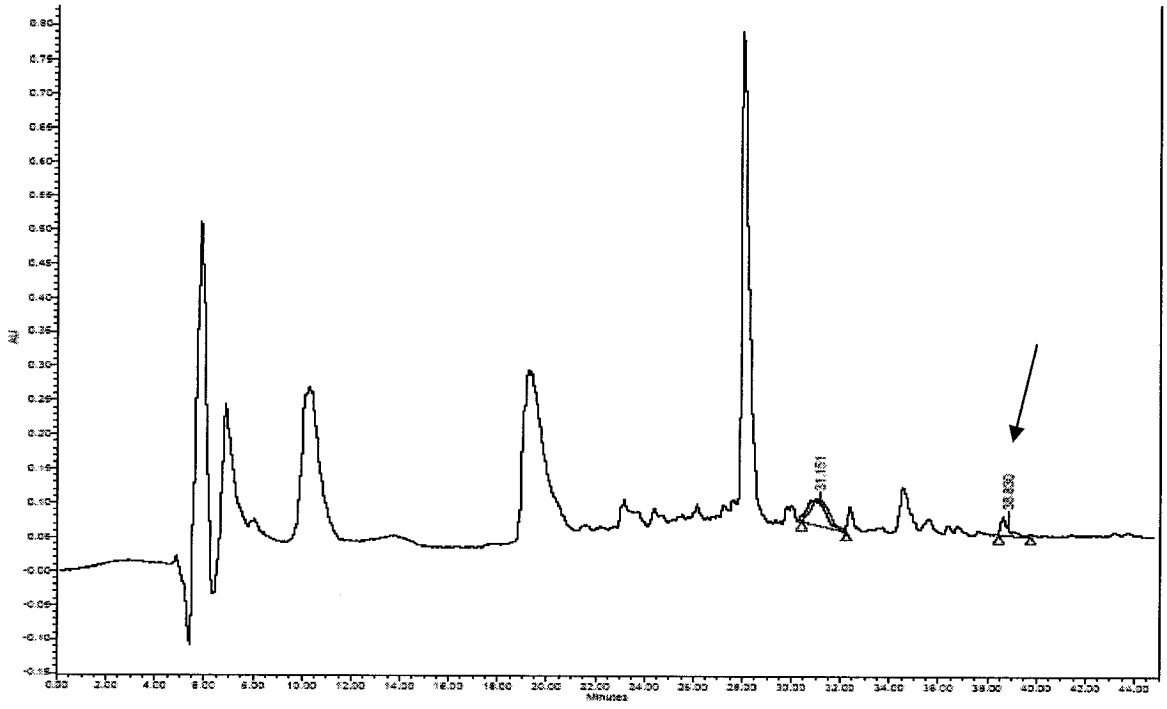


Figure 7 b. Reversed-phase HPLC chromatogram obtained via detection at 214 nm of peptides with MWCO less than 1 kDa derived from pepsin and pancreatin hydrolysis of native SPI. The arrow indicates area where differences exist between the native and the pressure-treated SPI.

Ferric Reducing Antioxidant Power (FRAP)

In the FRAP assay, the ferric-tripyridyltriazine complex can be reduced to the blue coloured ferrous form by an antioxidant with an absorption maximum at 593 nm. Thus, the change in absorbance is directly proportional to the combined or total reducing power of the electron-donating antioxidants present in the tested samples. The ferric reducing power of 40 mg/ml peptides (< 1 kDa) from native and pressurized SPI over a 21 h period is shown in Figure 8a. Various concentrations of peptides were tested and 40 mg/ml was found to be minimally effective (data not shown). At 0.5 h, the isolated peptides from the hydrolysates of native and pressurized SPI exhibited 494.3 μM and 586.0 μM of ferric reducing power abilities, respectively. There was a significant ($P < 0.05$) increase in ferric reducing power abilities at 2.0 and 4.0 h in comparison to 0.5, 1.0, 3.0 and 21.0 h in the

pressurized samples. There was a significant ($P < 0.05$) increase in ferric reducing power abilities at 1.0, 2.0, 3.0 and 4.0 h in comparison to 0.5 and 21.0 h in the native samples. The peptides from pressurized SPI demonstrated a significantly ($P < 0.05$) greater ferric reducing power abilities than the native group at 1.0, 2.0 and 3.0 h. The peptides isolated from the hydrolysates of native and pressurized SPI exerted a range of FRAP activity that corresponded to 1 to 10 μM of ascorbic acid equivalents per mg peptides over the 21 h period (data not shown).

The antioxidant activities over a 21 h period of unfiltered hydrolysates from native and pressurized (600 MPa) SPI is shown in Figure 8b. The unfiltered hydrolysates showed no increase with time in FRAP activity but rather showed a drop in FRAP by 3 and 4 h, which was not observed with the isolated peptide samples. At 0.5 h, the hydrolysates of native and pressurized SPI exhibited ferric reducing power abilities of 1194.3μM and 1070.8 μM, respectively. There was a significant ($P < 0.05$) decrease in ferric reducing power abilities of the native SPI hydrolysates at 3.0, 4.0 and 21.0 h incubation times in comparison to 0.5, 1.0 and 2.0 h. Similarly, peptides from the pressurized samples also demonstrated significantly ($P < 0.05$) greater ferric reducing power abilities at 3.0, 4.0 and 21.0 h incubation times in comparison to 0.5, 1.0 and 2.0 h. The unfiltered hydrolysates from native SPI demonstrated a significantly ($P < 0.05$) greater ferric reducing power abilities than the hydrolysates of pressurized SPI at 0.5, 1.0 and 2.0 h. Over the 21 h period, the unfiltered hydrolysates from the native and pressurized SPI exhibited a range of FRAP activity that corresponded to 2.5 to 11.25μM of ascorbic acid equivalents per mg peptides (data not shown).

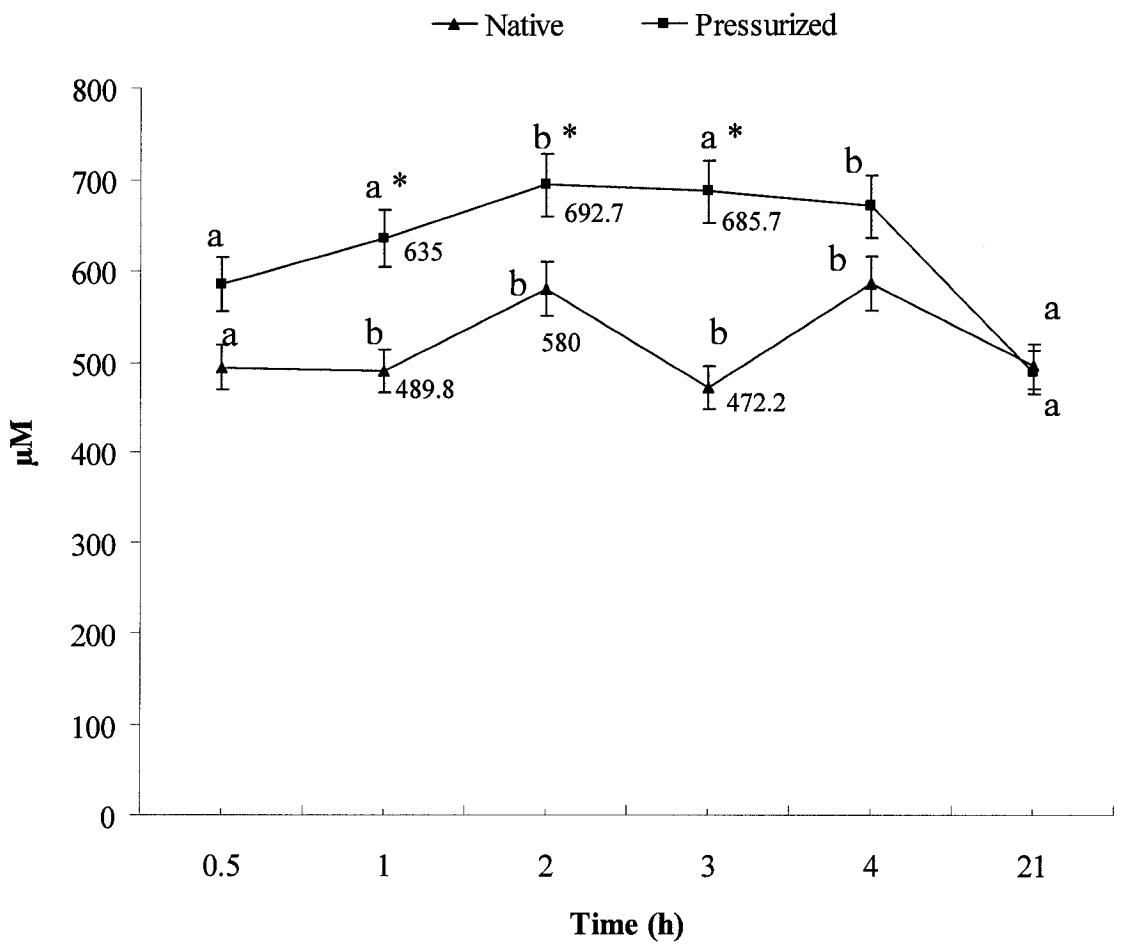
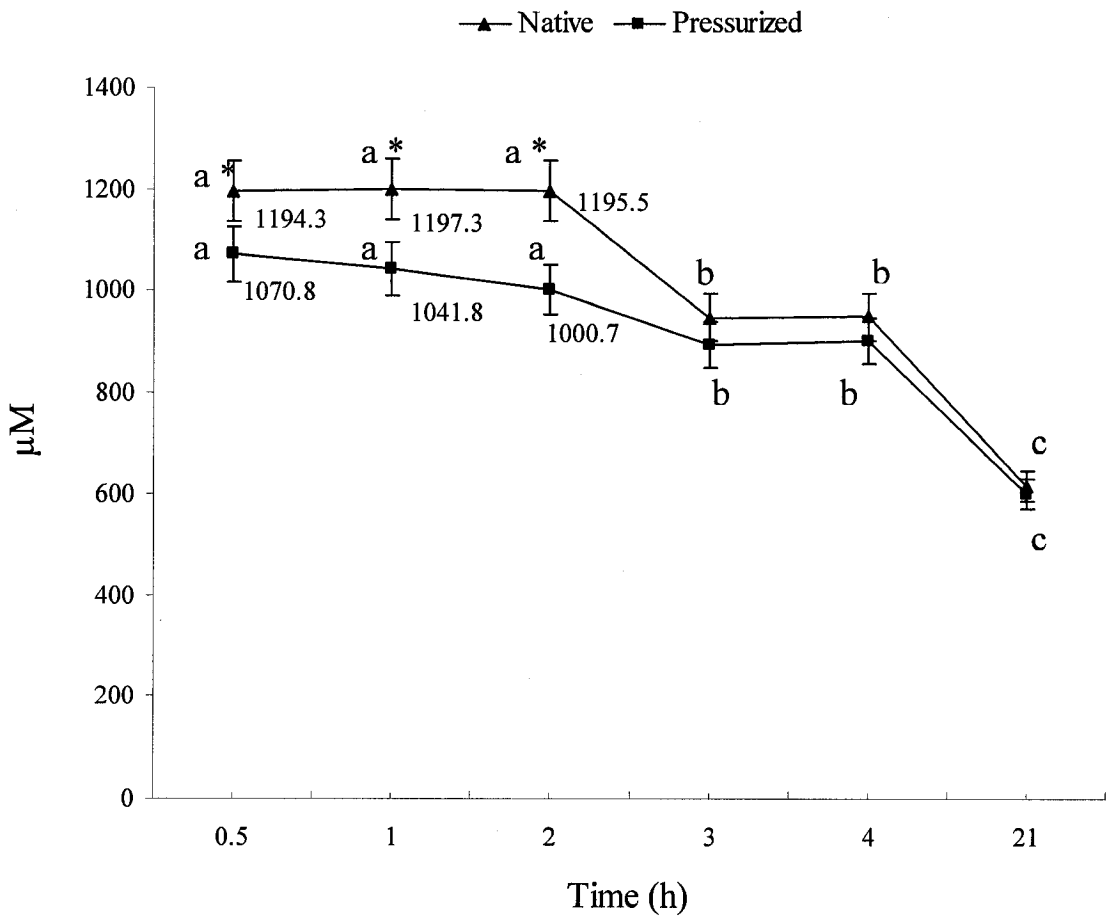


Figure 8a. Ferric reducing power of < 1 kDa native and pressurized (600 MPa) SPI hydrolysates over 21 h. The graph shows the antioxidant activity of 40 mg/ml of digested and filtered peptides smaller than 1 kDa obtained from either native or pressurized (600 MPa) SPI hydrolysates over a 21 h period. Bovine serum albumin was used to develop a standard curve at concentrations ranging from 100 - 1200 µM. The ferric reducing power is then expressed as µM. The absorbance is determined at 593 nm. The mean ± SD (n = 6) is presented for the numbers on the curve. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. The symbol (*) indicates significant differences ($P < 0.05$, ANOVA) between the treatments at the specific time point.



8b. Ferric reducing power of native and pressurized (600 MPa) SPI hydrolysates over 21 h. The graph shows the antioxidant activity of 40 mg/ml of digested and unfiltered SPI obtained from either native or pressurized (600 MPa) SPI hydrolysates over a 21 h period. BSA was used to develop a standard curve at concentrations ranging from 100 - 1200 µM. The ferric reducing power is then expressed as µM. The absorbance is determined at 593 nm. The mean ± SD (n = 6) is presented for the numbers on the curve. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. The symbol (*) indicates significant differences ($P < 0.05$, ANOVA) between the treatments at the specific time point.

DPPH radical-scavenging activity

DPPH is a proton-radical that can be stoichiometrically decolorized by antioxidants, which has been routinely used to test the free radical scavenging capabilities of various food extracts (Arnao, 2000). The reduction in the concentration of the DPPH is monitored via the decrease in its absorbance at a characteristic wavelength when it encountered proton-radical scavengers. The antioxidant activities over a 24 h period of isolated low molecular weight peptides (< 1 kDa) from the hydrolysates of native and pressurized (600 MPa) SPI is seen in Figure 9a. (The percent inhibition for the time 0 is 0%). The assay reaction time of DPPH with ascorbic acid was measured to be 5 min whereas the reaction time for the isolated peptides and SPI hydrolysates was measured over a 24 h period. Various concentrations of peptides were tested and 40 mg/ml was found to be minimally effective (data not shown). At 0.5 h, the isolated peptides from the hydrolysates of native and pressurized SPI exhibited similar DPPH scavenging activity of 34% and 33.7%, respectively. The scavenging activity; however, increased with increasing time of incubation (0.5 to 24 h). Thus, there was a significant ($P < 0.05$) increase in DPPH-scavenging activities at all the subsequent incubation times in comparison to 0.5 and 1.0 h in both the native and pressurized samples. The native peptide samples also showed significantly ($P < 0.05$) higher scavenging activities at 9.0, 12.0 and 24 h in comparison to the 2.0 h samples. Similarly, peptides from the pressurized samples demonstrated significantly ($P < 0.05$) greater DPPH scavenging activities at 6.0, 9.0, 12.0 and 24 h in comparison to the 2.0 and 3.0 h incubations. The peptides from native SPI demonstrated a significantly ($P < 0.05$) greater DPPH radical-scavenging activity than the pressurized group at 1.0, 3.0 and 6.0 h.

The unfiltered hydrolysates from the native and pressurized SPI exhibited a range of 47 - 72% of DPPH radical scavenging ability in comparison to the 34 - 59% of DPPH radical scavenging ability observed with the isolated < 1 kDa peptides (Figs. 9a and 9b). The antioxidant activities over a 24 h period of unfiltered hydrolysates from native and pressurized (600 MPa) SPI are shown in Figure 9b. Various concentrations of peptides were tested and 40 mg/ml was found to be minimally effective (data not shown). At 0.5 h,

the isolated peptides from the hydrolysates of native and pressurized SPI exhibited significantly different DPPH scavenging activity of 47.2% and 41.5%, respectively. Furthermore, the scavenging activity increased with increasing time of incubation (0.5 to 24 h). Thus, there was a significant ($P < 0.05$) increase in DPPH-scavenging activities at all the subsequent incubation times in comparison to 0.5 h in the native samples. The native peptide samples also showed significantly ($P < 0.05$) higher scavenging activities at 1.0 h in comparison to the 0.5 h samples.There were also significantly ($P < 0.05$) higher scavenging activities at 6.0, 9.0 h in comparison to the 2.0, 3.0 h samples. There were also significantly ($P < 0.05$) higher scavenging activities at 12.0 and 24.0 h in comparison to the 9.0 h samples. However, peptides from the pressurized samples demonstrated significantly ($P < 0.05$) greater DPPH scavenging activities at 6.0, 9.0, 12.0 and 24 h in comparison to the 2.0 h and 3.0 h incubations. The peptides from native SPI demonstrated a significantly ($P < 0.05$) greater DPPH radical-scavenging activity than the pressurized group at 0.5, 1.0 and 2.0 h.

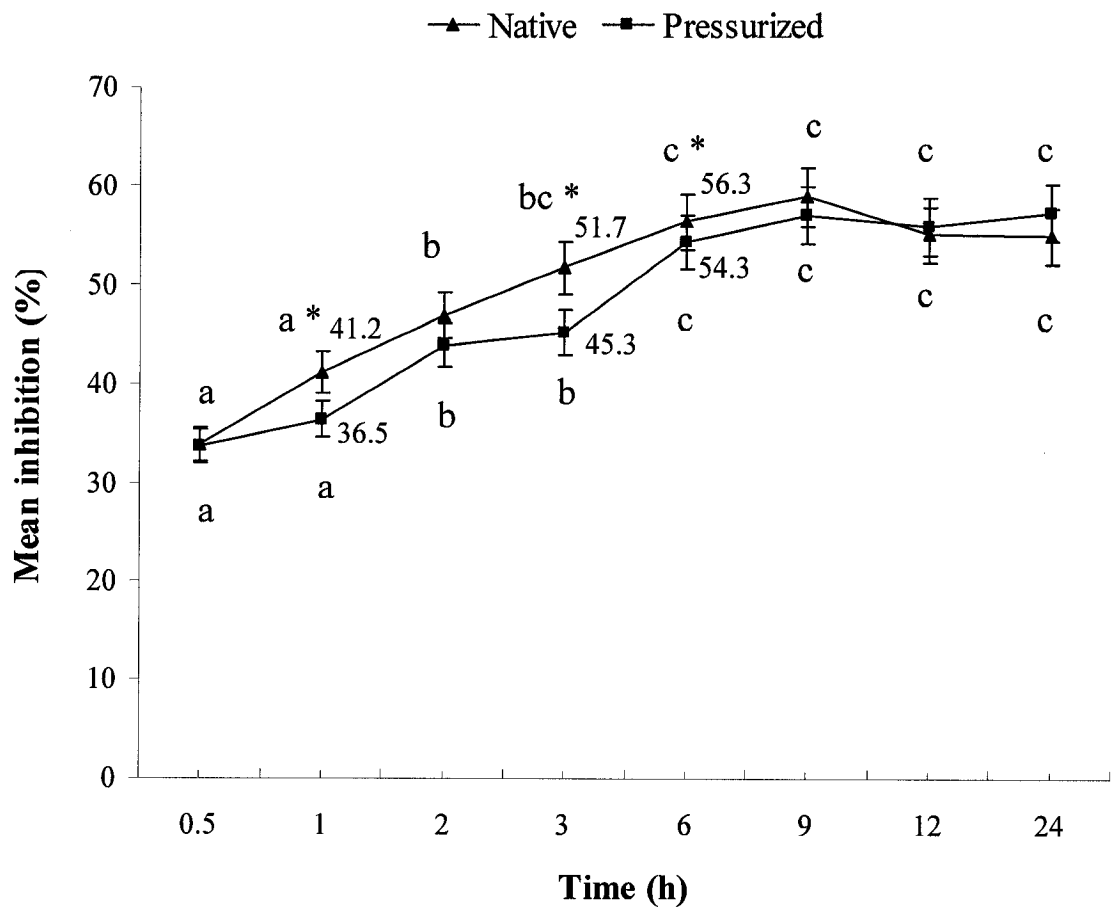


Figure 9a. DPPH radical scavenging ability of < 1 kDa native and pressurized (600 MPa) SPI hydrolysates over 24 h. The graph shows the antioxidant activity of 40 mg/ml of peptides smaller than 1 kDa obtained from either native or pressurized (600 MPa) SPI hydrolysates over a 24 h period. The percent inhibition is calculated as (OD control minus OD sample) / OD control x 100 and absorbance is determined at 520 nm. The mean ± SD (n = 6) is presented for the numbers on the curve. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. The symbol (*) indicates significant differences ($P < 0.05$, ANOVA) between the treatments at the specific time point.

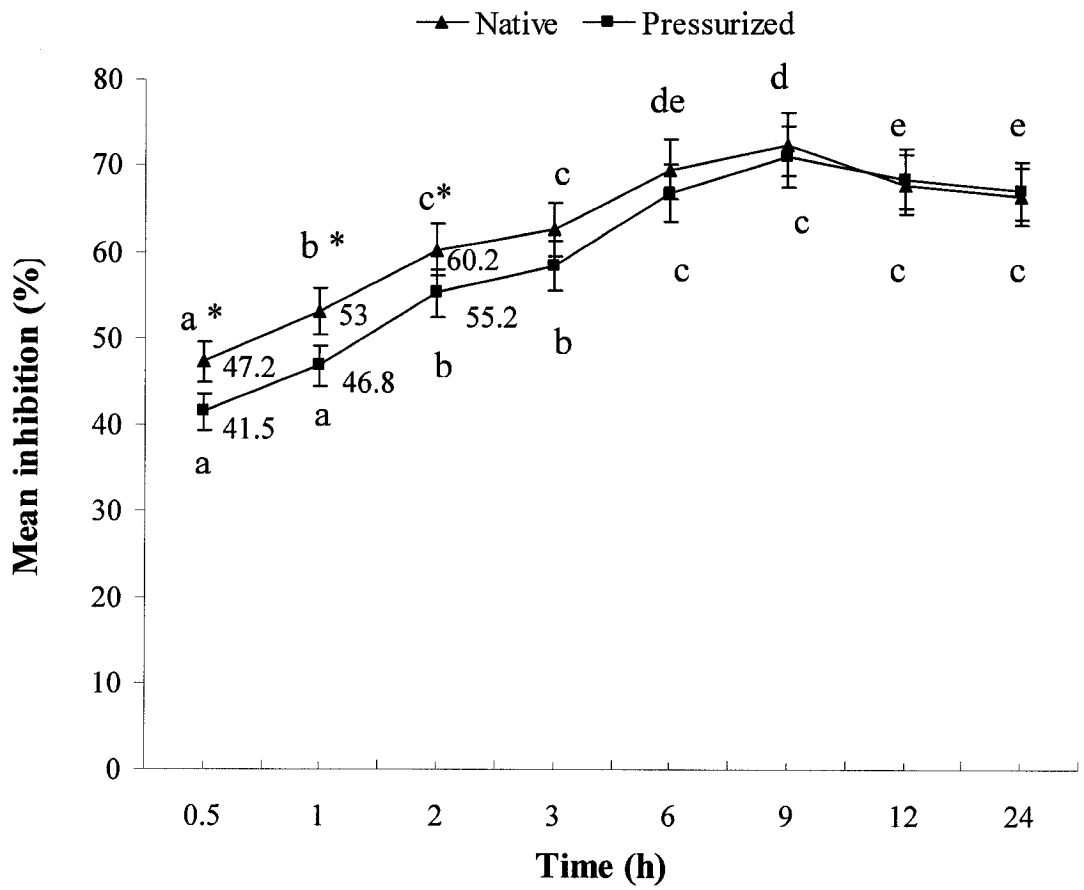


Figure 9b. DPPH radical scavenging ability of native and pressurized (600 MPa) SPI hydrolysates over 24 h. The graph shows the antioxidant activity of 40 mg/ml of digested and unfiltered SPI obtained from either native or pressurized (600 MPa) SPI hydrolysates over a 24 h period. The percent inhibition is calculated as (OD control minus OD sample) / OD control x 100 and absorbance is determined at 520 nm. The mean ± SD (n = 6) is presented for the numbers on the curve. Time points within the same treatment not sharing common letters differed significantly ($P < 0.05$; ANOVA) by Tukey's *post hoc* comparison. The symbol (*) indicates significant differences ($P < 0.05$, ANOVA) between the treatments at the specific time point.

3.4 DISCUSSION

The present study demonstrates that an increased pepsin-mediated digestibility of SPI occurred following high hydrostatic pressure processing. Significant decreases in protein content ($P < 0.05$) were detected in the pressurized SPI when compared to the native SPI at 10 and 20 min of pepsin digestion (Fig. 1) indicating a more rapid rate of hydrolysis of SPI. The increased digestibility as a result of pressure treatment also likely led to a greater release of peptides of < 3 kDa molecular weight since the Bradford assay used for protein determination can only detect peptides greater than 3 kDa (Sapan et al., 1999). The higher pepsin-mediated digestibility of pressurized SPI is likely due to unfolding of the soy protein with the exposure of the hydrophobic groups as Puppo et al. (2004) have shown that hydrostatic pressures greater than 200 MPa produce key structural changes in soy proteins. These structural changes include an increase in the surface hydrophobicity, a reduction of the free sulfhydryls, a partial unfolding of the 7s and 11s fractions and a change in secondary structure leading to a more disordered structure and aggregation of the proteins, especially the 11s fraction. Furthermore, Molina et al. (2001) showed that hydrostatic pressures of 400 MPa dissociated the 7s fraction of SPI into partially or totally denatured monomers that enhanced surface activity. The unfolding of the 11s polypeptides within the hexamer also led to aggregation, negatively affecting the surface hydrophobicity of the SPI. Therefore, it is likely that in the present study, the partial denaturation and subsequent exposure of hydrophobic groups and peptide bonds located in the interior of globular proteins such as β-conglycinin (7s fraction) could have increased accessibility for the proteolytic enzymes and lead to the increase in pepsin digestibility. Furthermore, other pressure levels, holding time, temperature and pressure cycles that were not tested in this study might also exert an effect on the digestibility of SPI.

No differences in protein content was observed between pressurized and native SPI following 30 min of pepsin digestion, which was likely due to the relatively high efficiency of pepsin digestion that was observed, i.e., >85% of soy protein was digested (Fig. 1). Similarly, the total content of < 1 kDa peptides isolated from hydrolysates of

native and pressure-treated SPI did not differ following either the pepsin or pancreatin hydrolyses (Figs. 2 and 3). Although trypsin inhibitors are present in soy protein isolates, these are in minimal quantities that are unlikely to impair the activity of trypsin (Friedmam and Brandon, 2001). Pancreatin enzymes including the endopeptidase, trypsin, chymotrypsin, elastase, exopeptidase, aminopeptidase and carboxypeptidases, are highly efficient in cleaving proteins and peptides into low molecular weight peptides, which could explain the similar peptide content (Biesalski and Grimm, 2005).

Although the ESI-MS data demonstrated a similar peptide profile for native and pressure-treated SPI following pepsin and pancreatin digestion, the fractionated hydrolysates clearly showed differences in the relative concentrations of isolated peptides from the native and pressurized SPI hydrolysates (Fig 4). The ESI-MS provided relatively good sensitivity from the femtomole to low picomole range and due to the lack of a matrix, there was no background matrix interference, and the multiple charging gave better mass accuracy (Cole, 1997). The CZE analyses that relied on differences in size and charge of the peptides further confirmed the differences in peptide profiles as the hydrolysates of the pressurized (600 MPa) SPI generated more peptide fragments than the native SPI hydrolysates as different migration patterns and peak areas were observed relative to the internal standard, BSA (Fig 5). Thus, a peptide that eluted at 8.75 min in the electropherogram of the <1 kDa peptides from the native SPI hydrolysates was not seen in the electropherograms of isolated peptides from pressurized SPI hydrolysates. Moreover, two additional peaks eluting later than BSA around 29.3 min were observed only in the hydrolysates of pressurized SPI. Such peptide profile differences might be attributable to the cleavage of the original peptides found in native SPI hydrolysates. Furthermore, the later elution time of the two additional peptide peaks in the pressurized SPI hydrolysates could also indicate the differences in the molecular weights and charges of the peptides produced in association with pressure treatment. Since the cations with the highest charge/mass ratio migrate first and the anions with greater charge/mass ratio migrate last (Chaiyasut, 2001), it is likely that the late eluting peptides seen in the pressurized SPI electropherograms are small anions since anions with the greatest electrophoretic mobilities migrate last. As BSA has an isoelectric point of 4.6 and the

peptides and amino acids isolated from the hydrolysates had molecular weights lower than BSA, it is reasonable to assume that peptides that eluted later than BSA also had isoelectric points higher than 4.6 and thus were more electropositive than BSA at pH 2.5.

The HPLC chromatograms obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm showed differences in intensity for < 1 kDa peptides isolated from hydrolysates of native versus pressurized (600 MPa) SPI, thereby indicating differing proportions of peptides (Fig 6). Since most peptide bonds absorb at wavelengths ranging from 200 and 220 nm (Becklin and Desiderio, 1995), further analysis of the peptide profiles using RP-HPLC at 214 nm was carried out, which showed different peptide peaks between the native and pressurized SPI samples (Fig. 7). Several factors can influence the elution time of peptides on a reversed phase column, which include the amino acid composition of the peptides, the hydrophobicity of the amino acid side chains in small peptides (< 15 residues) (Hearn et al., 1988) and the peptide length and molecular weight of the larger peptides (Chabanet and Yvon, 1992). Hence, it is difficult to determine the nature of the eluted peptides associated with pressure treatment based on the HPLC chromatograms.

Similar findings regarding an increase in pepsin-mediated digestibility have been observed following high hydrostatic treatment of whey protein isolates (Vilela et al., 2006). In concert with the present study, no differences in the total content of < 1 kDa peptides were observed following pepsin-pancreatin digestion of pressurized WPI whereas peptide compositional differences were noted relative to hydrolysates derived from native WPI. Taken together, these results indicate that pressure processing is effective in improving the digestibility and altering the release of peptides of disulfide-linked proteins such as soy and whey proteins, which are known to be relatively resistant to proteolysis (Smyth, 1967).

Although several clinical trials have suggested that soy protein intake results in potent antioxidant activity irrespective of isoflavone content (Ashton et al., 2000; Swain et al., 2002; Vega-Lopez et al., 2005; Heneman et al., 2006), surprisingly, little is known regarding the antioxidant bioactivity of peptides derived from digestive action. Soy derived peptides were previously shown to exert antioxidative effects via *in vitro* studies, but the peptides were synthetically produced to simulate the potential production of soy peptides produced from enzymatic hydrolysis (Chen et al., 1998; Saito et al., 2003). The present study indicates that peptides derived from *in vitro* digestive action that simulates *in vivo* digestion produce peptides that show potent antioxidant effects. Moreover, low molecular weight <1 kDa peptides that are primarily absorbed *in vivo* showed antioxidative effects, which could indicate that such peptides play a role in the *in vivo* antioxidative action of soy protein. It is well known that peptides can exhibit antioxidant properties. In particular, strong basic proteins and peptides rich in basic amino acids such as Lys and His have been shown to serve as potent antioxidants including the His-containing di-peptide carnosine (Chan and Decker, 1994). Recent studies; however, have emphasized the importance for the assessment of the resistance of bioactive peptides to degradation from gastrointestinal proteases to better assess their *in vivo* potential (Fujita et al., 2000). The antioxidant properties shown in the present study are thus attributable to peptides that resist digestion during simulated gastrointestinal digestion and are more likely to reflect *in vivo* antioxidant functionality. Although the specific nature of the isolated peptides was not characterized in the present study, previous work has shown that proline-containing peptides and peptides with moderate hydrophobicity are more resistant to proteolysis (Shimizu et al., 2004).

In the present study, the FRAP assay was more sensitive to the antioxidant properties of the low molecular weight < 1 kDa peptides isolated from the hydrolysates of pressurized SPI as compared to peptides obtained from native SPI hydrolysates. The impact of pressure processing of SPI on *in vitro* antioxidant activity demonstrated herein extends previous work showing that high hydrostatic pressure of WPI was associated with antioxidant improvements as assessed by cellular glutathione status (Vilela et al., 2006). Previous studies have shown that the pre-treatment of SPI via food processing steps such

as blanching and pasteurization can influence the potency of soy hydrolysates and peptides that have angiotensin converting enzyme (ACE) activities (Lo and Li-Chan, 2005). Similarly, the present work demonstrates that pressure processing can impact on the antioxidant properties of the hydrolysates and isolated peptides of soy proteins. In contrast to FRAP activity, low molecular weight peptides from native SPI showed relatively greater antioxidant effects in relation to the DPPH antioxidant assay. These results suggest that the altered proportion and types of peptides associated with pressure treatment can lead to differing functional antioxidant effects. The differential antioxidant effects of the isolated peptides observed with the FRAP versus the DPPH assays is not surprising since it is well known that different *in vitro* antioxidant assays can produce differing estimates of antioxidant capacities depending on the functional nature of the antioxidant and oxidant tested. In that regard, the DPPH assay operates by direct measurement of hydrogen atom donation whereas the FRAP assays depends on electron transfer from the potential antioxidant to free radical molecules in simple lipid free systems. Becker et al. (2004) have suggested that conflicting results obtained for antioxidative activities via different *in vitro* assays may be traced back to several factors that include: a) the physical structure of the test system; b) the nature of the substrate for oxidation; c) the presence of interacting components; d) the mode of initiating oxidation; and e) the analytical method for measuring oxidation. In the present work, most of the antioxidant activity in relation to the inhibition of DPPH activity could be attributed to the lower molecular <1 kDa peptides as there was a relatively small increase in the antioxidant capacity in the SPI hydrolysates in comparison to the isolated peptides (Figs. 9a and 9b). In contrast, a substantially greater amount of FRAP antioxidant activity was observed in the SPI hydrolysates as compared to the isolated low molecular weight peptides (Figs. 8a and 8b). It is likely that the compositional nature of the isolated peptides could account for these differences. For example, Chen et al. (1998) found that His-containing peptides in soy hydrolysates had a quenching activity on singlet oxygen but no effect was seen with respect to the DPPH radicals. Thus, it is likely that His-containing peptides were a relatively minor component of the < 1 kDa peptide fraction as these peptides showed a relatively greater potency with respect to DPPH. Saito et al (2003) found that tripeptides containing Trp or Tyr residues at the C-terminus had strong

radical scavenging activities in terms of the FRAP assay. Such peptides could have played an antioxidant role in the FRAP potency of the larger molecular weight peptides present in the SPI hydrolysates. The *in vivo* functional significance of the higher FRAP antioxidant capacities of the SPI hydrolysates is unclear since peptides with molecular weights larger than 1 kDa have not been observed in digestive juices following protein feeding in animal models (Qiao et al., 2004). On the other hand, the possibility of functional antioxidant effects attributed to higher molecular weight peptides cannot be discounted since a low bioavailability of larger molecular weight peptides has recently been characterized via a variety of mechanisms that include passive diffusion via paracellular spaces as well as cytotic and carrier-mediated uptake mechanisms (Shimizu et al., 2004; Herrera-Ruiz et al., 2003).

Interestingly, the antioxidant activities of both the filtered and unfiltered hydrolysates of native and pressurized (600MPa) SPI remained stable only after 9 h of incubation. This relatively long reaction time is unusual compared to other antioxidative molecules such as ascorbic acid that have only a 4 min reaction time. The present results; however, are comparable to previous findings with respect to the measured antioxidant activity of BSA via the FRAP assay (Benzie and Strain, 1996). Benzie and Strain (1996) found that BSA showed very low reaction kinetics, which would not allow an endpoint to be reached within the typical monitoring period of 8 min. This latter result was attributed to an effect of the low pH condititions on the functionality of the protein thiol groups, thereby requiring longer antioxidant monitoring periods.

A general limitation to the *in vitro* antioxidant assays is that they may not necessarily reflect the *in vivo* potency of antioxidant compounds (Becker et al., 2004). The DPPH scavenging assay is one of the most popular methods for determining the antioxidant capacity of foods and chemical compounds since the violet DPPH radical anions are easy to use, have a high level of sensitivity, and allow for analysis of a large number of samples (Kim et al., 2002). A limitation regarding the use of chromogens such as the DPPH radical to estimate the antioxidant activity of biological samples is that this assay tends to underestimate antioxidant capacity due to the interference of other absorbing

compounds at 517 nm (Arnao, 2000). The limitation for the FRAP assay is that the reducing capacity of the antioxidants tested may not necessarily correlate with actual *in vivo* antioxidant capacity (Becker et al., 2004). On the other hand, the FRAP assay gives fast and reproducible results with single or mixtures of antioxidants in aqueous solution (Benzie and Strain, 1996). Moreover, due to its sensitivity to various types of antioxidants, the FRAP test was selected to provide the most appropriate estimate of the antioxidant activity of foods and thus recently used to generate the largest published systematic ranked screening of antioxidants in food samples (Halvorsen et al., 2006). Nevertheless, further analysis of peptide and amino acid profiles and their functional antioxidative effects is warranted to help provide more insight regarding the compositional differences observed in pressurized and native SPI hydrolysates. Furthermore, it is difficult to transfer antioxidant mechanisms established in model systems and in foods to the *in vivo* situation as the absorbability and breakdown of peptides *in vivo* might limit their antioxidant efficacy (Becker et al., 2004). Therefore, additional research regarding an *in vivo* evaluation of the antioxidative effects of isolated peptides and hydrolysates of native and pressurized SPI is warranted. For instance, Heneman et al. (2006) used the PCA-ORAC (oxygen radical absorbance capacity using perchloric acid) method to measure the antioxidant activity of human plasma following a soy protein meal, which would allow measurement of the activity of absorbed low molecular weight antioxidants. Such human and animal feeding trials would be important to further clarify the antioxidative capacities of soy protein hydrolysates and isolated peptides.

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CHAPTER 4

SUMMARY AND FINAL CONCLUSIONS

One of the major findings of this thesis is that high hydrostatic pressure treatment increases the *in vitro* digestibility of soy proteins with the resulting in the differential production of low molecular weight peptides following dual pepsin and pancreatin digestion. The increased *in vitro* digestibility of pressure treated SPI could be attributed to unfolding of the protein with exposure of the hydrophobic groups and increased access to the peptide bonds located in the interior of globular proteins such as β-conglycinin (7s fraction) for the proteolytic enzymes (Johnston et al.,1992). Pressure processing of SPI at 600 MPa with a five minutes holding time also produced different peptide profiles and concentrations following pepsin and pancreatin digestion as examined by CZE, RP-HPLC and ESI-MS analysis. The CZE method was able to identify differences in peptide profiles; however, it did not allow further determination of peptide composition. Reversed-phase HPLC chromatogram obtained via diode array detection of a range of UV wavelengths from 210 to 285 nm allowed the detection of differences in peptide profiles. Since most peptides absorb at lower wavelength, additional RP-HPLC analysis of peptide profiles at 214 nm confirmed differences in peptide profiles between hydrolysates of native and pressured treated SPI. The HPLC analysis also allowed the isolation and collection of specific peptide fractions, which differed in peptide composition in the hydrolysates from the native and pressurized SPI. The collected peptide fractions were subjected to ESI-MS to determine the individual peptide masses showing that peptide differences existed between the native and pressurized samples; however, further amino acid analysis in future studies is needed to identify the composition of the specific peptides identified.

Due to the high costs and considerable time required for *in vivo* assessments of digestibility, *in vitro* techniques have been used as a standard approach to test protein digestibility since the 1980's. The *in vitro* dual enzymatic digestion method using pepsin and pancreatin has been well validated to simulate *in vivo* gastrointestinal digestion (Qiao

et al., 2004). An advantage of the pepsin and pancreatin digestion system is that the hydrolyzed peptide bonds are well characterized. Hence, pepsin hydrolyzes bonds with Phe, Tyr, Leu or Val residues, releasing peptides of molecular weight exceeding 10 kDa. Pancreatin digestion involves trypsin that is highly specific for the cleavage of peptide bonds linking the carboxylic groups of the basic amino acids, Lys and Arg, and chymotrypsin that breaks down the peptide bonds of aromatic and neutral amino acids at the C terminal end. Hence, pancreatin digestion results in the release of neutral or basic low molecular weight peptides (< 1 kDa) or free amino acids after the pepsin and pancreatin digestion. The present thesis work employed ultrafiltration to isolate and collect peptides with molecular weight < 1 kDa on the basis of previous studies by Qiao et al (2004) who found that peptides < 1 kDa are not absorbed *in vivo*. Similar approaches to isolate and collect < 1 kDa peptides from hydrolysates have been used by others to assess the bioactive properties of caseinophosphopeptides (Savoie et al., 2004).

Many peptides produced via *in vitro* digestion and have been found to have bioactive effects such as insulinotropic properties, anti-thrombotic effects and anti-aggregating action. Since *in vitro* digestion is less precise and less accurate than *in vivo* digestion, additional *in vivo* studies are required to validate effects observed *in vitro*. Another observed disadvantage of the *in vitro* digestive system was due to the lack of brush border peptidases such as aminopeptidases that could further digest insoluble peptides *in vivo* to either inactivate peptides or produce more bioactive peptides. Also, the *in vivo* solubility of peptides tends to be higher due to the continuous removal of peptides *in vivo*. The *in vitro* digestive system; however, has utility as a relatively simple approach to test for the presence of released bioactive soy peptides from digestion and to investigate the functional effects of the peptides such as their antioxidant roles.

Soy protein has been indicated to help lower blood cholesterol and reduce coronary heart disease risk (FDA, 1999). In that regard, the antioxidant activity of soy protein could play a role since SPI intake has been consistently seen to exert potent antioxidant effects in human studies (Ashton et al., 2000; Swain et al., 2002; Vega-Lopez et al., 2005; Heneman et al., 2006) and animal studies (Madani et al., 2000; Aoki et al., 2002),

including the lowering of tissue lipid peroxidation and increased cellular glutathione levels. The bioactive antioxidant component(s) in SPI are likely to be peptides since isoflavones have not been associated with the *in vivo* antioxidant action of SPI (Ashton et al., 2000; Swain et al., 2002; Vega-Lopez et al., 2005; Heneman et al., 2006). *In vitro* studies; however, have been limited to the testing of synthetic peptides or combinatorial tripeptide libraries to explore antioxidative properties of soy derived peptides containing either histidine or tyrosine residues (Chen et al., 1998; Saito et al., 2003). Hence, the results of this thesis are the first to demonstrate that peptides derived from SPI via a simulated *in vivo* digestion system can exert significant *in vitro* antioxidant activities. The thesis work; however, indicates that *in vitro* assessment of antioxidant capacity of peptides requires more prolonged reactions in the *in vitro* assays in comparison to other antioxidative molecules such as ascorbic acid. For example, a minimum of 30 min was required to demonstrate the antioxidant capacity with the DPPH assay, which is likely due to slow reaction kinetics of the peptides with the DPPH radical.

It is important to note that the radical scavenging assays operate primarily by direct measurement of hydrogen atom donation or electron transfer from the potential antioxidant to free radical molecules in simple lipid free systems. These assays thus lack oxidation substrates and so do not reflect the situation in foods undergoing peroxidation or the *in vivo* context (Becker et al., 2004). Bioavailability of the antioxidant is also an issue when simple attempts are made at extrapolating *in vitro* studies to *in vivo* situations (Azzi, 2004). Future studies could also utilize a variety of other *in vitro* antioxidant assays to test for different types of antioxidant reactions by the isolated soy peptides, which could include assessments of hydroxyl radical and superoxide anion radical scavenging abilities. Also, model systems involving low-density lipoproteins (LDL) or liposomes could be used for the assessment of antioxidant properties related to lipid peroxidation, since these systems allow investigation of the protection of a substrate by an antioxidant in membranes or lipoproteins (Becker et al., 2004). For *in vivo* studies, the PCA-ORAC (oxygen radical absorbance capacity using perchloric acid) method could be employed, as this method has been successfully used to measure the antioxidant activity of low

molecular weight antioxidants in human plasma following a soy protein meal (Heneman et al., 2006).

In conclusion, the results of the thesis work showed that hyperbaric pressure processing promoted a more rapid proteolysis of soy proteins resulting in an altered release of small bioactive peptides that had significantly greater ferric reducing power ability. In addition, both native and pressurized (600 MPa) SPI were shown to release peptides derived from enzymatic digestion that exert *in vitro* antioxidant activities, which might eventually be useful in the prevention or treatment of oxidative stress related diseases.

NOVEL FINDINGS

The novel findings demonstrated in this thesis are summarized below:

1) Hydrostatic pressure treatment (600 MPa) increases the *in vitro* digestibility of SPI leading to different peptide profiles of the hydrolysates produced via pepsin and pancreatin digestion.

2) Soy peptides isolated from pepsin-pancreatin hydrolysates of native and pressurized (600 MPa) SPI demonstrate significant antioxidant capacities in terms of the *in vitro* FRAP and DPPH antioxidant assays.

3) Soy peptides (< 1 kDa) isolated from hydrolysates of pressurized SPI show greater FRAP antioxidant activity in comparison to < 1 kDa peptides released from native SPI hydrolysates.

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Appendices

August 31, 2006

To Whom It May Concern:

The purpose of the present letter is to confirm that the co-authors (Larry Lands, Hosahalli Ramaswamy, Yasuo Konishi, Laurie Chan and Stan Kubow) agree that the candidate (Chia-Chien Chang) includes the manuscript entitled *Antioxidant activities of hydrolysates and peptides generated from high hydrostatic pressure-treated soy protein isolates* in her thesis.

The candidate's role in this study included helping in the experimental design, ordering equipment and materials, performing all experimental assays, organizing the data, analyzing the data and drawing conclusion from the analyzed data. The candidate wrote the manuscript under the guidance of the co-authors and made modification to it in response to their comments.

Chia-Chien (Carole) Chang

I, the co-author, agree that the candidate, Chia-Chien (Carole) Chang, include the manuscript entitled *Antioxidant activities of hydrolysates and peptides generated from high hydrostatic pressure-treated soy protein isolates* in her thesis.

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Dr. Larry Lands Dr. Hosahalli Ramaswamy

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Dr. Yasuo Konishi Dr. Laurie Chan

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Dr. Stan Kubow

SEE ATTACHMENTS FOR SIGNATURES

Research Compliance Certificates

This study took place solely in *in vitro* setting, not involving human or animal subjects or any biohazardous or radioactive materials; therefore, no ethics approval was required.