Bitterness of soy protein hydrolysates according to molecular weight of peptides

by

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ABSTRACT

The bitterness in soy protein hydrolysates is a major obstacle to acceptance of soy products by consumers. The primary objective of this research was to evaluate the bitterness of 4 different molecular weight peptide fractions obtained from Protex 7L-treated soy hydrolysate. First, a theoretical analysis was performed to predict hydrolytic cleavage points of 3 different bitter- and non-bitter-producing proteases on soy protein at 4% degree of hydrolysis (DH) and hypothesize how peptide size influences bitterness. Protex 7L-treated soy hydrolysate was fractionated by gel filtration, desalinated by ultra-filtration, freeze-dried, and re-diluted to 5% w/v in Milli-Q water for sensory evaluation. Molecular weight of the fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Panelists' sensitivity to Multifect Neutral (MN)-treated soy hydrolysate was compared to caffeine, quinine, leucine, and phenylalanine. Panelists' perceptions of the bitterness of MN-treated soy hydrolysate were compared to the bitterness of leucine and phenylalanine free amino acids. Panelists were screened for bitterness sensitivity and 15 highly sensitive panelists were selected to evaluate the Protex 7L-treated soy hydrolysate fractions, MN-treated soy hydrolysate, caffeine, quinine, leucine, and phenylalanine by using a modified triangle test. Comparison of panelist sensitivity was evaluated by Cohen's kappa coefficient and Fisher's exact test ($p \leq 0.05$). Comparison of bitterness was analyzed by Cohen's kappa coefficient and McNemar's test ($p \leq 0.05$).

A fraction consisting of low molecular weight peptides (estimated 1-5 kDa) was identified as bitter ($p = 0.009$) as well as the unfractionated hydrolysate ($p = 0.088$). The remaining 3 fractions (2 larger MW and one < 1 kDa) were not bitter. Panelists selected for bitterness sensitivity had an average threshold of 0.98 mM for caffeine, 8.9 µM for quinine,

5.3 mM for leucine, 3.4 mM for phenylalanine, and 5.2 g/100 mL for MN-treated soy hydrolysate. The kappa coefficient showed poor agreement between panelist sensitivity to soy hydrolysate in relation to caffeine, quinine, and phenylalanine, and fair agreement in relation to leucine. Fisher's exact test showed a non-significant p value for panelist sensitivity to soy hydrolysate in relation to caffeine, quinine, leucine, and phenylalanine, indicating that panelist sensitivity to soy hydrolysate was independent of panelist sensitivity to caffeine, quinine, leucine, and phenylalanine. While all panelists identified caffeine and quinine as bitter, 41%, 62%, and 86% identified leucine, phenylalanine, and soy hydrolysate as bitter, respectively. In both bitter perception relationships to MN-treated soy hydrolysate, leucine and phenylalanine showed poor agreement by the kappa coefficient and significance by McNemar's test, indicating that the bitterness in leucine and phenylalanine is different than the bitterness in soy hydrolysate. This suggests that free hydrophobic amino acids such as leucine and phenylalanine are not responsible for the bitterness of soy protein hydrolysate. In bitterness sensory studies of soy hydrolysate, neither leucine nor phenylalanine is recommended for panelist training. Although caffeine and quinine are recognized standards for bitterness, small peptides may be a better standard to use in bitterness training for soy hydrolysates.

Several factors are likely to be the cause for bitterness in protein hydrolysates. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptides tend to be inter-related and a combination of these factors is most likely responsible for bitterness. However, these bitterness models do not explain why certain proteases do not produce bitter hydrolysates. Further research on the bitterness of protein hydrolysates coupled with valid sensory analysis is still needed.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

From 1992 to 2008, sales of soy foods increased from $300 million to $4 billion, according to the Soyfoods Association of North America (SANA 2009). This dramatic increase can be attributed to the number of newly introduced soy food categories and increased number of customers choosing soy foods for health reasons. New soy food categories, such as soy-based drinks, drinkable cultured soy, soy frozen desserts, and energy bars, have experienced significant growth. Many of these soy products can help consumers meet the 2005 federal Dietary Guidelines, making it a healthy food choice.

Proteolytic enzyme hydrolysis of soy proteins can improve the chemical, functional, and nutritional properties of products. Improved functional properties including solubility, viscosity, emulsification, and gelation make soy hydrolysate ideal for use in many food applications. However, a major problem with consumer acceptance of soy hydrolysate products is their bitter taste caused by certain proteases. Much research has focused on determining the cause of bitterness in hydrolysates, although the exact cause is still not fully understood. Two of the main causes thought to be responsible for bitterness include hydrophobicity and molecular weight of the peptides in the hydrolysate. In this thesis, the cause of bitterness in soy protein hydrolysate will be reviewed.

RESEARCH OBJECTIVES

The goals for the present study were to make a soy hydrolysate using Protex 7L enzyme, separate the hydrolysate into fractions according to molecular weight of the peptides, and evaluate the hydrolysate fractions for bitterness by sensory evaluation. The three specific sensory evaluation objectives were to: (1) evaluate the unfractionated Protex

7L hydrolysate and its respective fractions using the bitter/not bitter test, (2) compare each panelist's sensitivity to caffeine, quinine, leucine, and phenylalanine in relation to MN soy hydrolysate, and (3) determine whether the bitterness in leucine or phenylalanine closely resemble the bitterness in MN soy hydrolysate.

THESIS ORGANIZATION

This thesis is organized into three chapters and an appendix. Chapter 1 provides a general introduction to soy hydrolysates and a literature review. The literature review includes topics relating to soy protein products and processing, production of protein hydrolysates, functional properties of hydrolysates, enzymes for protein hydrolysates, physiochemical properties of soy hydrolysates, and bitterness of hydrolysates. Chapter 2 contains the present study: Determination of bitterness in soy protein hydrolysates according to molecular weight of peptides. The paper in Chapter 2 will be condensed and submitted to the Journal of Food Science. Chapter 3 contains a general discussion and recommendations for future research. All references follow the format of the Council of Science Editors. Data for this research which is not reported in Chapter 2 is presented in the appendix.

LITERATURE REVIEW

Soy protein products and processing

Soybeans are protein-rich legumes which provide an important food source for humans as well as livestock. While most plant proteins need to be paired with grains to be complete in essential amino acids, soy protein is considered complete and equivalent to animal sources (Young 1991) in meeting the human essential amino acid requirement. Approximately 90% of soy's protein is storage protein, and the remaining 10% is comprised of intracellular enzymes, membrane protein, protein inhibitors, and lectins (Kinsella 1979).

The storage proteins can be described in four main groups by their mean sedimentation coefficients: 2S, 7S, 11S, and 15S (Table 1). The 11S and 7S protein fractions, glycinin and beta-conglycinin, make up the majority of the protein.

Table 1. Distribution of soy storage proteins[a]

Protein fraction by mean sedimentation coefficient	Molecular weight (kDa)	Percent of total protein	Principal components
2S	8-50	8	Trypsin inhibitor, cytochrome
7S	60-210	35	Lipoxygenase, amylase, globulins
11S	350	52	Globulins
15S	600	5	Polymers

[a]Adapted from Kinsella (1981)

Soy protein products are standard additives in the food industry. Soy flour, soy protein concentrate (SPC), and soy protein isolate (SPI) are the three protein preparations used in food systems to achieve desired functional properties (Figure 1). Table 2 explains the various functional properties of soy protein, ideal food systems for the property, and soy protein products in which the property is performed. Increased solubility, water absorption and binding, viscosity, gelation, cohesiveness-adhesiveness, elasticity, emulsification, fat adsorption, flavor binding, foaming, and color control are many of the functional properties that allow soy protein products to be desirable ingredients in a food system.

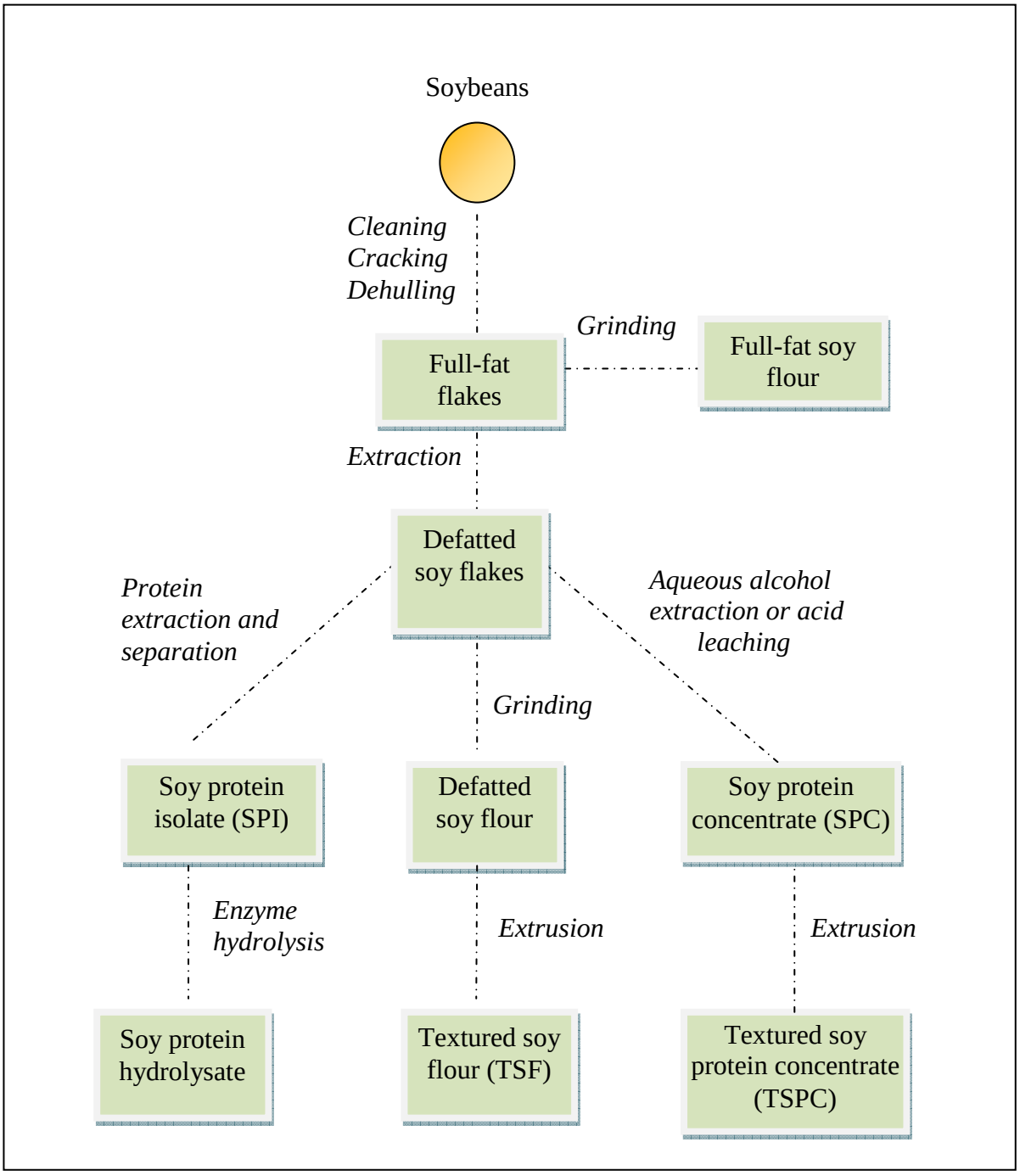

Figure 1. Soybean processing chart illustrating techniques used in producing various soy protein products[a]

[a]Adapted from U.S. Soybean Export Council (2008)

Table 2. Functional properties performed by soy protein preparations in actual food systems[a]

Functional property	Mode of action	Food system	Preparation used[b]
Solubility	Protein salvation, pH dependent	Beverages	F,C, I, H
Water absorption and binding	Hydrogen binding of water, entrapment of water, no drip	Meats, sausages, breads, cakes	F, C
Viscosity	Thickening, water binding	Soups, gravies	F,C, I
Gelation	Protein matrix formation and setting	Meats, curds, cheese	C, I
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked foods, pasta products	F, C, I
Elasticity	Disulfide links in gel deformable	Meats, bakery	I
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soup, cakes, meats, sausages, doughnuts	F, C, I
Fat absorption	Binding of free fat	Meats, sausages, doughnuts	F, C, I
Flavor-binding	Adsorption, entrapment, release	Simulated meats, bakery	C, I, H
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes	I, H
Color control	Bleaching of lipoxygenase	Breads	F

[a]Adapted from Kinsella (1979)

[b]F = soy flour; C = soy concentrate; I = soy isolate; H = soy hydrolysate

Soy flours are the simplest and least expensive type to produce, made by grinding and screening defatted bean flakes. However, the minimal processing makes soy flour the most variable form of soy protein in terms of quality. Soy flour ranges from 50-54 % protein content (Riaz 2005). The three forms of soy flour are full-fat, defatted, and lecithinated (Endres 2001). Full fat soy flour contains the soy oil present in the soybean, while the oil from defatted soy flour is removed during processing by solvent extraction. A blend of lecithin and soy oil is commonly added to the defatted soy flakes to improve emulsification and dispersion properties. Soy flour is high in oligosaccharides and cell wall carbohydrates, which give the flour a strong flavor profile. For this reason, soy flours are generally not used as ingredients in dairy systems and processed meats where a delicate flavor profile is desired (Hoogenkamp 2005). However, soy flours are still commonly used in baked goods, snack foods, pet foods, and other foods where their strong flavor profile is not an issue.

SPC was produced to overcome the flavor problems associated with soy flours (Lusas and Riaz 1995). SPC contains 65-72% protein on a dry basis. The major objective in SPC manufacturing is to remove the oligosaccharides and off-flavors in the defatted soy flour. Acid leaching and extraction with aqueous ethanol are the two main processes commercially used today for this purpose (Hoogenkamp 2005). Extraction with ethanol results in SPC with cleaner flavor profile and whiter color than acid leaching, but partly removes the soy isoflavones as well. Concentrates are low in sodium, retain much of the bean's dietary fiber, and have many of the same functional properties as isolates.

Textured protein products are commonly made by extruding soy flour, SPC or SPI. Textured soy proteins are designed for use in ground meat products. They can resemble beef, pork, poultry, or seafood when hydrated (Endres 2001). While textured SPC (TSPC)

has preferred physical and sensory properties compared to textured soy flour (TSF), TSF is used as a less expensive alternative in cost critical products. TSF, however, may cause undesirable flavor changes in finished products due to its soluble carbohydrates and has limited use as a filler in meat products because of its softening effect. TSC has an improved taste profile and can maintain textural integrity in complex meat systems compared to TSF (Hoogenkamp 2005).

SPI is produced by water extraction of protein from the flake at temperatures 25-80ºC through solubilization, separation, and isoelectric precipitation steps. The isolate may be dried at the pH of the precipitate or neutralized prior to drying, yielding SPIs with different properties. The protein content of SPI is generally around 90% on dry basis (Riaz 2005). The removal of nearly all the fat, fiber, and soluble carbohydrates makes SPI a premium quality protein ingredient with a low flavor profile. SPI has good gelation, emulsification, water absorption and foaming properties (Utsumi and others 1997), but solubility varies depending on the pH of the precipitate. Solubility in the pH 3-6 range is generally poor, and this limits the use of SPI in acidic foods (Utsumi and Kinsella 1985). The production of soy protein hydrolysates, through the enzymatic hydrolysis of SPI, is a way to greatly improve solubility in acidic conditions (Adler-Nissen 1976).

Production of protein hydrolysates

Protein hydrolysis can be achieved by either chemical or enzymatic means. Chemical hydrolysis may be carried out under acidic or basic conditions. Acid hydrolysis has been used for the production of flavoring products known as hydrolyzed vegetable protein (Olsman 1979). Production of acid hydrolyzed proteins, however, is limited due to randomness of the process and the risk of chloropropanol formation in the presence of

hydrochloric acid (Lawley and others 2008). Chloropropanols are side products of the reaction, which have possible toxic and carcinogenic effects. Alkali hydrolysis has been used in the production of foaming agents as substitutes for egg proteins and the production of fire extinguisher foams, but is not widely used (Kunst 2003). A disadvantage of hydrolysis under alkali or acidic conditions is that the reaction is not very specific (Synge 1945). Other problems include the destruction of L-form amino acids and the formation of D-form amino acids and toxic substances such as lysine-alanine (Lahl and Braun 1994). Enzymatic hydrolysis of protein occurs under mild processing conditions of pH 6-8 and temperature 40-60˚C, which minimizes side reactions (Clemente 2000). The use of selective protease in enzymatic hydrolysis makes the reaction much more specific than in alkali or acid hydrolysis, and the final hydrolysate contains less salt. This literature review will focus on enzymatically modified protein hydrolysates, which will be referred to as hydrolysates for simplicity.

Common protein sources for the production of hydrolysates include casein, whey, and soybeans. Since the 1940's, protein hydrolysates have been used for the special nutritional needs of individuals with difficulty digesting intact protein or poor absorption (Clemente 2000). These products are generally nutritionally complete formulas that are given to patients via enteral feeding tubes. Hydrolysates are also an essential part of the diet in individuals who suffer from phenylketonuria (PKU). PKU patients require hydrolysates very low in phenylalanine. These are generally extensively hydrolyzed proteins that involve extra purification treatments with charcoal or adsorption chromatography to remove the phenylalanine (Lopez-Bajonero and others 1991; Vasconcellos and others 1992).

For infants with special needs, hydrolysates are widely used in hypoallergenic infant formulas (Mahmoud and others 1992). In the case of food allergies, a specific antibody responds to the protein allergen by forming a bridge between two epitopes. Epitopes are small regions on the antigen that initiate antibody production. The hydrolysis of proteins reduces the amount of protein with two epitopes, which in turn reduces antigenicity (Cordle 1994). While there is no set molecular weight cutoff for peptides to be nonallergenic, research suggests a limit of 10-15 peptides for hypoallergenic benefits (Bindels 1992).

Hydrolysates are primarily used in sports nutrition products, diet control products, and nutrition-specific foods. These products are frequently found in beverage systems because of the ability of hydrolysates to be highly soluble in acidic solutions and withstand heat treatment applied in pasteurized shelf-stable drinks (Frøkjaer 1994).

Recently, there has been interest in the isolation of bioactive peptides from plant and animal protein hydrolysates for use in functional foods. Bioactive peptides are physiologically active peptides within the intact protein molecule sequence. They are generally 3-20 amino acid residues in length, and can be released by enzymatic hydrolysis during fermentation, enzymatic hydrolysis with selective protease, or gastrointestinal digestion (Korhonen and Pihlanto 2003). Bioactive peptides have been discovered with antimicrobial, antihypertensive, cholesterol-lowering, antithrombotic, mineral absorption enhancement, and immunomodulatory properties (Rutherfurd-Markwick and Moughan 2005).

Figure 2 shows a general processing flow diagram of the production of hydrolysates. The starting material is a protein solution or suspension. Most commercial productions use batch hydrolysis, which is carried out in a processing vessel that has the capability to control

agitation, temperature, and pH (Nnanna and Wu 2007). The hydrolysis generally takes a few hours (Lahl and Braun 1994). Hydrolysis depends on protease specificity, extent of protein denaturation, concentration of substrate and enzyme, temperature, pH, ionic strength, and the presence or absence of inhibitory substances (Kilara 1985).

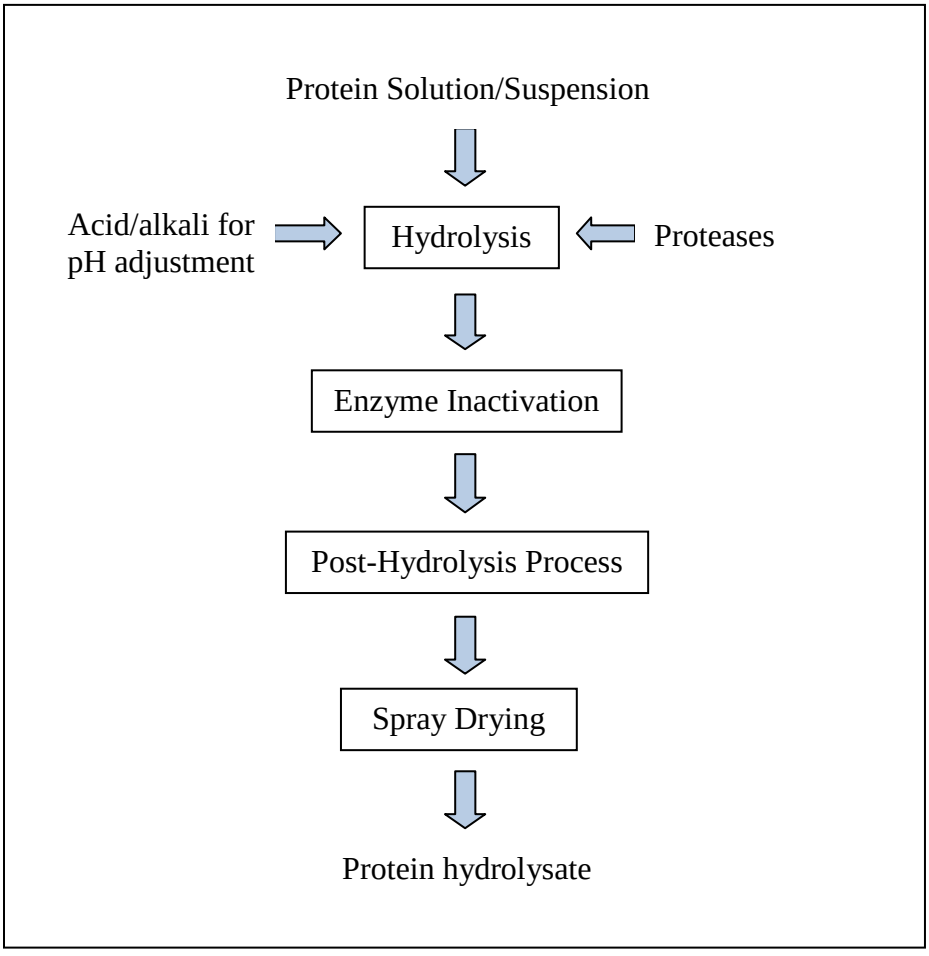

Figure 2. General processing flow diagram for the production of protein hydrolysate[a]

[a]Adopted from Nnanna and Wu (2007)

Depending on the pH during hydrolysis, hydrogen ions are either released or taken up due to enzyme action on the protein. The hydrolysate solution is titrated with alkali or acid to maintain an optimal pH for the enzyme. The rate at which alkali or acid is added corresponds to the rate of hydrolysis. The total volume of alkali or acid added to maintain pH during hydrolysis can be used in calculating the degree of hydrolysis (Adler-Nissen 1986). Once the desired degree of hydrolysis is reached, enzyme inactivation is necessary to ensure hydrolysis does not continue. Heat treatment is a common way to inactivate the enzyme, although the specific inactivation method will depend on the protease used. Posthydroysis processes may be employed after enzyme inactivation such as filtration, separation, or treatments with charcoal or ion-exchange chromatography (Lahl and Braun 1994). Filtration may be used to improve clarity by removing insoluble particles to remove larger particles which may be potential anitgenic material in hypoallergenic products, or to reduce endotoxin level in parenteral nutrition products (Kunst 2003). Fractionation may be performed to isolate specific peptides or remove undesired peptides (Nnanna and Wu 2007). Charcoal treatment may be used to remove off-color material, reduce off-odor and bitterness to some extent, or to remove phenylalanine in products for PKU patients. Spray drying is usually used to dry the hydrolysates, although roller drying and freeze drying may also be employed (Lahl and Braun 1994).

Functional properties of protein hydrolysates

During hydrolysis, physicochemical properties of the protein are altered which, in turn, modifies functional properties of the hydrolysate (Table 3). Molecular size of the peptides decreases as a result of hydrolysis, which has a major effect on many functional properties. Larger peptides of 2-5 kDa are ideal for functional ingredients in food, medium-

sized peptides of 1-2 kDa are ideal for sports nutrition (Frøkjaer 1994; Siemensma and Kunst 1999) and clinical nutrition (Schmidl and others 1994), while smaller peptides of < 1kDa are ideal for hypoallergenic infant formulas (Siemensma and others 1993). Besides molecular weight of the hydrolysate, surface activity, hydrophobicity, carbohydrate interaction, and mineral interaction influence various functional properties.

Table 3. Physiochemical and functional properties of protein hydrolysates in food products[a]

Chemical and Physiochemical properties	Functional properties
Molecular size	Immunogenicity (allergenicity) Solubility Osmolality Viscosity Gelation Emulsification Clarity (turbidity) Flavor
Surface activity and hydrophobicity	Emulsification Foaming
Carbohydrate interaction	Maillard browning Color formation Gelation Flavor formation
Mineral interaction	Solubility Thermal stability

[a]Adapted from Mahmoud (1994)

The desired functional properties of the hydrolysate depend on its application. For example, the osmolality of a hydrolysate is of great importance in infant and adult nutritional

formulas because of the influence on gastrointestinal side effects (Mahmoud and others 1994). Enhanced emulsification properties of the hydrolysate are essential in dressings, spreads, and emulsified meat products. Solubility of hydrolysates is ideal for many beverages in food industry applications.

Enzymes for protein hydrolysates

A protein may be hydrolyzed by one or more enzymes, and the selection of enzyme is critical for optimal results. Each enzyme has a certain activity and specificity. The activity of a protease is determined by whether it hydrolyzes a particular protein, while the specificity of the protease depends on which peptide bonds it cleaves in the protein. The catalytic efficiency of the protease is determined by the rate at which it hydrolyzes under standard pH, temperature, and enzyme concentration (Whitaker 2003). Enzyme selection may depend on parameters such as the amount of free amino acid or required degree of hydrolysis for the product. The proteins in enzymatic hydrolysates are only partially hydrolyzed due to the inability of most proteases to cleave glycoproteins, phosphoproteins, or protein domains that contain numerous disulfide bridges (Gibbs and others 2004).

A large variety of endo- and exopeptidases, also called endo- and exoproteases, are available for the production of hydrolysates. Endopeptidases hydrolyze amino acids of the interior of the polypeptide chain, while exopeptidases hydrolyze from either the N-terminal (aminopeptidases) or the C-terminal (carboxypeptidases) end of the protein. Proteases may also by mixtures of endo- and exopeptidases, although most commercial proteases are endopeptidases (Hamada 2000). Exopeptidases are most often produced in the form of crude extracts of microorganisms such as yeast or lactobacilli (Kunst 2003).

There are four main classes of proteolytic enzymes based on their active site. Table 4 lists each class of endopeptidase along with common endopeptidases used in industry, the EC number, and preferential cleavage. Table 5 lists common examples of exopeptidases along with their EC number. The enzyme used in the present study is primarily metalloendopeptidase (EC 3.4.24.28) with a small amount of subtilisin-like protease (EC 3.4.21.62). The Enzyme Commission number (EC number) is used to numerically classify enzymes based on the chemical reactions they catalyze. Enzymes that catalyze hydrolysis have 3 as the first digit of the EC number.

Table 4. Common endopeptidases used in industry[a]

Enzyme	EC number	Preferential cleavage
Serine proteases	3.4.21	
Chymotrypsin	3.4.21.1	Tyr, Trp, Phe, Leu,
Trypsin	3.4.21.4	Arg, Lys
Subtilisin	3.4.21.12	Mainly hydrophobic
Cysteine proteases	3.4.22	
Cathepsin B	3.4.22.1	Arg, Lys, Phe-X
Papain	3.4.22.2	Arg, Lys, Phe-X
Ficin	3.4.22.3	Phe, Tyr
Bromelain	3.4.22.4	Lys, Arg, Phe, Tyr
Aspartic proteases	3.4.23	
Pepsin	3.4.23.1	Aromatic, Leu, Asp, Glu
Chymosin	3.4.23.4	Cleaves Phe_{105}-Met_{106} bond in κ-casein
Metallo proteases	3.4.24	
Thermolysin	3.4.24.27	Ile, Leu, Val, Phe
Neutral proteinase	3.4.24.28	Leu, Phe, and others

[a]Adapted from Kunst (2003)

Table 5. Examples of common exopeptidases[a]

Enzyme	EC number
Leu-aminopeptidase	3.4.11.1
Lys-peptidehydrolase	3.4.11.15
Gly-leu dipeptidase	3.4.13.11
Di-peptidyl-peptide hydrolase	3.4.14.4
Gly-pro aminopeptidase	3.4.14.5
Carboxypeptidase C or Y	3.4.16.1
Glycine carboxypeptidase	3.4.17.4
Alanine carboxypeptidase	3.4.17.8
Carboxypeptidase S	3.4.17.9

[a]Adapted from Kunst (2003)

Physiological properties of soy hydrolysates

As previously discussed, there has been great interest in bioactive peptides. Although bioactive peptides have been isolated from milk, egg, fish, oyster, cereal, soybean, and radish seeds (Matsui and others 1993; Li and others; 2002; Yoshikawa and others 2003), there has been increasing interest in the health benefits of soy. Components of the soybean with anticarcinogenic effects been isolated (Kennedy 1995; Kennedy and others 2002; de Lumen 2005; Jeong and others 2003), and the main way to isolate these bioactive peptides is through enzyme hydrolysis. Table 6 shows examples of bioactive peptides which have been isolated from soy proteins. Besides soy's anticarcinogenesis effect, antihypertensive activity (Wu and Ding 2001; Kodera and Nio 2002; Kitts and Wiler 2003), cholesterol lowering effect

(Bakhit and others 1994), and plasma triglyceride levels reduction (Iritani and others 1996) have been reported.

Table 6. Examples of biologically functional peptides obtained from soy protein[a]

Source	Preparation	Peptides	Activity	Reference
Native and heated soy protein isolates	Hydrolysis with pepsin, papain, chymotrypsin, alacase, Protamex, Flavourzyme	Peptides obtained from 1.7- 20.6% degree of hydrolysis	Antioxidant activities, decreased serum thiobarbituric acid-reactive substances except for papain hydrolysates	Pena-Ramos and Xiong 2002
Soy protein concentrate	Hydrolysis with porcine, pepsin, and bovine pancreatic trypsin or only trypsin	Peptides separated by ultrafiltration	Up-regulate the uptake and degradation of LDL by HepG2 cell receptors	Amoldi and others 2001
Soy flour	Hydrolysis with papain or pronase enzymes	Soluble hydrolysate peptides separated by ultrafiltration	Growth-promoting and production enhancing activities	Franek and others 2000
Defatted soy protein	Hydrolysis with thermolase enzyme	X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr	Anticancer	Kim and others 2000
Defatted soy meal	Hydrolysis with alacase enzyme	Peptides obtained from soluble hydrolysate fractionated on cationic exchange resin	Hypotensive	Wu and Ding 2001
Soy protein	Hydrolysis with protease D3	(1) Tyr-Val-Val-Phe-Lys (2) Pro-Asn-Asn-Lys-Pro-Phe-Gln (3) Asn-Trp-Gly-Pro-Leu-Val (4) Ile-Pro-Pro-Gly-Val-Pro-Tyr-Trp-Thr (5) Thr-Pro-Arg-Val-Phe	Hypotensive	Kodera and Nio 2002
Soybean	Hydrolysis with enzyme from *Bacillus subtilis*	Pro-Gly-Thr-Ala-Val-Phe-Lys	Hypotensive	Kitts and Wiler 2003

[a]Adapted from Wang and Gonzalez de Mejia (2005)

Bitterness of soy hydrolysates

Despite the improved functional characteristics, a major problem concerning consumer acceptance of soy hydrolysates is their intense bitter taste. The intensity of the bitterness depends on the protease used and the degree of hydrolysis (DH) and protease used. While most proteases produce bitter hydrolysates, the bitterness of the hydrolysate varies according to DH. Generally, bitterness increases with DH for all protease-treated soy hydrolysates. However, certain proteases such as Flavourzyme (EC 3.4.11.1), a fungal protease from *Aspergillus oryzae* composed of both endo- and exoproteases, do not produce bitter peptides with increasing DH (Seo and others 2008). The bitterness of specific proteases may also not be perceived at low DH. Lock (2007) reported that the bitterness in bromelain-treated soy hydrolysate did not differ from soy protein isolate at 4% DH, while Seo and others (2008) reported significant increases in bitterness intensity in bromelain-treated soy hydrolysates at 10-14% DH.

Much research has focused on the cause of bitterness in soy hydrolysates. Bitterness is often attributed to the formation of low molecular weight peptides comprising primarily hydrophobic amino acids (Matoba and Hata 1972), although an exact cause has not been determined and conflicting research is present in the literature. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of the peptide have been studied as possible influences in the bitter taste of hydrolysates (Kim and others 2008).

Hydrophobicity

Ney (1971) proposed that hydrophobicity was the most important factor influencing bitterness of peptides and created the Q value as a means to theoretically measure it by Eq. 1:

Eq. 1 $$Q = \frac{\sum \Delta f}{n}$$

where Q = the average hydrophobicity of a peptide, $\sum \Delta f$ = the sum of free energy for the transfer of amino acid side chains from ethanol to water in cal/mol for each residue (Tanford 1962), and n = the number of amino acid residues. Tanford's free energy calculation can be summarized by Eq. 2:

Eq. 2 $$\sum \Delta f = -T\Delta S_{conf} + \sum \Delta f_u$$

where $-T\Delta S_{conf}$ is the change in conformational entropy of the polypeptide chain from ethanol to water and Δf_u is the change in free energy for the transfer of small component groups of the protein molecule from the native form to the unfolded form. Ney reported that peptides < 6 kDa in molecular weight with Q values > 1,400 cal/mol were bitter, while peptides with Q values < 1300 cal/mol of any molecular weight or peptides > 6 kDa with Q values > 1,400 cal/mol were not bitter. There were no reported correlations for peptides with Q values between 1300-1400 cal/mol.

Guigoz and Solms (1976) reviewed Ney's Q rule by comparing the bitterness and Q values of 206 different peptides. The majority of bitter peptides were > 1,300 cal/mol with the exception of some bitter peptides containing glycine. The authors proposed that glycine should be omitted from the Q value calculations, which would bring the Q value of the bitter glycine-containing peptides > 1,300 cal/mol. A weakness in Guigoz and Solms' evaluation of the Q rule was that a disproportionally larger amount of bitter peptides were evaluated than non-bitter peptides, although the research of Ney (1971) did include the evaluation of 41 non-bitter peptides which all obeyed the Q rule.

There has been concern about the validity of the Q rule for bitterness in protein hydrolysates. By extrapolation of the Q rule, Ney (1972) suggested that the bitterness of hydrolysates could be predicted by assessment of the intact protein. Proteins with a high Q value, such as casein (1605 cal/mol), soy protein (1540 cal/mol), and zein (1480 cal/mol) would produce bitter tasting hydrolysates. According to this extrapolation, the Q value of the bitter hydrolysate would be based on the average Q value of all peptides in the hydrolysate. However, Ney proposed certain strongly hydrophobic peptides with high Q values to cause bitterness, which cannot be predicted by simply using the average Q value of the hydrolysate (Adler-Nissen 1986). Another issue regarding Ney's Q values (Ney 1972; Ney 1979) is that they appear to be overestimated when compared to the hydrophobicity values given by Bigelow (1967) and Ricks and others (1978). Cho and others (2004) fractionated commercial soy hydrolysates and reported that bitterness depended on molecular mass, but not Ney's Q value of hydrophobicity. These differing results may be due to the fact that steric parameters and spatial structure, both important factors in bitterness intensity, are not taken into account with the Q value calculation (Kim and others 2008).

Matoba and Hata (1972) also suggested that hydrophobicity was responsible for the bitterness in protein hydrolysates. They reported that each hydrophobic amino acid contributed to the bitterness of the peptide, and that peptides with internally sited hydrophobic amino acids were more bitter than when located at N- or C-terminus. This was supported by the research of Ishibashi and others (1987) in which the hydrophobicity of leucine residues increased the bitterness of peptides and the strongest bitterness occurred when the leucine residue was located at the C-terminus end. Matoba and Hata (1972) explained the formation of bitterness by exposure to hydrophobic amino acids. Prior to

hydrolysis, the intact globular protein molecule does not taste bitter because hydrophobic side chains are concealed in the interior of the protein, preventing interaction with taste buds. As the protein is hydrolyzed, hydrophobic amino acids are exposed, allowing interaction with taste buds and elucidating a bitter response.

Adler-Nissen and Olsen (1979) proposed a qualitative relationship between DH and bitterness based on the Matoba and Hata's model of bitterness formation (Figure 3). Using this relationship, low bitterness can be expected at low DH because peptides are relatively large and able to mask hydrophobic side chains to a certain extent. Individuals may not perceive bitterness at low DH if it is below their threshold. As DH increases, more hydrophobic amino acids are exposed, increasing bitterness. At very high DH, however, peptides are degraded to small peptides with terminal hydrophobic amino acids or free amino acids, resulting in decreased bitterness.

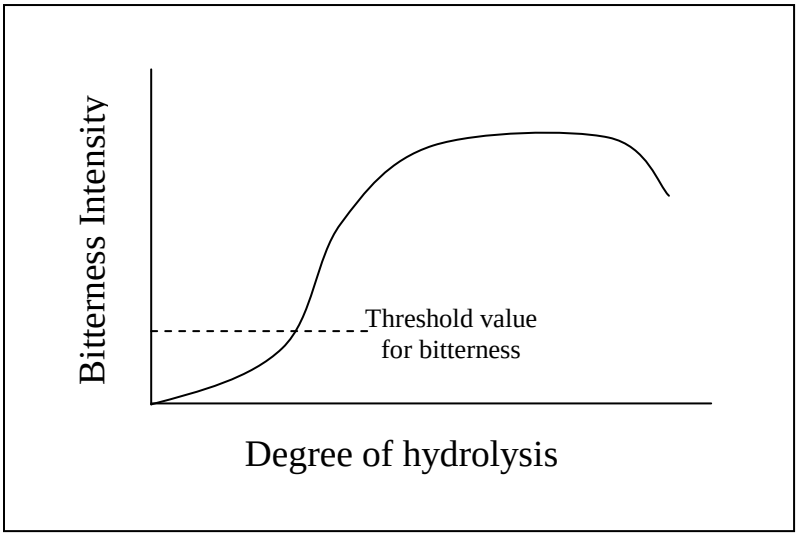

Figure 3. Qualitative relationship between bitterness and degree of hydrolysis[a]

[a]Adapted from Adler-Nissen and Olsen (1979)

Molecular weight

In general, small molecular weight peptides are thought to be responsible for bitterness. There is conflicting research, however, on the exact molecular weight range which constitutes a small bitter peptide. Lovsin-Kukman and others (1996) reported that bitterness in alacase-treated soy hydrolysates was due to hydrophobic bitter peptides of 1 kDa molecular weight. In contrast, Cho and others (2004) reported that the most intensely bitter fractions from two commercial soy protein hydrolysates included peptides of 1-4 kDa, while fractions with peptides < 1 kDa showed the least amount of bitterness. Kim and others (1999) expressed a gene encoding the A1aB1b glycinin subunit in *E. coli* and hydrolyzed it to generate bitter peptides. The fractions with the most intense bitter tasting peptides had average molecular weights < 1,700 Da. This supports earlier findings of Guigoz and Solms (1976) in which the majority of bitter peptides were reported to contain 2-15 amino acid residues, approximately equivalent to peptides with molecular weights less than 1,700 Da.

Bulkiness

Ishibashi and others (1988a) and Tamura and others (1989) have suggested that bulky basic groups or bulky hydrophobic groups may stimulate bitterness while hydrophobic groups may be the binding units for the mechanism of bitter taste perception in hydrolysates. It was proposed that these two groups needed to be adjacent to one another in the steric conformation of the peptide to elucidate bitterness (Ishibashi and others 1988b). Ishibashi and others (1988a) estimated the steric distance between these sites to be 4.1 Å, and Tamura and others (1990) estimated the pocket size to be 15 Å.

Debittering of hydrolysates

There has been great interest in determining ways to remove bitterness in hydrolysates and many options have been investigated. Procedures such as absorption of bitter peptides on activated carbon, chromatography, and selective alcohol extraction have helped remove bitterness in an extent, but also led to amino acid residue loss (FitzGerald and O'Cuinn 2006). Certain proteases have also been shown to have debittering effects. Arai and others (1970) reported that the bitterness of soy hydrolysates could be reduced by the addition of carboxypeptidase A and *Aspergillus* acid carboxypeptidase, which degrade the C-terminal structures. Flavourzyme (Novo Nordisk, Bagsværd, Denmark) is a mixture of endo- and exoproteases which has debittering effects on hydrolysates at 10 to 20% DH (Pommer 1995). Nishiwaki and others (2001) reported debittering effect of an aminopeptidase from the edible basidomycete *Grifola frondosa* on soy hydrolysate. This aminopeptidase appears to efficiently hydrolyze peptides containing hydrophobic amino acids at the N-terminal positions.

Sensory evaluation of bitterness in hydrolysates

Previous studies on the bitterness of soy hydrolysates vary greatly in method of sensory evaluation. These sensory methods included taste dilution analysis (Lioe and others 2006; Seo and others 2008), category scaling (Lovsin-Kukman and others 1995; Nishiwaki 2002), caffeine equivalency methods (Cho and others 2004; Kodera and others 2006), line scaling (Aaslyng and others 1999; Kim and others 1999, 2003), and phenylthiourea comparison (Yamashita 1969; Fujimaki and others 1970). Taste dilution analysis (TDA) is a technique based on serial dilutions of sample in which the relative taste thresholds of compounds is determined (Frank and others 2001). In category scaling, the bitterness

intensity is assigned a value on a limited numerical scale (Meilgaard and others 2007). Caffeine equivalency methods are very similar to category scaling, in which the panelist assigns a caffeine concentration from several standards which best describes the bitterness of the sample. In line scaling, the panelists rates the perceived bitterness intensity on a horizontal line scale. Comparison by phenylthiourea (PTU) is a method similar to caffeine equivalency. PTU is perceived as either extremely bitter or tasteless depending on the genetic makeup of an individual (Bartoshuck and others 1994). The use of PTU in present sensory studies, however, has been largely abandoned due to concerns about its toxicity (Nelson and others 2003).

Table 7 compares the sample concentration, bitterness standard, number of panelists, absence or presence of bitterness sensitivity screening, and sensory method for various studies evaluating the bitterness of soy protein hydrolysates. In many studies, important information was not specified, such as the concentration of sample, bitterness standard, number of panelists, and whether or not sensitivity screening was conducted. The number of panelists also varied greatly in these studies. There is no set minimum number of panelists for each method, but 6-12 screened and trained panelists is typically recommended for intensity scales (Kilcast 2000). However, in many of these studies on soy hydrolysate bitterness, no screening procedure is specified and very few panelists are used. Screening is essential because sensitivity to bitterness is genetic and inherent for each individual (Cornelis and others 2007). The bitterness standard used in screening is also important due to variation in an individual's bitterness sensitivity between bitter compounds (Delwiche and others 2001). Most studies on the bitterness evaluation of soy hydrolysates use either caffeine or quinine as a bitterness standard. No previous studies on soy hydrolysate, however, have

screened panelists with more than one bitter compound. Another challenge in the evaluation of bitter hydrolysates is the lingering aftertaste in the mouth. A waiting period between evaluation of samples and the use of palate cleansers is important in limiting carry-over effects from residual bitterness.

Table 7. Comparison of sensory studies on the bitterness of soy protein hydrolysate

Material studied	Concentration of sample	Bitterness standard	Sensory method	# of panelists	Screened?	Reference
Enzymatic soy hydrolysate	1.0%	not specified	line scale	9	not specified	Aaslyng and others 1999
Enzymatic soy hydrolysate	0.15-1.0%	caffeine	caffeine equivalent	9 to 12	not specified	Cho and others 2004
Enzymatic soy hydrolysate	0.1 mM	PTU	ΓU comparison	not specified	not specified	Fujimaki and others 1970
Soy hydrolysate peptides expressed in *E. coli*	not specified	quinine	line scale	5	not specified	Kim and others 1999
Bitter peptides from soy glycinin hydrolysate	0.01%	quinine	line scale	3	not specified	Kim and others 2003
Enzymatic soy hydrolysate	20 mg/mL (2.0%)	caffeine	caffeine equivalent	7	yes, 18 screened	Kodera and others 2006
Enzymatic soy hydrolysate	2.0%	quinine	line scale	19	not specified	Li and others 2008
Soy sauce peptides	not specified	caffeine	TDA	8	yes, 16 screened	Lioe and others 2006
Enzymatic soy hydrolysate	0.5-3.0%	not specified	category scale	3	not specified	Lovsin-Kukman and others 1995
Enzymatic soy hydrolysate	not specified	caffeine	TDA	10	yes	Seo and others 2008
Enzymatic soy hydrolysate	0.1 mM	PTU	PTU comparison	not specified	not specified	Yamashita and others 1969

Several factors are likely to be the cause for bitterness in protein hydrolysates. Ney's Q rule, which does not account for steric parameters and spatial structure, is too simplistic to be an absolute measure of bitterness in peptides. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of peptides tend to be inter-related and a combination of these factors is most likely responsible for bitterness. However, none of these bitterness models explain why certain proteases, such as Flavourzyme, do not produce bitter hydrolysates. Further research on the bitterness of protein hydrolysates coupled with valid sensory analysis is still needed.

REFERENCES

Aaslyng MD, Poll L, Nielsen PM, Flyge H. 1999. Sensory, chemical and sensometric studies of hydrolyzed vegetable protein produced by various processes. Eur Food Res Technol 209:227-36.

Adler-Nissen J. 1986. Methods in food protein hydrolysis. In: Adler-Nissen J, editor. Enzymic hydrolysis of food proteins. New York: Elsevier. p 110-31.

Adler-Nissen J. 1976. Enzymatic-hydrolysis of proteins for increased solubility. J Agric Food Chem 24(6):1090-3.

Arai S, Noguchi M, Kurosawa S, Kato H, Fujimaki M. 1970. Applying proteolytic enzymes on soybean. Part 6. Deoderization effect of aspergillopeptidase-A and debittering effect of aspergillus acid carboxypeptidase. J Food Sci 35(4):392-5.

Arnoldi A, D'Agostina A, Boschin G, Lovati MR, Manzoni C, Sirtori CR. 2001. Soy protein components active in the regulation of cholesterol homeostasis. Biologically active phytochemicals in food. R Soc Chem Spec Pub 269:103–6.

Bakhit R, Kelin B, Essex-Sorlie D, Ham J, Erdman J, Potter S. 1994. Intake of 25 g of soybean protein with or without soybean fiber alters plasma lipids in men with elevated cholesterol concentrations. J Nutr 124:213-22.

Bartoshuck L, Duffey V, Miller I. 1994. PTC/PROP tasting: anatomy, phychophysics, and sex effects. Physiol Behav 56:1165-71.

Bigelow C.C. 1967. On the average hydrophobicity of proteins and the relation between it and protein structure. J Theor Biol 16:187-211.

Bindels J. 1992. Peptiden in de Voeding. Voeding 53(10)253-7.

Cho M, Unklesbay N, Hsieh F, Clarke A. 2004. Hydrophobicity of bitter peptides from soy protein hydrolysates. J Agric Food Chem 52:5895-901.

Clemente A. 2000. Enzymatic protein hydrolysates in human nutrition. Trends Food Sci Technol 11:254-62.

Cordle C. 1994. Control of food allergies using protein hydrolysates. Food Technol 48(10):72-6.

Cornelis M, El-Sohemy A, Campos H. Genetic polymorphism of the adenosine A2A receptor is associated with habitual caffeine consumption. Am J Clin Nutr 86(1):240-4.

de Lumen BO. 2005. Lunasin: a cancer-preventive soy peptide. Nutr Rev 63(1):16-21.

Delwiche J, Buletic Z, Breslin P. 2001. Covariation in individuals' sensitivities to bitter compounds: evidence supporting multiple receptor/transduction mechanisms. Percept Psychophys 63(5):761-76.

Endres J. 2001. Definitions and methods of preparation. In: Endres J, editor. Soy protein products: characteristics, nutritional aspects, and utilization. Champlain, Ill.: AOCS Press. p 4-9.

FitzGerald RJ, O'Cuinn G. 2006. Enzymatic debittering of food protein hydrolysates. Biotechnol Adv 24(2):234-7.

Franek F, Hohenwarte O, Katinger H. 2000. Plant protein hydrolysates: preparation of defined peptide fractions promoting growth and production in animal cells cultures. Biotechnol Prog 16(5):688-92.

Frank O, Ottinger H, Hofmann T. 2001. Characterization of an intense bitter-tasting 1H,4H-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods. J Agric Food Chem 49:231-8.

Frøkjaer S. 1994. Use of hydrolysates for protein supplementation. Food Technol 48(10):86-8.

Fujimaki M, Yamashita M, Okazawa Y, Arai S. 1970. Applying proteolytic enzymes on soybean. Part 3. Diffusable bitter peptides and free amino acids in peptic hydrolysate of soybean protein. J Food Sci 35:215-8.

Gibbs B, Zougman A, Masse R, Mulligan C. 2004. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Res Int 37(2):123-31.

Guigoz Y, Solms J. 1976. Bitter peptides, occurrence and structure. Chem Senses Flavor 2(1):71-84.

Hamada J. 2000. Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. J Food Sci 65(2):305-10.

Hoogenkamp H. 2005. Soy protein essentials. In: Hoogenkamp H, editor. Soy protein and formulated meat products. Oxfordshire, UK: Cabi Publishing. p 7-18.

Iritani N, Hosomi H, Fukuda H, Tada K, Ikeda H. 1996. Soybean protein suppresses hepatic lipogenic enzyme gene expression in Wistar fatty rats. J Nutr 126:380-8.

Ishibashi N, Arita Y, Kanehisa H, Kouge K, Okai H, Fukui S. 1987. Bitterness of leucine-containing peptides. Agric Biol Chem 51(9):2389-94.

Ishibashi N, Kouge K, Shinoda I, Kanehisa H, Okai H. 1988b. A mechanism for bitter taste sensibility in peptides. Agric Biol Chem 52:819-27.

Ishibashi N, Kubo T, Chino M, Fukui H, Shinoda I, Kikuchi E, Okai H, Fukui S. 1988b. Taste of proline-containing peptides. Agric Biol Chem 52(1):95-8.

Jeong HJ, Park JH, Lam Y, de Lumen BO. 2003. Characterization of lunasin isolated from soybean. J Agric Food Chem 51(27):7901–6.

Kennedy AR.1995. The evidence for soybean products as cancer preventive agents. J Nutr 125:733–43.

Kennedy AR, Wan XS. 2002. Effects of the Bowman-Birk inhibitor on growth, invasion, and clonogenic survival of human prostate epithelial cells and prostate cancer cells. Prostate 50(2):125–33.

Kilara A. 1985. Enzyme-modified Protein Food Ingredients. Process Biochem 20:149-58.

Kilcast D. 2000. Sensory evaluation methods for shelf life assessment. In: Kilcast D, Subramaniam P, editors. The stability and shelf-life of food. Great Abington, U.K.: Woodhead Publishing. p 79-106.

Kim MR, Choi SY, Kim CS, Kim CW, Utsumi S, Lee CH. 1999. Amino acid sequence analysis of bitter peptides from a soybean progycinin subunit synthesized in *Escherichia coli.* Biosci Biotechnol Biochem 63(12):2069-74.

Kim SE, Kim HH, Kim JY, Kang YI, Woo HJ, Lee HJ. 2000. Anticancer activity of hydrophobic peptides from soy proteins. BioFactors 12(1–4):151–5.

Kim MR, Kawamura Y, Lee CH. 2003. Isolation and identification of bitter peptides of tryptic hydrolysate of soybean 11S glycinin by reverse-phase high-performance liquid chromatography. J Food Sci 68(8):2416-22.

Kim MR, Kawamura Y, Kim KM, Lee CH. 2008. Tastes and structures of bitter peptide, asparagines-alanine-leucine-proline-glutamate, and its synthetic analogs. J Agric Food Chem 56(14):5852-8.

Kinsella J. 1979. Functional properties of soy proteins. J Am Oil Chem Soc 56(3):242-58.

Kinsella J. 1981. Functional properties of proteins: possible relationships between structure and function in foams. Food Chem 7:273-88.

Kitts DD, Weiler K. 2003. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Curr Pharm Des 9(16):1309–23.

Kodera T, Nio N. 2002. Angiotensin converting enzyme inhibitors. PCT Int. Appl.WO 2002055546 A1 18 43 p. Kokai Tokkyo Koho.

Kodera T, Asano M, Nio N. 2006. Characteristic property of low bitterness in protein hydrolysates by a novel soybean protease D3. J Food Sci 71(9):609-14.

Korhonen H, Pihlanto A. 2003. Bioactive peptides: new challenges and opportunities for the dairy industry. Aust J Dairy Technol 58:128-34.

Kunst T. 2003. Protein modification in optimize functionality: protein hydrolysates. In: Whitaker J, Voragen A, Wong D, editors. Handbook of food enzymology. New York: Marcel Dekker. p 222-36.

Lahl W, Braun S. 1994. Enzymatic production of protein hydrolysates for food use. Food Technol 48:68-71.

Lawley R, Davis J, Curtis L. 2008. Non-biological contaminants. In: Lawley R, Davis J, Curtis L, editors. Food safety hazard guidebook. London: RSC Publishing. p 288-348.

Li C, Matsui T, Matsumoto K., Yamasaki R, Kawasaki T. 2002. Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein. J Pept Sci 8(6):267–74.

Li L. Yang Z, Yang X, Zhang G, Tang S, Chen F. 2008. Debittering effect of *Actinomucor elegans* peptidases on soybean protein hydrolysates. J Ind Microbiol Biotechnol 35:41-7.

Lioe H, Takara K, Yasuda M. 2006. Evaluation of peptide contribution to the intense umami taste of Japanese soy sauces. J Food Sci 71(3):277-83.

Lock S. 2007. Flavor characteristics of soy products modified by proteases and alpha-galactosidase. [MSc thesis]. Ames, IA.: Iowa State University. 95 p. Available from: Iowa State Park Library, Ames, IA.

Lopez-Bajonero L, Lara-Calderon P, Galvez-Mariscal A, Valazques-Arellano A, Lopwz-Munguia A. Enzymatic production of low-phenylalanine product from skim milk powder and caseinate. J Food Sci 56(4):938-42.

Lovsin-Kukman K, Zelenik-Blatnik, Abram V. 1995. Isolation of low-molecular-mass hydrophobic bitter peptides in soybean protein hydrolysates by reversed-phase high-performance liquid chromatography. J Chromatogr A 704:113-20.

Lovsin-Kukman K, Zelenik-Blatnik, Abram V. 1996. Bitterness intensity of soybean protein hydrolysates - chemical and organoleptic characterization. Z Lebensm Unters Forsch 203(3):272-6.

Lusas E, Riaz M. 1995. Soy protein products: processing and use. J Nutr 125(3):573-80.

Mahmoud M, Malone W, Cordle C. 1992. Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. J Food Sci 57(5):1223-9.

Matsui T, Matsufuji H, Seki E, Osajima K, Nakashima M, Osajima Y. 1993. Inhibition of angiotensin I-converting enzyme by Bacillus-licheniformis alkaline protease hydrolysates derived from sardine muscle. Biosci Biotechnol Biochem 57(6):922–5.

Matoba T, Hata H. 1972. Relationship between bitterness of peptides and their chemical structures. Agric Biol Chem 36:1423-31.

Meilgaard M, Civille G, Carr B. 2007. Measuring responses. In: Meilgaard M, Civille G, Carr B, editors. Sensory evaluation techniques. 4th ed. Boca Raton, Fla.: CRC Press. p 39-61.

Ney KH. 1971. Prediction of bitterness of peptides from their amino acid composition. Z Lebensm Unters Forsch 147(2):64-8.

Ney KH. 1972. Amino acid composition of proteins and bitterness of their peptides. Z Lebensm Unters Forsch 149:321–323.

Ney KH. 1979. Bitterness of peptides- amino acid composition and chain length. In: Boudreau JC, editor. Food Taste Chemistry (ACS Sym Ser 115). Washigton DC. Am Chem Soc 149-73.

Nishiwaki T, Yoshimizu S, Furuta M, Hayashi K. 2002. Debittering of enzymatic hydrolysates using an aminopeptidase from the edible basidiomycete *Grifola frondosa*. J Biosci Bioeng 93(1):60-3.

Nnanna I, Wu C. 2007. Dairy protein hydrolysates. In: Hui Y, editor. Handbook of food products manufacturing: health, meat, milk, poultry, seafood, and vegetables. New York: Wiley. p 537-91.

Olsman H. 1979. Hydrolyzed and autolyzed vegetable proteins as functional food ingredients. J Am Oil Chem Soc 56(3):375-6.

Pena-Ramos EA, Xiong YL. 2002. Antioxidant activity of soy protein hydrolysates in a liposomal system. J Food Sci 67(8):2952–6.

Pommer K. 1995. New proteolytic enzymes for the production of savory ingredients. Cereal Foods World. 40:745-8.

Riaz M. 2005. Processing of soybeans into ingredients. In: Riaz M, editor. Soy applications in food. Boca Raton, Fla.: CRC Press. p 41-62.

Ricks E, Ridling B, Iacobucci G, Myers D. 1978. Approaches to analyze and optimize protein hydrolyzates. In: Adler-Nissen J, editor. Biochemical aspects of new food proteins. New York: Pergamon. p 53-62.

Rutherfurd-Markwick K, Moughan P. 2005. Bioactive peptides derived from food. J AOAC Int 88(3):955-66.

Schmidl M, Taylor S, Nordlee J. 1994. Use of hydrolysate based products in special medical diets. Food Technol 48(10):77-85.

Seo W, Lee H, Baek H. 2008. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. J Food Sci 73:41-6.

Siemensma A, Weijer W, Bak H. 1993. The importance of peptide length in hypoallergenic infant food formulae. Trends Food Sci Technol 4:16-21.

Siemensma A, Kunst A. 1999. Sports nutrition: the recovery sports drink. World of Ingredients. October 53-6.

Soyfoods Association of North America. 2009. Sales and trends. Available from: www.soyfoods.org. Accessed Aug 10, 2009.

Synge R. 1945. The kinetics of low temperature acid hydrolysis of gramicidin and some related dipeptides. Biochem J 39:351-5.

Tanford C. 1962. Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. J Am Chem Soc 84:4240-7.

Tsumura K, Saito T, Tsuge K, Ashida H, Kugimiya W, Inouye K. 2005. Functional properties of soy protein hydrolysates obtained by selective proteolysis. Lwt-Food Sci Technol 38:255-61.

U.S. Soybean Export Council. 2008. Soy protein concentrate technical bulletin. Available from: www.ussoyexports.org/resources/SPCforaquaculture.pdf. Accessed Aug 10, 2009.

Utsumi S, Kinsella J. 1985. Forces involved in soy protein gelation: effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S and soy isolate. J Food Sci 50(5):1278–82.

Utsumi S, Matsumura Y, Mori T. 1997. Structure-function of soy proteins. In: Damodaran S, Paraf A, editors. Food proteins and their applications. Boca Raton, Fla.: CRC Press. p 257-92.

Vasconcellos A, Oliveria C, Grassiano D, Sentos-Neto A. 1992. Adsorption of phenylalanine from casein hydrolysates. Appl Biochem Biotechnol 37:69-80.

Wang W, Gonzalez de Mejia E. 2005. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. Compr Rev Food Sci F 4(4):63-78.

Whitaker J. 2003. Proteolytic enzymes. In: Whitaker J, Voragen A, Wong D, editors. Handbook of food enzymology. New York: Marcel Dekker. p 993-1018.

Wu J, Ding X. 2001. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. J Agric Food Chem 49(1):501-6.

Yamashita M, Arai S, Fujimaki M. 1969. Applying proteolytic enzymes on soybean. Part 4. A ninhydrin-negative bitter peptide in peptic hydrolysate of soybean protein. Agric Biol Chem 33(3):321-30.

Yoshikawa M, Takahashi M, Yang S. 2003. Delta opioid peptides derived from plant proteins. Curr Pharm Des 9(16):1325-30.

Young VR. 1991. Soy protein in relation to human protein and amino acid nutrition. J Am Diet Assoc 91:828-35.

CHAPTER 2. BITTERNESS IN SOY PROTEIN HYDROLYSATE ACCORDING TO MOLECULAR WEIGHT

A paper to be submitted to the *Journal of Food Science.*

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ABSTRACT

Bitterness of soy protein hydrolysates is a major obstacle to acceptance of soy products. The objective of this sensory evaluation research was to compare the bitterness of Protex 7L-treated soy hydrolysate and 4 fractions of varying molecular weight. A fraction of low molecular weight peptides (estimated 1-5 kDa) and the unfractionated hydrolysate were identified as bitter, $p = 0.009$ and $p = 0.088$, respectively. Cohen's kappa coefficient showed fair agreement between panelist sensitivity to soy hydrolysate and leucine, but poor agreement to caffeine, quinine, and phenylalanine. Panelist sensitivity to soy hydrolysate by Fisher's exact test was independent of sensitivity to caffeine, quinine, leucine, and phenylalanine. McNemar's test indicated that bitterness in leucine and phenylalanine was different than the bitterness in soy hydrolysate. Free hydrophobic amino acids may not be responsible for the bitterness of soy hydrolysate. Small peptides may be a better standard than caffeine or quinine for bitterness training.

INTRODUCTION

Soy protein hydrolysates have many advantages over the intact soy proteins in terms of improved functional properties. Proteolytic enzyme hydrolysis of soy proteins is widely used in many food applications to modify the solubility, viscosity, emulsification, and gelation properties. The degree of hydrolysis determines the size of peptides in a

hydrolysate, and is greatly influenced by the product application. Larger peptides of 2-5 kDa are ideal for functional food ingredients, medium-sized peptides of 1-2 kDa are ideal for sports nutrition (Frøkjaer 1994; Siemensma and Kunst 1999) and clinical nutrition (Schmidl and others 1994), while smaller peptides of < 1kDa are ideal for hypoallergenic infant formulas (Siemensma and others 1993).

However, a major objective limiting consumer acceptance of soy hydrolysate products is their bitter taste. Bitterness is often attributed the formation of low molecular weight peptides made up of primarily hydrophobic amino acids (Matoba and Hata 1972), although an exact cause is not fully understood and conflicting research exists in the literature. The hydrophobicity, primary sequence, spatial structure, molecular weight, and bulkiness of the peptide have been studied as possible influences in the bitter taste of hydrolysates (Kim 2008).

Many studies on the bitterness of soy hydrolysates vary in method of sensory evaluation. Taste dilution analysis based on compound thresholds in serial dilutions (Lioe and others 2006; Seo and others 2008), category scaling (Lovsin-Kukman and others 1995; Nishiwaki 2002), caffeine equivalency methods (Cho and others 2004; Kodera and others 2006), line scaling (Aaslyng and others 1999; Kim and others 1999, 2003), and phenylthiourea comparison (Yamashita 1969; Fujimaki and others 1970) are some of the sensory methods that have been used. The number of panelists and the presence or absence of screening for bitterness sensitivity varies greatly in published literature and is important when assessing the validity of the research. Most studies on the bitterness evaluation of soy hydrolysates use either caffeine (Cho and others 2004; Kodera and others 2006; Seo and others 2008) or quinine (Kim and others 1999, 2003, 2008; Li and others 2008) as a

bitterness standard. However, previous studies have not looked at the correlation of sensitivity to caffeine or quinine in relation to soy hydrolysate.

The purpose of this study was to evaluate the bitterness of different molecular weight fractions obtained from Protex 7L-treated soy hydrolysate and compare various bitter compounds for differences in sensitivity and bitterness in relation to soy hydrolysate by a screened panel.

MATERIALS AND METHODS

A challenge in this research was ensuring all protein hydrolysis, fractionation, and concentration procedures were food-grade. The research was performed in a food-grade laboratory using a food grade enzyme. The compounds in the gel filtration buffer were adjusted to be safe for use in foods, and nearly all of these compounds were filtered out of the samples during ultrafiltration. It was also important to limit microbial growth. The gel filtration buffer was filtered prior to loading onto the column to limit initial load of microorganisms. Both the gel filtration column and buffer were refrigerated between uses, and all hydrolysate fractions were stored at -20˚C. These adjustments for food-grade procedures will be discussed in detail throughout this section.

Theoretical Analysis

The goal of the theoretical analysis was to determine how differences in hydrolytic cleavage between 3 different proteases at 4% degree of hydrolysis (DH) influence the bitterness of soy protein hydrolysate. Bromelain (BR)-treated soy protein, which does not differ in bitterness from a control soy protein, was compared to Multifect Neutral (MN) (Genencor International Inc., Rochester, N.Y., U.S.A.) and Experimental Exopeptidase-C

(EEC) (Genencor International Inc., Rochester, N.Y., U.S.A.), which are bitter tasting (Lock 2007).

Proteolytic hydrolysis

The objective of the proteolytic hydrolysis was to obtain 4% DH of soy protein treated with a protease known to produce bitter-tasting hydrolysate. DH describes the extent to which peptide bonds are hydrolyzed by the enzyme reaction and is measured as the percentage of cleaved peptide bonds out of the total number of peptide bonds present in the intact protein. DH is calculated by the following formula: $DH = [V_{NaOH} * N_{NaOH}) / (\alpha * MP * h_{tot})] * 100\%$ (Adler-Nissen 1986). V_{NaOH} is the volume of NaOH added by the pH-stat in mL, N_{NaOH} is the normality of the NaOH, α is the dissociation of the α-amino groups, MP is the mass of the protein in grams, and h_{tot} is the total number of peptide bonds in the protein substrate (meqv/g protein). A value of 0.44 for α describes the degree of dissociation of the α-amino groups at the hydrolysis temperature of 50˚C and pH 7.0. The h_{tot} value for soy protein is 7.8 (Adler-Nissen 1986).

Profam 825 (ADM Co., Decatur, Ill., U.S.A.), a commercially available soy protein isolate (SPI), was used as the substrate for the hydrolysis. Profam 825 has a proximate moisture content of 3.9% at pH 7.0 and 3.6% at pH 5.4 on a dry protein content basis, with a fat content < 4%. The protein dispersibility index was 66.2, determined by Eurofins Scientific Inc. (Des Moines, Iowa, U.S.A.).

A 10% (w/w) suspension of Profam 825 in distilled water was prepared at 50˚C and the pH was adjusted to 7.0 with 2N NaOH. The suspension was held at a constant temperature of 50˚C in a water bath with stirring for 10 min. Protex 7L was added to the suspension at a 1:10 enzyme-to-substrate ratio (E/S), expressed as grams of enzyme per gram

of protein. A pH stat (718 Titrino, Metrohm Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.) was used to moniter the hydrolysis. The pH stat added NaOH to maintain a constant pH of 7.0 as hydrogen ions were released due to enzyme action on the protein. The volume of NaOH added corresponded to % DH according to the DH formula (Adler-Nissen 1986). To inactivate the enzyme at 4% DH, the hydrolysate was adjusted to pH 5.8 with 3N citric acid and chilled in an ice water bath for 20 min. Three replications of hydrolysates were produced. The hydrolysate was stored at -20˚C.

Although each replication was calculated to reach 4% DH, the length of time and the amount of enzyme necessary to achieve 4% DH varied. A malfunction of the pH stat was suspected due to noticeable corrosion of machine parts by NaOH, disagreement between the monitor reading and the amount of NaOH being dispensed, and failure to operate after the replications were completed. To determine whether malfunction occurred, the 3 replications of Protex 7L hydrolysates were analyzed for similarity in protein amount and peptide size. If all the replications were the same, this would indicate the pH stat was working correctly and all the replications were at 4% DH. If the replications were not the same, this would indicate a pH stat malfunction in which the hydrolysates were not correctly hydrolyzed to 4% DH. Although differing DHs would not mean the results are invalid, an exact % DH could not be stated and the replications would not be as similar in hydrolysate composition as desired.

The Biuret assay (Gornall and others 1949) was completed to determine the amount of soluble protein present in each replication. The Biuret assay causes polypeptides and proteins with at least 2 peptide bonds to turn purple when treated with dilute copper sulfate in an alkaline solution. The color change results from the formation of a complex of a copper(II) ion with 4 nitrogen atoms, 2 from each of the 2 peptides. In this method, 4.0 mL

of Biuret reagent was added to 1 mL of sample, mixed, and allowed to stand for 30 min. The absorbance of each sample was read at 540 nm and compared to a standard curve of known dilutions of 10.0 mg/mL bovine serum albumen (BSA) (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.) to determine protein concentration.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to compare the molecular weight of peptides for each hydrolysate replication. SDS-PAGE is a technique used to separate proteins based on molecular weight.

Gel filtration

The hydrolysate was separated using a 2.5 cm × 100 cm gel filtration column (Pharmacia Biotech, Uppsala, Sweden) packed with Sephacryl 200-HR (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.) at 20˚C by gravity to separate peptide fractions by molecular size. Between gel filtration runs, the column was stored at 4˚C to inhibit microbial growth.

Sucrose (0.5 g) was run through the gel filtration column to determine V_T, the total amount of buffer necessary to elute all components. The Dubois method for carbohydrate detection (Dubois and others 1956) was performed to determine which test tube samples contained sucrose. In this method, 1 mL of sample was mixed with 0.2 mL of 6% phenol solution and 1 mL of concentrated sulfuric acid. After 20 min, the absorbance of samples was measured at 490 nm. Dilutions of 5 mg/mL D-mannose were used as standards. The total volume of buffer before sucrose eluted was calculated to determine the total amount of eluant necessary to pass through the column. Sucrose was used because it is a food-grade alternative to gel filtration molecular weight markers. Sucrose has a molecular weight of 365 Da, which would elute at a similar volume on the column as an average peptide with 8 amino acid residues.

A buffer consisting of 1.4 mM sodium bisulfate, 2.6 mM monopotassium phosphate, 9.2 mM sodium sulfite, 33 mM dipotassium phosphate, and 400 mM sodium chloride at pH 7.6 was used. β-Mercaptoethanol, which is commonly used in eluting buffer as a reducing agent and has microbial growth inhibition properties, could not be used because it was not food-grade. Sodium bisulfate was used as a food-grade alternative to limit microbial growth and act as a reducing agent. The buffer was filtered by a 47-mm glass vacuum filtration system (Millipore Corp., Bedford, Mass., U.S.A.) under a 0.2-µm filter to limit the initial microbial load before elution on the column.

To ensure the hydrolysate moved efficiently through the column, the hydrolysate was adjusted so it had similar composition and ionic strength as the eluting buffer. All compounds present in the buffer were added to the hydrolysate at equal concentrations and the pH of the hydrolysate was adjusted to 7.6. The hydrolysate was centrifuged at 10,000 X g for 15 min at 20˚C. The supernatant, which contained the soluble protein, was retained and the precipitate was discarded. The hydrolysate (0.5 mL) was loaded onto the column for each gel filtration run. Gel filtration was performed at 20˚C and the gel was refrigerated at 4˚C between runs.

The hydrolysate was filtered through the column and 5-9 mL of sample was collected in each test tube, depending on the flow rate of the column. A Retriever 500 fraction collector (Teledyne Isco Inc., Lincoln, Neb., U.S.A.) was used to collect samples. Initially, the Folin-Lowry method (Lowry and others 1951) was performed to determine the amount of protein present in each test tube. The Folin-Lowry method is similar to the Biuret procedure, but is a more sensitive method of protein determination. In this method, Lowry Reagent B was prepared with 50 mL of 0.1 M sodium hydroxide containing 2% sodium carbonate, 1.0

mL of 0.5% copper sulfate pentahydrate, and 1.0 mL 1% sodium potassium tartrate. Lowry Reagent B (5.0 mL) was added to 0.5 mL of sample and allowed to stand for 10 min. Lowry Reagent A (0.5 mL), consisting of Folin-Ciocalteu's phenol reagent 1:1 with water, was mixed with the sample and allowed to sit for 30 min. The absorbance of the samples was read at 750 nm and compared to a standard curve using 300 µg/mL BSA dilutions to determine protein concentration. Unfortunately, the Lowry method consumed sample needed for sensory evaluation. Conservation of the sample was important because only a limited amount of protein (0.5 g) could be loaded on the column for each gel filtration run. As an alternative to the Folin-Lowry method, the ultraviolet (UV) absorbance of the samples was measured at 254 nm using a Beckman DU 520 spectrophotometer (Beckman Coulter Inc., Fullerton, Calif., U.S.A). Aromatic amino acids are absorbed best at 254 nm (Randall and others 1991).

In addition to analyzing for protein content by UV absorbance, the free amino acid contents of the samples were determined by using the ninhydrin assay (Moore and Stein 1948). In this assay, ninhydrin reagent was added to all samples and the mixture reacted at 100˚C for 10 min. Absorbance values were read at 570 nm and compared to a standard curve of aspartic acid solutions to determine free amino acid concentration.

Fractions corresponding to UV absorbance peaks were determined in each chromatogram. Samples tubes corresponding to these fractions were combined for each gel filtration run. A standard molecular weight kit with molecular markers 12.4 kDa (cytochrome c from horse heart), 29 kDa (carbonic anhydrase from bovine erythrocytes), 66 kDa (BSA), 150 kDa (alcohol dehydrogenase from yeast), and 200 kDa (β-amylase from sweet potato) was used to calibrate the elution rates (Sigma-Aldrich Corp., St. Louis, Mo.,

U.S.A.). Blue Dextran, a very high molecular weight gel filtration marker, was used to determine the void volume. Void volume (V_o) is the amount of buffer needed to elute from the column before a very large molecular weight compound, such as Blue Dextran, elutes. Elution volume (V_e), the amount of buffer necessary to elute before known protein standards elute, was also calculated. A standard curve of molecular weight verses V_e/V_o was produced.

Ultrafiltration

Pooled fractions were ultrafiltered under nitrogen gas by a 44.5-mm diameter ultrafiltration stirred cell (Millipore Corp., Bedford, Mass., U.S.A.) to concentrate the samples and remove the buffer components, which would otherwise impart an extremely salty taste to the hydrolysate fractions. Saltiness from the high concentration of sodium chloride in the unfiltered buffer was overpowering and masked other flavors in the hydrolysates. Peptides with molecular weight smaller than membrane pores diffuse while peptides larger than the membrane pores are retained. Ultrafiltration was performed at 4˚C. Figure 4 illustrates a diagram of the ultrafiltration process.

A 1-kDa nominal molecular weight limit (NMWL) regenerated-cellulose filter (Millipore Corp., Bedford, Mass., U.S.A.) was used to retain protein > 1 kDa, while diffusing the buffer salts. Although it would have been ideal to use a filter which retained peptides < 1 kDa, this was the lowest molecular weight ultrafiltration filter commercially available. After ultrafiltration, each fraction was boiled at 100˚C for 10 min to eliminate any harmful microbial growth. Samples were stored at 4˚C.

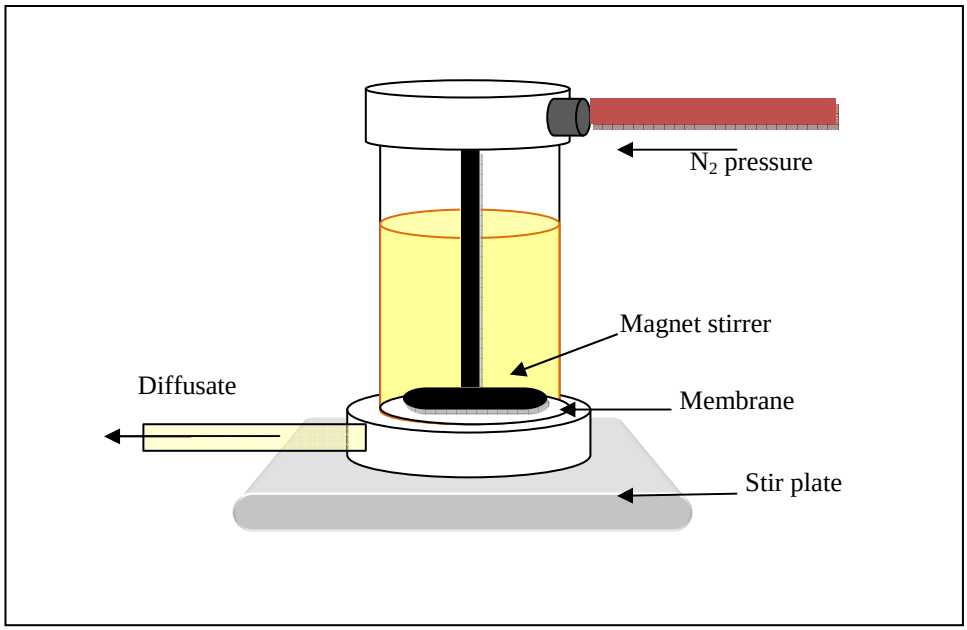

Figure 4. Diagram of an ultrafiltration stirred cell with sample under nitrogen pressure. Particles with molecular weight smaller than membrane pores diffuse while particles larger than the membrane pores are retained.

Freeze-drying

The retentate from the 3 pooled fractions, the diffusate from the lowest molecular weight pooled fraction, as well as the unfractionated hydrolysate were placed in the freeze-dryer (FreeZone 4.5, Labconco Corp., Kansas City, Mo., U.S.A.). The mass of each dried fraction was recorded after freeze-drying. Freeze-dried samples were stored in glass containers covered in Parafilm (Pechiney Plastic Packaging Co., Chicago, Ill., U.S.A.) at 4˚C.

Gel electrophoresis

SDS-PAGE was performed to determine the molecular weight (MW) of fractions 1-4 as well as the unfractionated Protex 7L soy hydrolysate according to the methods of Laemmli (1970). Different concentrations of resolving gels were made to obtain a gel with optimal resolution of the hydrolysate. SDS-PAGE was performed using a SDS-Tris–glycine buffer

system with 4% stacking gel and either 8-18 gradient, 13, 15, or 18% resolving gel (Bio-Rad, Hercules, Calif., U.S.A.). Coomassie blue or silver staining according to the method of Morrissy (1981) was used to visualize the protein bands. Standards included a low-range MW marker from 66- 6.5 kDa (M3913; Sigma, St. Louis, Mo., U.S.A.), a low-range MW silver stain marker from 97.4- 14.4 kDa (161-0314; Bio-Rad, Hercule, Calif., U.S.A.) an ultra-low MW marker from 26.6- 1.06 kDa (M3546; Sigma, St. Louis, Mo., U.S.A.), and Profam 825.

Recruitment of panelists for sensory evaluation of hydrolysate fractions

Panelists were recruited from Iowa State University students and faculty by email message (Appendix A). They were selected based on availability, interest, and lack of allergies to soy protein. The study was approved for human subjects by the Iowa State University Institutional Review Board (IRB), and each panelist consented to take part in this research study (Appendix B).

Training of sensory evaluation panelists

The goal of sensory training was to find optimal procedures (tasting small quantities and cleansing) and evaluate testing methods (3-Alternate forced choice, magnitude estimation, and modified triangle test) of soy hydrolysate and its separated fractions. Initial sensory training was conducted over a period of 6 wks with 11 panelists. Panelists were evaluated for bitterness sensitivity with various bitter compounds including caffeine, quinine, tea, and unsweetened chocolate. Caffeine purchased from Sigma-Aldrich Corp. (St. Louis, Mo., U.S.A.) was chosen as a reference for bitterness in training sessions.

Threshold test. Each panelist completed two 3-alternate forced choice (3-AFC) threshold test to determine his/her caffeine threshold (Appendix C). Samples were presented to the panelists in 3 mL amounts in ascending concentration from 40 to 500 mg/L.

Magnitude estimation. Panelists were trained in the magnitude estimation sensory method in which a 1 g/L caffeine standard was used to represent 100 for bitterness. Panelists were given samples and asked to assign a bitterness value relative to the standard. For example, if the sample was half as bitter as the standard, it would be given a value of 50. Unfortunately, this technique was abandoned after determining panelists could not determine magnitude with the very small sample amounts of hydrolysate available.

Modified triangle test. As an alternative, a modified version of the triangle test was used to evaluate both sensitivity and bitterness. Panelists were first presented with samples in a triangle test and asked to identify the odd sample. Secondly, they were to decide whether the odd sample was bitter or was different due to another sensory attribute. For simplicity, this test will be referred to as the "bitter/not bitter test" throughout the paper (Appendix D).

Tasting small quantities. Tasting small quantities was necessary because only a limited amount of sample could be fractionated by gel-filtration for optimal separation. Panelists were trained in bitterness detection of small sample quantities (Kim and others 2008, Lee 1996, Weiffenbach and others 1983) by using a medicine dropper (2 mL). The panelists determined they needed a minimum of 2-3 drops (125-188 µL) to detect bitterness. After discussion of various procedures during training, panelists determined the procedure for evaluation. The drops of sample were placed at the back of the tongue and pushed up

towards the roof of the mouth. Panelists were instructed to wait at least 15 sec before responding.

Palate cleansing procedures. Palate-cleansing was especially important because bitterness has a lingering effect and should not carry-over to the next sample. Panelists experimented with cleansing by using a rinse of distilled or tap water, a 0.10% solution of carboxymethylcellulose (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.), eating unsalted saltine cracker, and a combination of methods. Panelists determined that cleansing with an unsalted saltine cracker and tap water was the best method. Before tasting a sample, a cracker was eaten and the mouth was rinsed with water. Panelists waited a total of 30 sec after cleansing to evaluate the sample, and the procedure was repeated for each sample. A waiting period of 1 min was required between each set of triangle test samples.

Although training was helpful in developing tasting and palate-cleansing procedures, it was evident that there was a wide range of bitter sensitivities among the panelists in the training group and that each panelist's bitterness threshold could not be changed with additional training. This supported the fact that sensitivity to bitter-tasting compounds is genetic and inherent for each individual (Cornelis and others 2007). A panel of only those highly sensitive to bitterness would improve the consistency of bitter responses during evaluation while using minimal amount of sample. Screening a larger number of panelists and selecting those most sensitive to bitterness would be a better method for this study because of the small amount of sample available.

Screening of panelists

A total of 25 panelists were screened over 4-6 sessions for bitterness sensitivity. Each panelist filled out a survey on his/her age, gender, ethnicity, and food consumption

habits. Panelists completed 3-AFC threshold tests for caffeine (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.), quinine sulfate (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.), and MN-treated soy hydrolysate (prepared by Lock, 2007, Iowa State University). Screening with MN-treated soy hydrolysate helped determine which panelists were sensitive at a 5% (w/v) hydrolysate/water solution (the concentration of Protex 7L fractions during evaluation). Both caffeine and quinine were used in screening to determine whether panelists were consistently sensitive to both compounds or highly sensitive to one but not the other.

Panelists evaluated 5 sets of caffeine solutions ranging from 50-400 mg/L, 4 sets of quinine solutions ranging from 2.3-14 μM, and 4 sets of MN soy protein hydrolysate ranging from 5-8% (w/v) in ascending order of concentration. Each set was served in randomized order labeled with 3-digit random codes. Samples were served at room temperature (20 ± 3ºC). Water was used as the control for caffeine and quinine solutions, while Profam 825 SPI was used as the control for the MN soy hydrolysate solutions. Panelists tasted 0.5 mL of solution contained in microcentrifuge tubes using an eyedropper to place 2-3 drops on the tongue. It was necessary for panelists selected from the screening process to be sensitive at small volumes due to the small quantity of Protex 7L soy hydrolysate fractions available for evaluation. Between samples, the eyedropper was rinsed at least twice in water. Panelists cleansed their palate with a portion of unsalted saltine cracker and water, and waited 30 sec between samples and 1 min between sets.

Fifteen panelists were selected from the screening process. Generally, 20-40 panelists are optimal for a triangle test (Meilgaard and others 2007). However, 15 panelists were employed in this study due to the selective nature of the screening process as well as time and budget constraints. Panelists selected for the bitterness evaluation panel met at least

one of the following requirements: caffeine threshold ≤ 150 mg/L, quinine threshold ≤ 6 µM, or MN soy hydrolysate threshold ≤ 5% (w/v). The caffeine and quinine threshold requirements were chosen because they are less than the reference threshold values for the general population (Delcour and others 1984) and include the highest one-third of panelist sensitivities determined in screening of each compound.

Sensory evaluation

Panelists (15) evaluated 4 sets of leucine solutions ranging from 3.8-22.8 µM and 4 sets of phenylalanine solutions ranging from 6.1-24.2 µM by the bitter/not bitter test twice for each compound. These tests supplemented the evaluation of caffeine, quinine, and MN soy hydrolysate completed during screening. Two replications provided an average threshold for each panelist and were sufficient since panelists tended to be consistent in their responses. These tests provided threshold data and bitterness perception data for each compound and for all panelists. The soy hydrolysate fractions and unfractionated hydrolysate were evaluated in one session at 5% (w/v) in Milli-Q water obtained from a Milli-Q SP reagent water system (Millipore Corp., Bedford, Mass., U.S.A.). Milli-Q water served as a control in the bitter/not bitter test for the hydrolysate.

Sensory evaluation was conducted in the Sensory Evaluation Unit, Human Nutritional Sciences Bldg, Iowa State University. Evaluation took place in the sensory testing facility consisting of 10 individual computerized booths using the CompuSense™ Five computer program (version 4.4.8, Guelph, Ontario, Canada). White incandescent lighting was used in the evaluation of caffeine, quinine, leucine, and phenylalanine. Red lighting was used to mask the slight color differences MN soy hydrolysate and Protex 7L soy hydrolysate.

Statistical Analysis

Protex 7L hydrolysate bitterness. Results from the sensory evaluation of unfractionated Protex 7L hydrolysate and its respective fractions were analyzed for significance based on the critical number of correct responses in a triangle test (Meilgaard and others 2007). The p value was calculated with a one-tailed binomial test based on the null hypothesis that the probability of selecting the correct sample by chance is 1/3 (O'Mahony 1986).

Comparison of sensitivity. In order to compare sensitivity to soy hydrolysate in relation to sensitivity to caffeine, quinine, leucine, and phenylalanine, threshold data from each panelist was converted into yes/no sensitivity data. A few panelists in the bitter-sensitive group were highly sensitive to all compounds during screening, but most were highly sensitive to certain ones and not as sensitive to others. A “yes” meant the panelist was sensitive to the particular compound, while a “no” meant he/she was not sensitive to that compound. Sensitive panelists had thresholds lower than the literature average threshold for that compound.

Cohen's kappa coefficient and Fisher’s exact test were used to analyze panelist sensitivity to soy hydrolysate in relation to sensitivity to caffeine, quinine, leucine, and phenylalanine. The statistical analysis was analyzed at $p \leq 0.05$ using the PROC FREQ procedure in SAS statistical program (version 9.1, SAS Institute, Inc., 2007). Contingency tables showing yes/no sensitivity data in a tabular form were produced along with the analysis. An example of a contingency table is shown in Figure 5, in which panelist responses to two questions are compared.

Response to question #1

Response to question #2	No	Yes	Total
No	5	3	8
Yes	3	4	7
Total	8	7	15

Figure 5. Example of a 2x2 contingency table

Cohen's kappa coefficient is a measure of inter-rater agreement between categorical groups while accounting for agreement expected by chance alone. For the example shown in Figure 5, the kappa coefficient would measure the amount of agreement in panelist responses to both questions while accounting for the number of similar responses expected by chance. The kappa value ranges from 0 to1, with 0 indicating low agreement and 1 indicating perfect agreement (Kline 1995).

The Fisher's exact test is used in place of the chi-square test when sample sizes are small. It is used in the analysis of contingency tables with two nominal variables and is based on the null hypothesis that the relative proportions of one variable are independent of the second variable (McDonald 2008). For the example shown in Figure 5, a statistically significant result would indicate that the responses to question #1 and question #2 are not independent of one another. The probability is two tailed in this study's comparison of sensitivity because both bitter sensitive and not sensitive categories are important.

Comparison of bitterness. To determine whether the bitterness in leucine or phenylalanine resembled the bitterness in soy hydrolysate, yes/no bitterness data for leucine,

phenylalanine, and soy hydrolysate were produced based on panelist responses to the bitter/not bitter test. A "yes" meant the panelist considered the compound bitter, while a "no" meant he/she did not.

Cohen's kappa coefficient and McNemar's test were used to analyze the bitter/not bitter designation in leucine or phenylalanine compared to soy hydrolysate. The statistical analysis was analyzed at $p \leq 0.05$ using the PROC FREQ procedure in SAS statistical program (version 9.1, SAS Institute, Inc., 2007). Contingency tables showing yes/no bitterness perception data in a tabular form were produced along with the analysis. McNemar's test is used to compare two population proportions that are related to each other. It is based on the null hypothesis that two proportions are equal in paired data (Petrie and Watson 2006). The concordant responses, such as Yes/Yes and No/No, are ignored. In the example shown in Figure 5, McNemar's test would test whether the proportion of panelists who responded No/Yes and Yes/No to question #1 and question #2 are equal. This eliminates responses from panelists who have a tendency to evaluate one way, such as rating not bitter for all compounds in this study (personal communication with Dr. Philip Dixon May 18, 2009).

RESULTS AND DISCUSSION

Prior to laboratory research, a theoretical analysis was conducted in order to predict differences in hydrolytic cleavage sites between 3 different proteases in soy protein at 4% DH and hypothesize how these differences influence bitterness. In 2007, Lock reported that Genencor Multifect Neutral and Genencor Experimental Exopeptidase-C (EEC) produced bitter soy hydrolysates while bromelain did not. The theoretical analysis of differences in hydrolysis sites between these proteases led to our hypothesis: low molecular weight peptides

are responsible for the bitterness of bitter-tasting soy hydrolysates. The reasoning for our hypothesis which led to the present study is summarized by Figure 6. To test this hypothesis, a soy hydrolysate (prepared with a protease similar to Multifect Neutral and assumed to produce a bitter product) was separated into different molecular weight fractions and evaluated for bitterness by sensory panelists. This section describes the procedures for this research in detail.

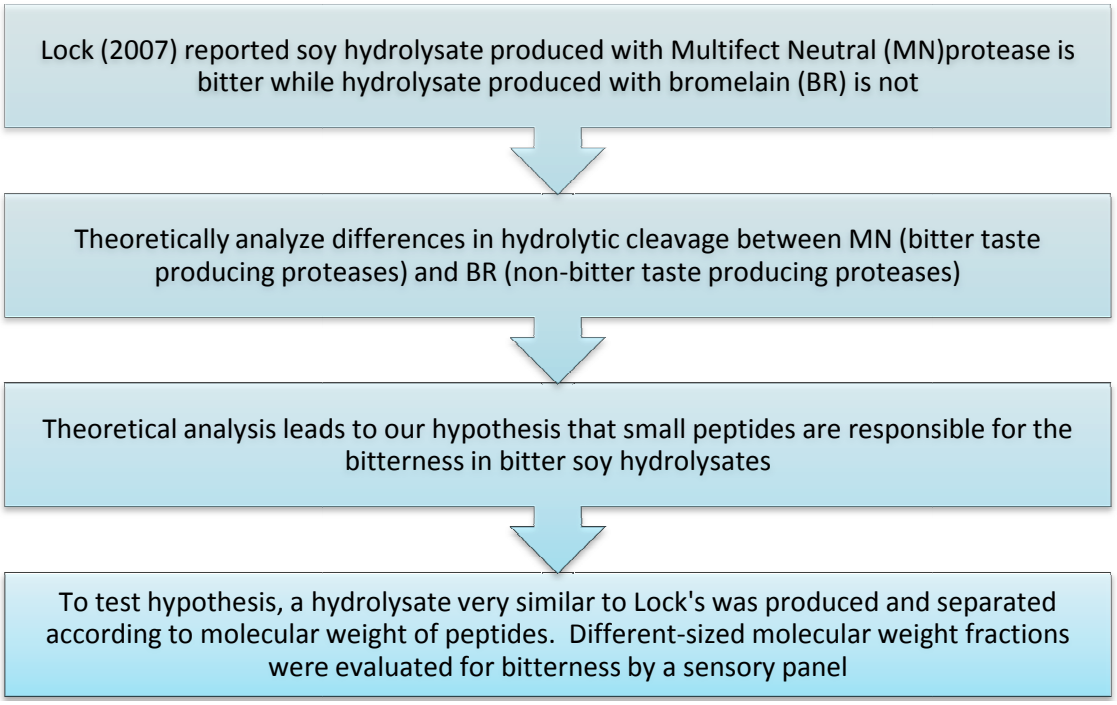

Figure 6. Reasoning for our hypothesis and materials and methods of this research

Theoretical Analysis

The proteases in this theoretical analysis included fruit bromelain (BR), Genencor Multifect Neutral (MN), and Genencor Experimental Exopeptidase-C (EEC). These proteases were chosen because they were studied in previous research (Lock 2007), which

concluded that soy hydrolysates treated with BR at 4% DH did not differ in bitterness from a control soy protein, while hydrolysates treated with MN and EEC at 4% DH were significantly more bitter.

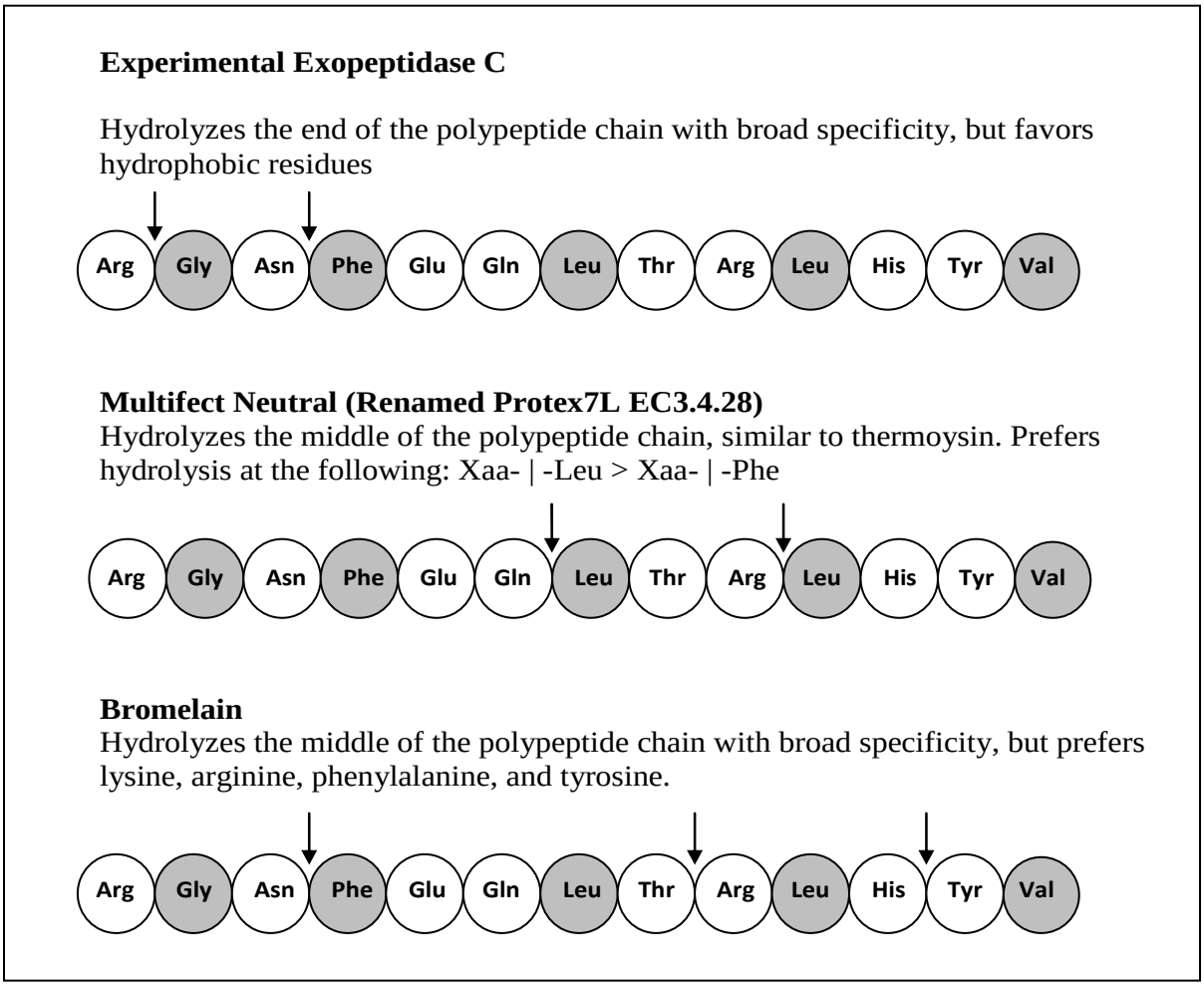

Figure 7. Hydrolytic cleavage preference for Experimental Exopeptidase C, Multifect Neutral, and Bromelain proteases. Shaded amino acid residues represent hydrophobic amino acids.

BR and MN are endoproteases, which hydrolyze peptide bonds randomly from the middle of the polypeptide chain. EEC is an exoprotease, which hydrolyzes peptide bonds from the end of the polypeptide chain (Figure 7). BR has broad specificity, but prefers to

cleave the peptide before lysine, arginine, phenylalanine, and tyrosine residues. MN also has broad specificity, but prefers to cleave before leucine residues. EEC also has broad specificity, but prefers to cleave before hydrophobic amino acids.

Several assumptions were made (Table 8). The proportion of soy storage proteins, glycinin to β-conglycinin, was assumed to be equal for this analysis. In actuality, glycinin content is slightly larger, but varies according to soybean variety. Carrão-Panizzi and others (2008) reported the ratio of glycinin/β-conglycinin ranged from 1.17 to 2.78 in 90 Brazilian soybean cultivars. Based on 0.5 glycinin/β-conglycinin ratio and 4% DH, 2% DH was assumed for glycinin and 2% DH for β-conglycinin. Since glycinin has 5 subunits, each subunit would have 0.4% DH. Each of β-conglycinin's 3 subunits would have 0.67% DH. This calculation resulted in approximately 2 points of hydrolytic cleavage for each glycinin subunit and 3-4 points of hydrolytic cleavage for each β-conglycinin subunit, depending on the number of amino acid residues in the subunit. The proteases were assumed to attack only the acidic portions of the glycinin subunits because acidic portions are hydrolyzed first (Wilson and others 1988; Govindarajuk and Srinivas 2007).

Table 8. Assumptions for the theoretical analysis

#	Assumption	Reasoning
1	Proportion of glycinin to beta-conglycinin is equal	They are approximately equal, but may differ according to soybean variety[a]
2	2% DH for glycinin, 2% DH for β–conglycinin	Based on Assumption #1 and a 4% DH total, % DH would be half of the total (2%) each for glycinin and β–conglycinin
3	0.4% DH for each glycinin subunit, 0.67% DH for each β–conglycinin subunit	Based on Assumption #2, each of 5 glycinin subunits would go to 0.4% DH (2% DH/5) and each of 3 β–conglycinin subunits would go to 0.67% DH (2% DH / 3)
4	Proteases attack only the acidic portions of the glycinin subunits	Acidic portions are hydrolyzed first[b]
5	Proteases most likely to cleave random coils, rather than β-sheets or α-helices	Due to their structure, random coils would be easier for protease to attack[c]

[a] Carrão-Panizzi and others (2008)
[b] Wilson and others (1988), Govindarajuk and others (2007)
[c] Solgaard and others (2008)

Three-dimensional (3-D) structures for the subunits in soy protein, available from the online RCSB Protein Data Bank (RCSB 2009), were used to help limit the possibilities for hydrolysis based on structural constraints. Proteases would most likely cleave random coils rather than β-sheets or α-helices because they are readily exposed and easier to attack (Solgaard and others 2008). This assumption was used to reduce the number of sites predicted only from the primary structure. Unfortunately, 3-D structures through the Protein Data Bank were not available for the glycinin subunits $A_{1b}B_2$, A_2B_{1a}, and $A_5A_4B_3$, or the α subunit of β-conglycinin at the time of this study.

Based on the assumptions in Table 8, the sites of hydrolysis for EEC-treated soy protein could be predicted. It was not possible to specifically predict where the protease would attack at a 4% DH for the 2 endoproteases, BR and MN. The number of possible sites of hydrolysis, however, was narrowed based the assumptions in Table 8, protease preference of hydrolysis site, and 3-D modeling. Figure 8 illustrates the predicted hydrolysis sites of the 3 proteases using glycinin subunit A1aB1b as an example.

Figure 8. Predicted hydrolysis sites for Experimental Exopeptidase-C treated- (shown in red), Multifect Neutral treated- (blue), and bromelain treated- (green) glycinin subunit A1aB1b. Acidic portion shown in bold. Cleavage occurs at the right side (C-terminal direction) of marked amino acid. 3-D structure was available for this subunit to reduce number of possible hydrolytic cleavage sites.

Table 9 lists the number of possible hydrolysis sites which could be predicted based on the assumptions (Table 8) and hydrolysis preferences for each protease (Figure 7) as well

as the number of hydrolysis sites predicted at 4% DH for this subunit. BR was the most difficult protease to predict because of its broad preference for hydrolysis sites. For example, 51 possible sites of hydrolytic cleavage could be narrowed down for BR treated glycinin subunit A1aB1b, while only 2 hydrolytic sites were predicted at 4% DH (Table 9).

Table 9. Number of possible hydrolysis sites for protease treated glycinin subunit A1aB1b

Enzyme used for hydrolysis	# possible hydrolysis sites	# predicted hydrolysis sites at 4% DH
Experimental Exopeptidase-C (EEC)	2	2
Multifect Neutral (MN)	15	2
Bromelain (BR)	51	2

[a] based on assumptions for theoretical analysis and number predicted to occur at 4% DH

Although it was not possible to determine the specific points of hydrolysis for all proteases, the theoretical analysis revealed important differences in the hydrolysate products. BR-treated SPI, which does not differ in bitterness from untreated SPI (Lock 2007), had a much broader range of hydrolytic cleavage sites. This type of broad and random attack could tend produce peptides of similar molecular weight. It would not be likely for BR-treated hydrolysate to have very large or very small peptides with this type of hydrolysis preference. In contrast, it would be more probable for EEC- and MN-treated hydrolysates to produce a wider range of peptide sizes. The EEC and MN proteases were shown to produce bitter-tasting hydrolysates (Lock 2007). The fact that EEC is an exoprotease means that it would always produce very small and very large peptides since it hydrolyzes from the end of the

polypeptide chain. MN is more selective than BR, preferring to cleave at leucine residues (Figure 7). Since amino acids such as leucine do not occur at equally spaced intervals in protein, MN-treated hydrolysate would be more likely to have peptides of differing sizes. The theoretical analysis results support our hypothesis that low molecular weight peptides are responsible for bitterness. Hydrolysis by BR may not produce bitter hydrolysate due to the production of medium-sized peptides, while MN and EEC treated hydrolysates may produce bitter hydrolysate due to the production of small peptides. Additionally, Ishibashi and others (1987) found that the strongest bitterness occurred when the leucine residue was located at the C-terminus end of the peptide. MN preferentially hydrolyzes peptide bonds at leucine residues, which would expose this position and could explain the intense bitterness.

Proteolytic hydrolysis

Profam 825 SPI was chosen as a substrate because it is commercially available in the food industry, widely used, and has ideal physical properties for this project. Profam 825 is very bland, with low viscosity, high dispersibility, and high solubility properties. These physical properties were ideal for filtering the SPI hydrolysate through the gravity filtration column. In the food industry, Profam 825 is used for various products such as low viscosity beverages, nutritional bars, and extruded cereals.

Protex 7L protease was chosen because it behaves very similarly to MN, which produces bitter-tasting hydrolysates at 4% DH (Lock 2007). Ideally, MN would have been used for this hydrolysis; however, Genencor discontinued the production and replaced it with Protex 7L. Protex 7L is primarily composed of metallo-endopeptidase produced by *Bacillus amyloliquefaciens* (EC 3.4.24.28) and a lesser amount of subtilisin-like protease (EC 3.4.21.62). Subtilisins are serine proteases, which nucleophilically attack the peptide bond

through a serine residue at the active site. The activity of Protex 7L is >1600 AU, which is a measure of azo units/g (AU) based on the hydrolysis of Azo-casein substrate at pH 7.5 for 5 minutes at 30˚C. Protex 7L is active between pH 6.0-8.0 and 40-60˚C, with optimal working conditions at pH 7.0-7.5 at 50˚C. It functions best at a 1:10 E/S.

To determine the % DH similarity of hydrolysate replications, the 3 batches of Protex 7L hydrolysate were analyzed for protein amount and peptide size. The amount of protein present in each replication was measured by using the Biuret assay and the molecular weight size of the peptides was estimated by SDS-urea-PAGE. The results of the Biuret assay are shown in Table 10. This was possible indication that the % DH was not consistent across replications. As DH increases, the size of the peptides decreases because of the additional protease action. If the hydrolysate had a portion of very small peptides or free amino acids due to the large DH, they would not show up as protein in the Biuret assay. The Biuret assay only reacts with peptides longer than 2 residues (Hortin and Meilinger 2005).

Table 10. Amount protein in each hydrolysate replication by Biuret assay

Protex 7L hydrolysate replication	Amount protein (mg/mL)
1	56.1
2	54.6
3	60.8

SDS-PAGE profiles of the 3 Protex 7L hydrolysis replications revealed the protein had been hydrolyzed > 4% DH. Lamsal and others (2006) produced a similar SDS-PAGE of MN-treated soy protein hydrolysate, which only showed hydrolysis of the α'-, α-, and β-

subunits of β-conglycinin at 4% DH. The SDS-PAGE in the present study, however, showed complete hydrolysis of all acidic glycinin subunits for each replication and the basic glycinin subunits were extensively hydrolyzed in replication 2. Evaluation of the size of peptides by gel electrophoresis confirmed that the 3 replications underwent different DHs. The protein bands on an 8-18% gradient urea SDS-PAGE were very different across replications (Figure 9).

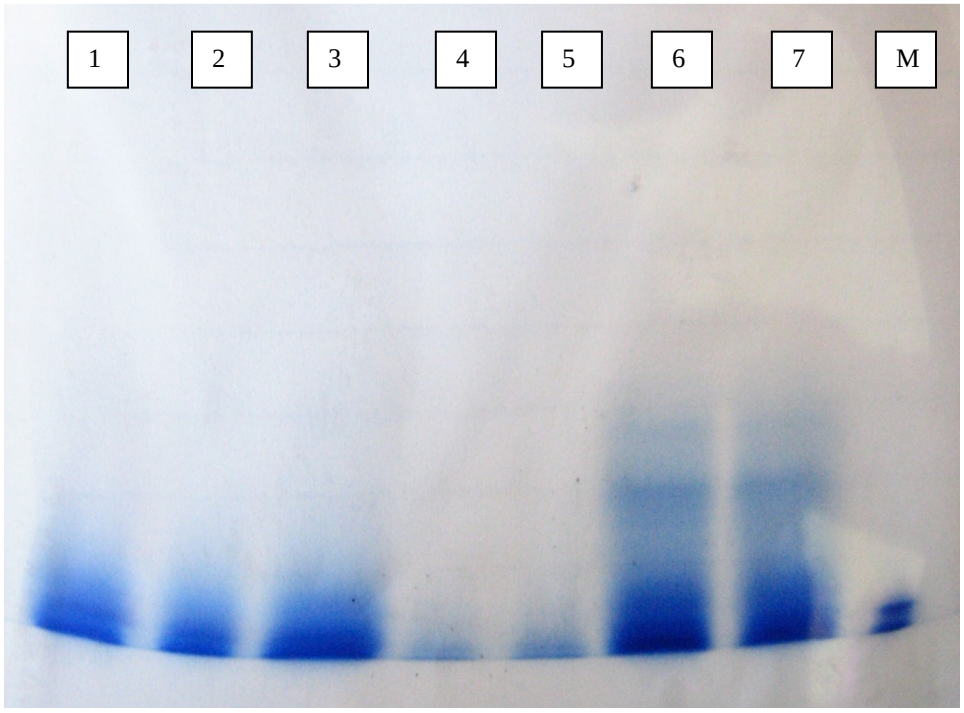

Figure 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of Protex 7L soy protein hydrolysate replications at unknown % DH. Lanes 1-3 = replication 1; lanes 4-5 = replication 2, lanes 6-7 = replication 3; 100µg protein/lane. M = ultra-low molecular weight markers 26.6, 17, 14.2, 6.5, 3.5, 1.06 kDa from top down, respectively.

The third replication had the widest range of molecular weight bands, indicating the least amount of hydrolysis among the three replications. The second replication showed only very small molecular weight bands, indicating extensive hydrolysis. The first replication went to an intermediate DH between the other two replications, and was chosen as the one replication used for the remainder of the study. Each gel filtration run would serve as a replication.

Gel filtration

The gel filtration standard curve of known molecular weight markers (Figure 10) was used to determine the average weight of the hydrolysate fractions and V_T was known from elution with sucrose.

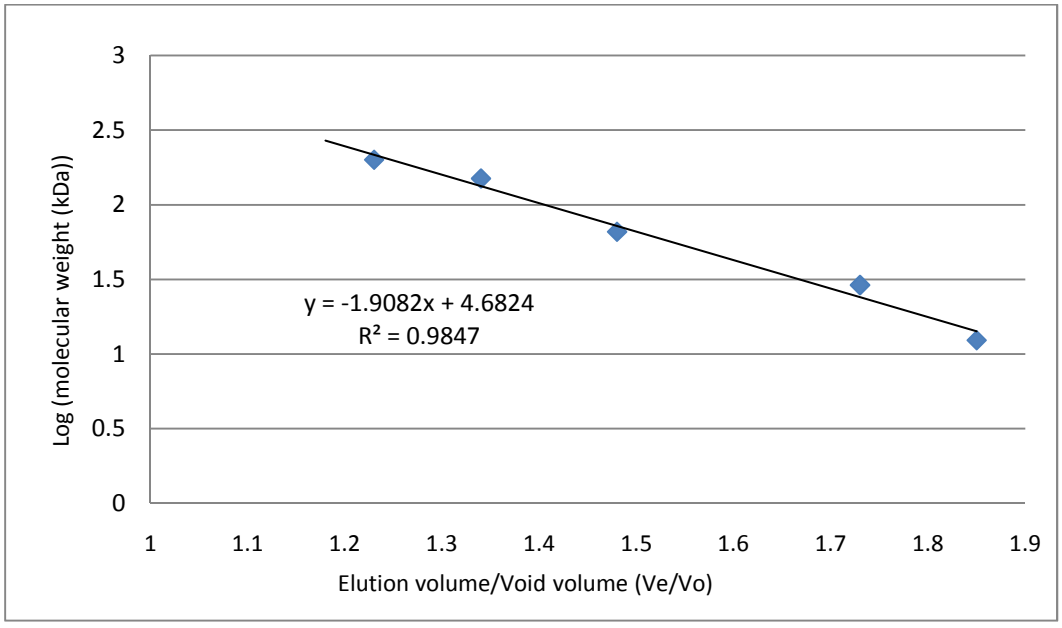

Figure 10. Gel filtration calibration standard curve of known molecular weights: 12.4 kDa (cytochrome c from horse heart), 29 kDa (carbonic anhydrase from bovine erythrocytes), 66 kDa (BSA), 150 kDa (alcohol dehydrogenase from yeast), and 200 kDa (β-amylase from sweet potato). Blue Dextran was used to determine the void volume (V_o).

Absorbance of the hydrolysate was plotted against the total volume of eluant to provide a plot of protein peaks, which revealed the relative protein content in the eluant samples (Figure 11). A standard curve of known Protex 7L hydrolysate concentrations at 254 nm was produced to quantify the protein content of the eluated fractions. An approximate recovery rate was calculated by the total protein in test tubes divided by the amount of protein loaded onto the column (0.5 g).

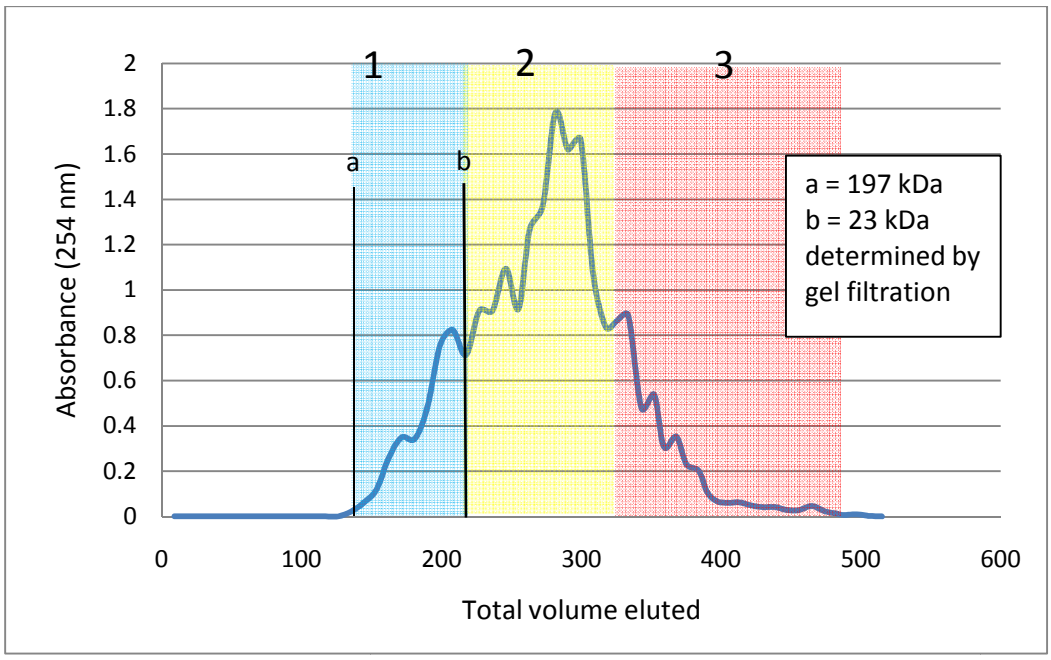

Figure 11. Sephacryl 200-HR gel filtration chromatogram of Protex 7L soy hydrolysate fractions 1-3 based on protein absorbance at 254 nm. Blue region= fraction 1; yellow region= fraction 2; red region= fraction 3

The purpose of analyzing for free amino acid content by the ninhydrin assay was to determine whether samples collected towards the end of the gel filtration run had been hydrolyzed to very small peptides which would not show peaks by UV absorbance. Free amino acids were not detected beyond the end of the gel filtration run, so the UV absorbance

plot was used to determine fractions. Three distinct sections were determined in each chromatogram. Samples tubes corresponding to these 3 areas from 12 gel filtration runs were combined. These fractions were labeled as fraction 1, 2, and 3 and corresponded to the largest to smallest molecular weight peptide fractions, respectively.

Ultrafiltration

It was evident that peptides < 1 kDa were present in fraction 3 because sucrose (MW 365 Da) eluted before the fraction peak was finished. Fraction 3 was filtered and divided into 2 portions. Buffer salts could not be removed from the diffusate of fraction 3 due to the 1 kDa ultrafiltration membrane. The retentate with peptides >1 kDa without buffer components was considered fraction 3, while the diffusate with peptides < 1-kDa and buffer components was considered fraction 4.

Freeze drying

The recovered dry mass of each fraction after freeze drying was compared to the initial amount of hydrolysate loaded onto the gel filtration column in Table 11. Some of fraction 2 was lost due to a spill prior to ultrafiltration, which could explain the low mass of this fraction compared to the others. The high mass of fraction 4 was primarily due to the components in the eluting buffer, which could not be removed during ultrafiltration. The initial mass of the buffer components in fraction 4 was calculated based on the amount of eluant in fraction 4 and the mass of components in the buffer (31 mg/mL). Mass was most likely lost throughout several of the processes steps including gel filtration, ninhydrin assay, freeze drying or accidental spills, which could account for the low recovery rate.

Table 11. Initial and recovered mass of Protex-7L hydrolysate fractions

	Sample	Mass (g)
Initial	Protein loaded	6.0
	Buffer components in fraction 4	36.3
Recovered	Fraction 1	1.54
	Fraction 2	0.94
	Fraction 3	1.39
	Fraction 4	19.86
	Total	23.73
Percentage mass recovered		56.09%

Gel electrophoresis

Fractions were analyzed by gel electrophoresis to see if molecular weight information corresponded with the gel-filtration standard curve and provided more information on fractions 2 and 3. An 18% urea SDS-PAGE stained with Coomassie blue provided molecular weight information, but it was difficult to see all of the molecular weight bands (Figure 12).

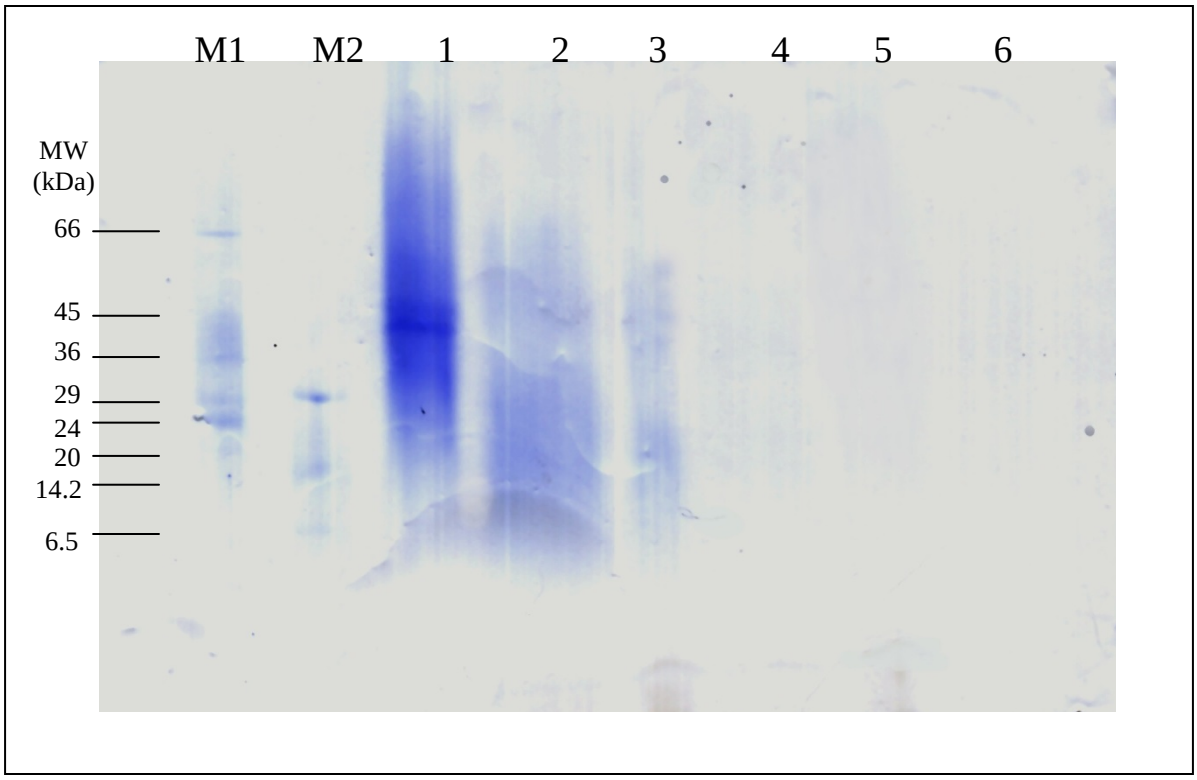

Figure 12. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of Protex 7L soy protein hydrolysate fractions. M1 = low molecular weight marker; M2 = ultra low molecular weight markers 26.6, 17, 14.2, 6.5, 3.5, 1.06 kDa from top down respectively; 1 = Profam 825 SPI; 2 = unfractionated hydrolysate; 3 = fraction 1; 4 = fraction 2; 5= fraction 3; 6 = fraction 4. 170 µg protein/lane.

Figure 12 showed that the unfractionated hydrolysate ranged in size from approximately 70 kDa to < 5 kDa. The molecular weight of peptides in fraction 1 ranged from approximately 60 kDa to < 5 kDa. The 3.5 kDa and 1.06 kDa bands of the ultra low molecular weight marker (M2) did not appear, which made it difficult to predict the lowest molecular weight of the hydrolysate.

To gain more information on the lower molecular weight bands that did not appear using Coomassie blue staining, an 18% SDS-PAGE urea gel using silver staining was made (Figure 13). Silver staining is 100-fold more sensitive than Coomassie blue staining, so protein present in smaller amounts can be detected (Switzer 1979).

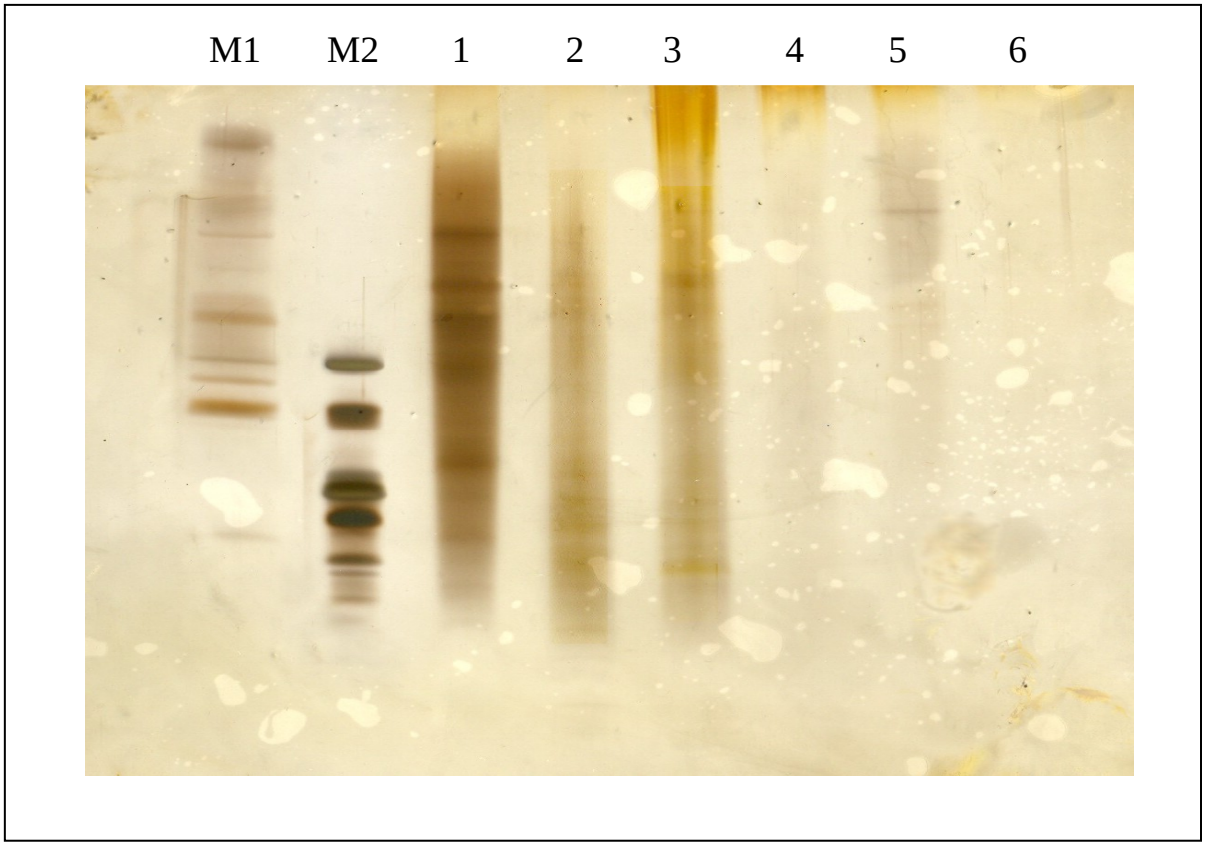

Figure 13. Silver stained SDS-PAGE profile of Protex 7L soy protein hydrolysate fractions. M1 = low molecular weight markers 97.4, 66.2, 45, 31, 21.5, 14.4 kDa from top down respectively; M2 = ultra low molecular weight markers 26.6, 17, 14.2, 6.5, 3.5, 1.06 kDa from top down respectively; 1 = Profam 825 soy protein isolate; 2 = unfractionated hydrolysate; 3 = fraction 1; 4 = fraction 2; 5= fraction 3; 6 = fraction 4. 150 ng protein/lane.

Figure 13 showed that the unfractionated hydrolysate ranged in size from approximately 80 kDa to < 1 kDa. The molecular weight of peptides in fraction 1 ranged from approximately 80 kDa to 1 kDa. The average molecular weight of the fraction 1 as determined by using the gel-filtration standard curve was 57 kDa, which was within the molecular weight range in determined in SDS-PAGE. The fact that peptides as small as 1 kDa were found in fraction 1 is unexpected because fractions 2 and 3 did not appear, but

contained peptides > 1 kDa retained by the 1 kDa ultrafiltration membrane. A summary of the composition and estimated molecular weight of each pooled fraction is summarized in Table 12.

Table 12. Characterization of pooled peptide fractions

Peptide fraction	Composition of fraction	Estimated mol. weight[a]	Estimated mol. weight[b]
1	First peak eluted from column of Protex 7L treated soy hydrolysate, 12 gel filtration runs pooled	5 - 60 kDa	1- 80 kDa
2	Second peak eluted from column of Protex 7L treated soy hydrolysate, 12 gel filtration runs pooled	<5 kDa	unknown
3	Third peak eluted from column of Protex 7L treated soy hydrolysate and retentate from 1kDa MW ultrafiltration, 12 gel filtration runs pooled	< fraction 2, >1 kDa	unknown
4	Third peak eluted from column of Protex 7L treated soy hydrolysate and diffusate from 1kDa MW ultrafiltration, 12 gel filtration runs pooled duplication =2	<1 kDa	<1 kDa

[a]Molecular weight of fraction 1, 2, and 3 estimated from figure 12; fraction 4 estimated from ultrafiltration
[b]Molecular weight of fraction 1, 2, and 3 estimated from figure 13; fraction 4 estimated from ultrafiltration

The standard curve of known molecular weight markers (Figure 10) indicated that fraction 1 of the hydrolysate had a molecular weight of 23-197 kDa. Because the molecular weight markers range from 12-200 kDa, it could not accurately predict the size of peptides < 12 kDa. Fractions 2 and 3 were below the molecular weight estimation limit by the standard curve (Figure 10), indicating they had average molecular weights was smaller than the can be

accurately predicted by this gel filtration molecular weight standards. Fraction 4, which was composed of the diffusate from fraction 3 after ultrafiltration, had a molecular weight of < 1 kDa due to the 1 kDa pore size of the ultrafiltration membrane.

Screening of panelists

Comparison of bitter sensitive and insensitive panelists. From the 25 panelists screened for bitterness sensitivity, the selected group of 15 were termed 'bitter sensitive', while the group of 10 panelists not selected were termed 'bitter insensitive'. Table 13 summarizes the age, gender, ethnicity, and food consumption habits of the bitter sensitive group and bitter insensitive group. Although our study did not have enough panelists to make any statistical correlations between panelist characteristics, overall patterns for these particular groups can be noted.

Table 13. Comparison of demographics and consumption habits between bitter sensitive and insensitive groups

Bitter sensitive group[a]							
Age range	**Gender**	**US born or International**	**Moderate caffeine consumer**	**High caffeine consumer**	**Moderate quinine consumer**	**Moderate soy consumer**	**High soy consumer**
20-30	Female	international	N	N	N	N	N
30-40	Female	international	Y	N	N	Y	Y
20-30	Female	international	N	N	N	Y	N
30-40	Female	international	N	N	Y	Y	Y
30-40	Female	international	N	N	N	Y	N
20-30	Male	international	Y	Y	Y	N	N
20-30	Female	US	N	N	N	N	N
50-60	Female	US	Y	Y	N	N	N
20-30	Female	US	Y	N	N	N	N
50-60	Female	US	Y	N	N	N	N
20-30	Female	US	Y	N	N	N	N
30-40	Female	international	N	N	N	N	N
20-30	Female	international	Y	N	N	Y	Y
30-40	Female	international	Y	N	N	Y	N
20-30	Male	US	N	N	N	N	N
Bitter insensitive group[b]							
20-30	Male	US	N	N	N	N	N
50-60	Male	US	Y	Y	N	N	N
20-30	Female	US	N	N	N	Y	Y
20-30	Female	US	Y	Y	N	N	N
20-30	Male	US	N	N	N	N	N
40-50	Female	US	Y	Y	Y	N	N
50-60	Female	US	N	N	N	N	N
60-70	Female	US	Y	Y	N	N	N

[a]Bitter sensitive group included panelists who met at least one of the following threshold requirement: ≤ 0.8 mM caffeine, ≤ 6 µM quinine sulfate, ≤ 5% soy hydrolysate in water

[b]Bitter insensitive group did not meet the requirements for the bitter sensitive group

There was a wide variety of age in both groups, ranging from panelists in their 20s to those in their 60s. Although studies have reported sensitivity to bitterness decreases with age (Murphy and Gilmore 1989; Frank and others 1992), more specific studies have found taste sensitivity remains largely unimpaired until the late 50s and begins to decline at age 60 (Cooper and others 1959; Schiffman 1993). All panelists were < 60 in the sensitive group, which may explain why there were no clear patterns with age.

There were not enough male participants in the bitterness screening process to notice any trends across gender. Past studies on the relationship between gender and bitterness have shown variable results, which may be due to the different composition of population studied (Cubero-Castillo and Noble 2004). Yamauchi and others (1995) reported lower bitter thresholds (higher sensitivity) in females than males over age 20, whereas the opposite trend was true for men and women in their late teens aged 18-19. Mojet and others (2001) studied detection thresholds at different ages and reported no effect for bitterness sensitivity and gender, but a significant interaction between gender and age. It has also been reported that a higher percentage of women are supertasters compared to men (Bartoshuck and others 1994). Supertasters are individuals who experience taste with much greater intensity than average. More research needs to be done before any conclusive relationship between gender and bitterness sensitivity can be drawn.

The bitter sensitive group includes a much greater percentage of international panelists than U.S.-born with the insensitive group. Bitterness sensitivity is largely genetic, which could be related to nationality. Two chemically related compounds, phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), taste extremely bitter to some individuals (tasters) but are tasteless to others (nontasters) (Cohen and Ogdon 1949). While

nontasters exist in all global populations, the frequency of nontasters varies greatly by race and ethnicity. Approximately 30% of Caucasians are nontasters, while populations in China, Japan, and sub-Saharan Africa are approximately 10%–20%. Additionally, the frequency of nontasters is exceeds 50% in some subgroups studied in India (Guo and Reed 2001). The cause of differences in sensitivity to PTC and PROP unclear, but is not a reliable indicator to sensitivity in other bitter substances (Delwiche and others 2001).

Although our data did not show any clear trends with between sensitivity and bitter food consumption, food consumption habits can influence sensitivity (Mennella and others 2005). Individuals who consume bitter foods regularly are probably not bothered by the bitter taste, which suggests lower sensitivity. Tanimura and Mattes (1993) studied the effects of beer and caffeine consumption on bitterness sensitivity. Non-consumers of caffeine had lower caffeine thresholds than moderate or heavy consumers while slight beer consumers had lower iso-alpha-acid (beer bittering agent) thresholds than heavy beer consumers. Studies have also found that PTC tasters tend to avoid raw cruciferous vegetables which contain bitter compounds such as broccoli, cabbage, and brussels sprouts (Fischer and others 1961; Kalmus 1971). Glanville and Kaplan (1961) reported that PROP tasters tend to prefer mild tasting foods and disliked sharp tasting foods such as black coffee, grapefruit juice, lemon juice, and horseradish. These studies suggest taste sensitivity plays a role in food consumption habits.

Sensitivity among various bitter compounds. The bitter sensitive group evaluated various bitter compounds to determine their detection thresholds. These compounds included caffeine, quinine sulfate, leucine, phenylalanine, and MN soy hydrolysate. Table 14 lists the mean detection threshold of each compound, the percentage of panelists who considered the

compound bitter, along with a comparison of literature data on threshold and taste. Threshold values among the bitter sensitive group were less than the literature thresholds for the general population. This indicated the screening process was successful for selecting panelists who had greater bitterness sensitivity than average. While all compounds evaluated were reported as bitter tasting in the literature, panelists in this group were not in agreement on the bitter taste. Descriptors such as rancid for phenylalanine, medicinal for leucine, and sweet for MN soy hydrolysate were some examples of taste sensations reported by panelists who did not consider the compounds bitter. The greater sensitivity of the bitter sensitive group may have influenced whether they considered the compounds bitter or not. Highly sensitive panelists may notice other contributing flavors in the compound that an insensitive panelist may not, which could complicate the categorization of bitterness.

Studies on bitterness sensitivity to various compounds have reported large individual variation to each of the compounds. In an intensity evaluation of 11 bitter compounds, Delwiche and others (2001) reported a significant correlation between bitterness intensity ratings of caffeine and quinine, although the correlation was not strong. Tryptophan, a hydrophobic amino acid like leucine, showed a significant moderate correlation with phenylalanine. Neither caffeine and phenylalanine or quinine and phenylalanine showed a significant bitterness intensity correlation. Sensitivity to PROP and PTC do not correlate with sensitivity to other bitter compounds (Bartoshuk and others 1979; Hall and others 1975; Yokomukai and others 1993).

Table 14. Threshold data and taste of bitter compounds by bitter-sensitive group and literature reference data

Compound taste	Group threshold	Standard Deviation	Literature threshold	Literature reported taste	% panelists reporting taste as bitter
Caffeine (mM)	0.98	0.62	1.5[a]	bitter[a]	100
Quinine sulfate (μM)	8.2	6.8	18[a]	bitter[a]	100
Leucine (mM)	5.3	4.9	19[b]	bitter[b]	41
Phenylalanine (mM)	3.4	2.7	20[b]	bitter[b]	62
MN treated soy hydrolysate (g/100mL)	5.2	0.5	NA	bitter[c]	86

[a]Delcour and others (1984)
[b]Kato and others (1989)
[c]Lock (2007)

Sensory evaluation

The objectives of the sensory evaluation were to evaluate the unfractionated Protex 7L hydrolysate and its respective fractions using the bitter/not bitter test, to compare each panelist's sensitivity to caffeine, quinine, leucine, and phenylalanine in relation to the MN soy hydrolysate, and to determine whether the bitterness in leucine or phenylalanine closely resemble the bitterness in MN soy hydrolysate.

Protex 7L soy hydrolysate bitterness. All hydrolysate samples were evaluated at 5% w/v with Milli-Q water. The bitter/not bitter evaluation results for the hydrolysate fractions and p value calculations are tabulated (Table 15). Individual responses and comments are included in Appendix D and E.

Table 15. Results from bitter/not bitter evaluation of hydrolysate fractions[a]

Sample	# panelists identified odd sample	# panelists identified odd sample as bitter	p value
Fraction 1	13	4	0.791
Fraction 2	12	1	0.998
Fraction 3	14	10	0.009
Fraction 4	15	3	0.921
Unfractionated hydrolysate	15	8	0.088

[a]15 panelists

Fraction 3, a low molecular weight fraction (estimated 1-5 kDa), was identified as bitter tasting (Table 12). This fraction may be responsible for the bitterness of the unfractionated Protex 7L hydrolysate. The high p value (> 0.05) for the unfractionated hydrolysate (p = 0.088) could be explained by the difference in bitterness intensity compared to fraction 3. If fraction 3 was responsible for all of the bitterness in the hydrolysate, it would be more intensely bitter than the entire hydrolysate. The lower bitterness intensity combined with the small quantity of hydrolysate used for evaluation may have caused difficulty in the detection of bitterness.

Although fraction 4 was not identified as bitter, this fraction contained all of the buffer components which could not be filtered. The high NaCl concentration from the buffer made fraction 4 extremely salty and may have masked any bitterness. The suppression of bitterness by sodium has been studied. Breslin and Beauchamp (1995) reported differential suppression of bitterness by sodium across several bitter compounds. The bitterness in urea

was almost completely suppressed, caffeine and quinine were moderately suppressed, and magnesium sulfate was mostly unaffected by the sodium. The suppression of bitter by sodium on soy hydrolysate has not been studied, but may have affected the bitterness results of fraction 4.

Lock (2007) reported that MN hydrolysate, which was produced by a very similar protease to Protex 7L, was bitter. Both MN and Protex 7L hydrolysates were evaluated at 5% (w/v) in water. However, there were differences in sample quantity, method of tasting, centrifugation of hydrolysate, and protease (Table 16). Although the DH was greater in this study than in Lock's, this would have most likely increased the bitterness so it is not a possible explanation for non-significant result. However, the 100-fold difference in sample quantity probably made bitterness detection much more difficult. Lock's panel could sample up to 40 mL of hydrolysate, while this panel had 0.4 mL of sample available. Method of tasting also differed. The panelists in Lock's study swirled the hydrolysate around in their mouth, which helped coat all portions of the tongue and taste buds. Panelists in the present study had to taste the sample with droplets placed on the center of the back of the tongue. If placement on the tongue is not centered, the bitterness sensation may be difficult to detect. The hydrolysate in this study was centrifuged to remove insoluble solids for smooth elution during gel filtration. The hydrolysate in Lock's study was not centrifuged, so the texture was also different.

Table 16. Differences in hydrolysate preparation between present study and study by Lock (2007)

Difference	Lock's study	Present study
Amount of sample	40 mL	0.4 mL
Method of tasting	Swirl to coat in mouth	Use eyedropper to place drops on back center of tongue
Centrifugation	Not centrifuged, insoluble solids dispersed within sample	Centrifuged, no insoluble solids
Protease	Multifect Neutral	Protex 7L
Degree of hydrolysis	4%	> 4%

The bitter/not bitter test was not a standard sensory evaluation method, but was ideal for this study because it provided information on whether the panelist was able to distinguish the compound from control samples as well as information on whether the compound was perceived as bitter. It was very similar to the method used by Mossman (1986), in which taste thresholds for salt, sweet, sour and bitter were measured using a forced choice 3-drop test with increasing concentration. In Mossman's method, 2 drops of water and 1 drop of the solution of interest were placed in the mouth. Panelists chose the drop that was different from the others and decided the taste of the odd sample. The main difference from Mossman's technique is that the present study evaluated the hydrolysate fractions at a single concentration (5% w/v) due to the limited amount of available sample. There are also 2 types of modified triangle tests in the literature (Gacula and Signh 1984). In the Bradley-Harmon method, (Bradley and Harmon 1964), the panelist identifies the odd sample in the usual triangle test and then rates the difference between the odd sample and the remaining pair of samples. A "confidence scale" may be used as an alternative to rating the difference,

in which the panelist rates the confidence of his/her identification of the odd sample from a pure guess to absolutely confident. The Bradley-Harmon method is not widely used in sensory research, possibly due to the complex calculations in the analysis (Bi 2006). In the Gridgeman model, (Gridgeman 1964, 1970), the panelist identifies the odd sample in the usual triangle test followed by answering whether he/she thinks the odd sample is less flavorsome or more flavorsome than the remaining pair of samples. The validity of this modified triangle test, however, is questionable due to bias in the preference judgments (Bi 2006).

Comparison of sensitivity. Results comparing panelist sensitivity to caffeine, quinine, leucine, and phenylalanine in relation of MN-treated soy hydrolysate are summarized (Table 17) and shown in contingency tables (Figure 14).

Table 17. Panelist sensitivity of bitter compounds in relation to soy hydrolysate[a]

Test statistic	Caffeine and hydrolysate	Quinine and hydrolysate	Leucine and hydrolysate	Phenylalanine and hydrolysate
Kappa coefficient	0.20 ± 0.50	0.10 ± 0.38	0.36 ± 0.35	-0.27 ± 0.34
Fisher's exact test	NS p= 0.62	NS p= 1.0	NS p= 0.20	NS p= 0.20

[a]15 panelists, S = significant at $p \leq 0.05$, NS = not significant

Hydrolysate sensitive

Caffeine sensitive	No	Yes	Total
No	5	3	8
Yes	3	4	7
Total	8	7	15

Hydrolysate sensitive

Quinine sensitive	No	Yes	Total
No	2	1	3
Yes	6	6	12
Total	8	7	15

Hydrolysate sensitive

Leucine sensitive	No	Yes	Total
No	3	0	3
Yes	5	7	12
Total	8	7	15

Hydrolysate sensitive

Phe sensitive	No	Yes	Total
No	0	2	2
Yes	8	5	13
Total	8	7	15

Figure 14. Contingency tables for panelist sensitivity to soy hydrolysate in relation to caffeine, quinine, leucine, or phenylalanine (phe)

According to kappa value interpretations in Altman (1991), the kappa coefficient showed poor agreement between panelist sensitivity to soy hydrolysate in relation to caffeine, quinine, and phenylalanine and shows fair agreement in relation to leucine. Fisher's exact test showed a non-significant p value for panelist sensitivity to soy hydrolysate in relation to caffeine, quinine, leucine, and phenylalanine. This indicated that panelist sensitivity to soy hydrolysate is independent of panelist sensitivity to caffeine, quinine, leucine, and phenylalanine.

The non-significant Fisher's exact test results and poor/fair kappa coefficient agreement suggested that neither caffeine, quinine, leucine, nor phenylalanine was an ideal screening compound for selecting panelists sensitive to soy hydrolysate. Caffeine or quinine has been used as a screening compound in nearly all studies on soy hydrolysate bitterness (Cho and others 2004; Kodera and others 2006; Li and others 2008; Seo and others 2008). However, studies that screen panelists using caffeine or quinine may be selecting a portion of panelists who are not sensitive to soy hydrolysate while dismissing a portion of panelists that are sensitive. If the panelists in the present study were selected based on caffeine sensitivity alone, 33% of the panelists chosen would not have been sensitive to soy hydrolysate and 20% of those dismissed would have been. If selection was based on quinine sensitivity alone, 40% of the panelists chosen would not have been sensitive. Clearly, a better screening reference for sensitivity in hydrolysate studies is necessary.

Comparison of bitterness. Results for the comparison of bitterness in leucine or phenylalanine to soy hydrolysate are summarized in Table 18 and contingency tables are shown in Figure 15. Leucine and phenylalanine taste bitter in their free amino acid form (Kato and others, 1989) and are part of a class of bitter-tasting amino acids not frequently used in bitterness studies (Delwiche and others 2001). Hydrolysate peptides are comprised of amino acids, so there was interest in whether bitter amino acids would make a better reference for soy hydrolysate bitterness compared to caffeine or quinine.

The kappa coefficient showed poor agreement in panelist identification of bitterness between leucine and soy hydrolysate and between phenylalanine and soy hydrolysate. The p value for the McNemar's test was significant in both relationships. This indicated that the bitterness in leucine and phenylalanine was different than the bitterness in soy hydrolysate.

In sensory studies evaluating the bitterness of soy hydrolysate, neither leucine nor phenylalanine would be a good compound to use during training.

Table 18. Panelist's bitterness perceptions of leucine and phenylalanine in relation to soy hydrolysate[a]

Test statistic	Leucine and hydrolysate	Phenylalanine and hydrolysate
Kappa coefficient	0.14 ± 0.21	-0.017 ± 0.33
McNemar's test	S P = .0047	S P = 0.034

[a]15 panelists, S = significant at $p \leq 0.05$, NS = not significant

Identify leucine bitter

Identify hydrolysate bitter

	No	Yes	Total
No	2	8	10
Yes	0	5	5
Total	2	13	15

Identify phenylalanine bitter

Identify hydrolysate bitter

	No	Yes	Total
No	1	7	8
Yes	1	6	7
Total	2	13	15

Figure 15. Contingency tables for panelist bitterness perception of soy hydrolysate in relation to caffeine, quinine, leucine, or phenylalanine

CONCLUSIONS

Protex 7L-treated soy hydrolysate was successfully separated into different molecular weight fractions. Although the UV chromatograms of gel filtration samples differed slightly for each replication, similar peaks were found in chromatograms for each run. The ultrafiltration procedure helped concentrate the samples and remove buffer components in fractions 1-3. Unfortunately, fraction 4 contained salt and other buffer components due to the 1 kDa membrane pore size. The small quantity of hydrolysate which could be loaded onto the gel filtration column limited the final quantity of hydrolysate fractions available for sensory evaluation, which was another challenge in this study.

It was demonstrated that the bitter/not bitter test could be applied to small sample quantities for bitterness evaluation. Although larger quantities of sample were preferred, a screened panel with adequate amount of training for tasting small quantities was able to obtain reliable sensory evaluation results. It was difficult to obtain information on the magnitude of bitterness with small sample quantities, but the presence or absence of bitterness in the sample was assessed. Fraction 3 of the Protex 7L hydrolysate (estimated 1-5 kDa) was identified as bitter tasting. This supports research that low molecular weight peptides are bitter. Peptides in this molecular weight range may be responsible for the bitterness of the whole unfractionated hydrolysate. Neither caffeine, quinine, leucine, nor phenylalanine was found to be an ideal screening compound for selecting panelists sensitive to soy hydrolysate. Free hydrophobic amino acids are not ideal for training a soy hydrolysate bitterness panel. A better reference is needed for bitterness sensitivity screening and training of soy hydrolysate sensory evaluation panels.

This research demonstrated that the bitterness in soy hydrolysate is affected by the molecular weight of the peptide and that small molecular weight peptides are responsible for this bitterness. Molecular weight is not the only cause of bitterness, since hydrolysates produced with certain proteases do not taste bitter but still contain small molecular weight peptides. A combination of the peptide's molecular weight, hydrophobicity, primary sequence, spatial structure, and bulkiness is most likely responsible for bitterness. Further research on the bitterness of protein hydrolysates along with valid sensory analysis is needed to determine a clear cause.

REFERENCES

Adler-Nissen J. 1986. Methods in food protein hydrolysis. In: Adler-Nissen J, editor. Enzymic hydrolysis of food proteins. New York: Elsevier. p 110-31.

Altman DG. 1991. Comparing groups: categorical data. In: Altman DG, editor. Practical statistics for medical research. London, U.K.: Chapman and Hall. p 229-76.

Bartoshuck L, Duffey V, Miller I. 1994. PTC/PROP tasting: anatomy, phychophysics, and sex effects. Physiol Behav 56:1165-71.

Bi J. 2006. Modified discrimination tests. In: Bi J, editor. Sensory discrimination tests and measurements. New York: Wiley-Blackwell. p 45-56.

Bier M. 1959. In: Bier M, editor. Electrophoresis: theory, methods and applications. 3rd ed. New York: Academic Press. p 225-40.

Bradley R, Harmon T. 1964. The modified triangle test. Biometrics 20:608-25.

Breslin P, Beauchamp G. Suppression of bitterness by sodium: variation among bitter taste stimuli. Chem Senses 20:609-23.

Carrão-Panizzi M, Kwanyen P, Erhan S, Lopes I. 2008. Genetic variation and environmental effects on beta-conglycinin and glycinin content in Brazilian soybean cultivars. Pesq Agropec Bras 43(9):1105-14.

Cho M, Unklesbay N, Hsieh F, Clarke A. 2004. Hydrophobicity of bitter peptides from soy protein hydrolysates. J Agric Food Chem 52:5895-901.

Cohen J, Ogdon D. 1949. Taste blindness to phenyl-thio-carbamide and related compounds. Psychol Bull 46:490-8.

Cooper R, Bilash I, Zubek J. 1959. The effect of age on taste sensitivity. J Gerontol 14:56-8.

Cornelis M, El-Sohemy A, Campos H. Genetic polymorphism of the adenosine A2A receptor is associated with habitual caffeine consumption. Am J Clin Nutr 86(1):240-4.

Cubero-Castillo E, Noble A. 2004. Relationship of 6-n-propylthiouracil status to bitterness sensitivity. In: Prescott J, Tepper B, editors. Genetic variation in taste sensitivity. New York, NY: Marcel Dekker. p 105-16.

Delcour J, Vandenberghe M, Corten P, Dondeyne P. 1984. Flavor thresholds of polyphenolics in water. Am J Enol Vitic 35(3):134-6.

Delwiche J, Buletic Z, Breslin P. 2001. Covariation in individuals' sensitivities to bitter compounds: evidence supporting multiple receptor/transduction mechanisms. Percept Psychophys 63(5):761-76.

Dubois M, Gilles K, Hamilton J, Rebers P, Smith E. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-6.

Frank M, Hettinger T, Mott A. 1992. The sense of taste: neurobiology, aging, and medication effects. Crit Rev Oral Biol Med 3:379-93.

Fischer R, Griffin F, England S, Garn SM. 1961. Taste thresholds and food dislikes. Nature 1291:1328.

Frøkjaer S. 1994. Use of hydrolysates for protein supplementation. Food Technol 48(10):86-8.

Gacula MC, Singh J. 1984. Statistical methods in food and consumer research. New York: Academic Press. 505 p.

Gornall A, Bardwil C, David M. 1949. Determination of serum proteins by means of the biuret reactions. J Biol Chem 177:751-66.

Govindarajuk K, Srinivas H. 2007. Controlled enzymatic hydrolysis of glycinin: susceptibility of acidic and basic subunits to proteolytic enzymes. Lwt-Food Sci Technol 40(6):1056-65.

Gridgeman N. 1964. Sensory comparisons: the two-stage triangle test with simple variability. J Food Sci 29:112-7.

Gridgeman N. 1970. A reexamination of the two-stage triangle test for the perception of sensory differences. J Food Sci 35:87-91.

Guo S, Reed D. 2001. The genetics of phenylthiocarbamide perception. Ann Hum Biol 28:111-42.

Hortin G, Meilinger B. 2005. Cross-reactivity of amino acids and other compounds in the biuret reaction: interference with urinary peptide measurements. Clin Chem 51:1411-9.

Humiski L, Aluko R. 2007. Physiochemical and bitterness properties of enzymatic pea protein hydrolysates. J Food Sci 72(8):605-10.

Ishibashi N, Arita Y, Kanehisa H, Kouge K, Okai H, Fukui S. 1987. Bitterness of leucine-containing peptides. Agric Biol Chem 51(9):2389-94.

Ishibashi N, Kubo T, Chino M, Fukui H, Shinoda I, Kikuchi E, Okai H, Fukui S. 1988. Taste of proline-containing peptides. Agric Biol Chem 52(1):95-8.

Kato H, Rhue M, Nishimura T. 1989. Role of free amino acids and peptides in food taste. In: Teranishi R, Buttery TG, Shahidi G, editors. Flavor chemistry: trends and developments. Washington D.C.: American Chemical Society. p 158-74.

Kalmus H. 1971. Genetics of taste. In: Beidler LM, editor. Handbook of sensory physiology. Berlin: Springer. p 165–79.

Kim MR, Kawamura Y, Lee CH. 2003. Isolation and identification of bitter peptides of tryptic hydrolysate of soybean 11S glycinin by reverse-phase high-performance liquid chromatography. J Food Sci 68(8):2416-22.

Kim MR, Kawamura Y, Kim KM, Lee CH. 2008 Tastes and structures of bitter peptide, asparagines-alanine-leucine-proline-glutamate, and its synthetic analogs. J Agric Food Chem 56(14):5852-8.

Kline T. 1995. Reliability of raters. In: Kline T, editor. Psychological testing: a practical approach to design and evaluation. Thousand Oaks, Calif.: SAGE Publications. p 185-200.

Kodera T, Asano M, Noriki N. 2006. Characteristic property of low bitterness in protein hydrolysates by a novel soybean protease D3. J Food Sci 72(9):609-13.

Randall CS, Malefyt TR, Sternson LA. 1991. Approaches to the analysis of peptides. In: Lee V, editor. Peptide and protein drug delivery. New York: Marcel Dekker. p 203-46.

Laemmli U K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227:680–5.

Li L. Yang Z, Yang X, Zhang G, Tang S, Chen F. 2008. Debittering effect of *Actinomucor elegans* peptidases on soybean protein hydrolysates. J Ind Microbiol Biotechnol 35:41-7.

Lim S. 1996. A sensory evaluation of the bitter compounds from *Ixeris dentate* Nakai. Korean J Soc Food Sci 12:115-21.

Lioe H, Takara K, Yasuda M. 2006. Evaluation of peptide contribution to the intense umami taste of Japanese soy sauces. J Food Sci 71(3):277-83.

Lock S. 2007. Flavor characteristics of soy products modified by proteases and alpha-galactosidase. [MSc thesis]. Ames, IA.: Iowa State University. 95 p. Available from: Iowa State Park Library, Ames, IA.

Lowry O, Rosebrough N, Farr A, Randall R. 1951. Protein measurement with the folin phenol reagent. J Biol Chem 193:265-75.

Matoba T, Hata H. 1972. Relationship between bitterness of peptides and their chemical structures. Agric Biol Chem 36:1423-31.

McDonald, J. 2008. Tests for nominal variables. In: McDonald J, editor. Handbook of biological statistics. Baltimore, Md.: Sparky House Publishing. p 64-8.

Meilgaard M, Civille G, Carr B. 2007. Statistical tables. In: Meilgaard M, Civille G, Carr B, editors. Sensory Evaluation Techniques. 4th ed. Boca Raton, Fla.: CRC Press. p 419-39.

Mennella J, Pepino Y, Reed D. 2005. Genetic and environmental determinants of bitter perception and sweet preferences. Pediatrics 115(2):216-22.

Mojet J, Christ-Hazelhof E, Heidema J. 2001. Taste perception with age: generic or specific losses in threshold sensitivities to the five basic tastes. Chem Senses 26:845-60.

Moore S, Stein W. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J Biol Chem 176:367-88.

Morrissey JH. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307-10.

Mossman KL. 1986. Gustatory tissue injury in man: Radiation dose response relationships and mechanisms of taste loss. Br J Cancer 53(7):9-11.

Murphy C, Gilmore M. 1989. Quality-specific effects of aging on the human taste system. Percept Psychophys 45:121-8.

O'Mahony M. 1986. The binomial test: applications in sensory difference and preference testing. In: O'Mahony M, editor. Sensory evaluation of food: statistical methods and procedures. New York: Marcel Dekker. p 57-90.

Otagiri K, Nosho Y, Shinoda I, Fukui H, Okai H. 1985. Studies on a model of bitter peptides including arginine, proline, and phenylalanine residues. Part 1. Bitter taste of di- and tripeptides and bitterness increase of the model peptides by extension of the peptide chain. Agric Biol Chem 49(4):1019-26.

Petrie A, Watson P. The chi-squared test: comparing proportions. In: Petrie A, Watson P, editors. Statistics for veterinary and animal science. 2nd ed. New York: Wiley-Blackwell. p 107-20.

RCSB, 2009. Protein Data Bank: An Information Portal to Biological Macromolecular Structures. Research Collaboratory for Structural Bioinformatics. Available from: http://www.rcsb.org. Accessed Jan 22, 2008.

Schiffman S. 1993. Perception of taste and smell in elderly persons. Crit Rev Food Sci Nutr 33(1):17-26.

Schmidl M, Taylor S, Nordlee J. 1994. Use of hydrolysate based products in special medical diets. Food Technol 48(10):77-85.

Seo W, Lee H, Baek H. 2008. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. J Food Sci 73:41-6.

Siemensma A, Weijer W, Bak H. 1993. The importance of peptide length in hypoallergenic infant food formulae. Trends Food Sci Technol 4:16-21.

Siemensma A, Kunst A. 1999. Sports nutrition: the recovery sports drink. World of Ingredients. October 53-6.

Solgaard G, Haug I, Draget K. 2008. Proteolytic degradation of cold-water fish gelatin solutions and gels. Int J Biol Macromol 43(2):192-7.

Spellman D, O'Cuinn G, FitzGerald R. 2005. Physiochemical and sensory characteristics of whey protein hydrolysates generated at different total solids levels. J Dairy Res 72:138-43.

Spellman D, O'Cuinn G, FitzGerald R. 2009. Bitterness in Bacillus proteinase hydrolysates of whey proteins. Food Chem 114:440-6.

Switzer R, Merril C, Shifrin S. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem 98(1):231-7.

Tanimura S, Mattes R. 1993. Relationships between bitter taste sensitivity and consumption of bitter substances. J Sens Stud 8(1):31-41.

Weiffenbach J, Wolf R, Benheim A, Folio C. 1983. Taste threshold assessment: a note on quality specific differences between methods. Chem Senses 8:151-9.

Wilson K, Papastoitsis G, Hartyl P, Tan-Wilson A. 1998. Survey of the proteolytic activities degrading the kunitz trypsin inhibitor and glycinin in germinating soybeans. Plant Physiol 88:355-60.

Yamauchi Y, Endo S, Yoshimura I. 1995. Whole mouth gustatory test. Effect of aging, gender and smoking on the taste threshold [in Japanese]. Nippon Jibi Inkoka Gakkai Kaiho 98**(**7):1125-34.

CHAPTER 4. GENERAL CONCLUSIONS

A theoretical analysis for the prediction of hydrolytic cleavage points of three different bitter- and non-bitter-producing proteases on soy protein at 4% degree of hydrolysis (DH) led to our hypothesis that bitterness of hydrolysates is caused by low molecular weight peptides. To test this hypothesis, Protex 7L-treated soy hydrolysate was fractionated by molecular weight using gel filtration, ultra-filtrated, freeze dried, and re-diluted to 5% w/v in Milli-Q water for sensory evaluation. Molecular weight of the fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Due the fact that bitterness sensitivity is genetic and inherent for each individual (Cornelis and others 2007), a panel of 15 highly sensitive individuals were selected for evaluation of the hydrolysate fractions. Using a panel of only those highly sensitive to bitterness improved the agreement of bitter responses during evaluation while using minimal amount of sample. Although evaluating with a larger quantity of sample would have been ideal, only a small amount of sample was available due to quantity limitations in the gel filtration procedure. Due to the small sample size, it was very difficult for panelists to quantify the intensity of bitterness. The bitter/not bitter test was designed to evaluate whether panelists could detect a difference between the sample and two controls and determine if the difference was due to bitterness or another sensory attribute.

The primary sensory evaluation objective was to evaluate the unfractionated Protex 7L hydrolysate and its respective fractions using the bitter/not bitter test. This was accomplished and our hypothesis that small molecular weight peptides are responsible for bitterness was found to be correct. Fraction 3 of the Protex 7L hydrolysate, a low molecular weight fraction, was identified as bitter tasting. This fraction may be responsible for the

bitterness of the hydrolysate. The initially determined molecular weight range of 1-5 kDa for fraction 3 agrees with the research of Cho and others (2004) who reported that the most intensely bitter fractions from two commercial soy protein hydrolysates included peptides of 1-4 kDa. Although fraction 4, containing peptides < 1 kDa, was not identified as bitter, this fraction contained all of the buffer components which may have masked any bitterness. For future studies, it is recommended that fraction 3 include an amino acid profile and determine a more accurate range of molecular weight. If the sensory evaluation of the hydrolysate fractions were repeated it would be interesting to see if the same bitterness conclusions are found with different sensory methods and greater sample quantities.

The second sensory objective was to compare each panelist's sensitivity to caffeine, quinine, leucine, and phenylalanine in relation to MN-treated soy hydrolysate. It was determined that neither caffeine, quinine, leucine, nor phenylalanine was an ideal screening compound for selecting panelists sensitive to soy hydrolysate. Caffeine or quinine is used as a screening compound in nearly all studies on soy hydrolysate bitterness (Cho and others 2004; Kodera and others 2006; Li and others 2008; Seo and others 2008). However, studies on the evaluation of soy hydrolysate that screen panelists using caffeine or quinine may be selecting a portion of panelists who are not sensitive to soy hydrolysate while dismissing a portion of panelists that are sensitive. More research is needed to find an ideal compound for screening. Synthetic bitter peptides may be a possible screening compound to study.

The third sensory evaluation objective was to determine whether the bitterness in leucine or phenylalanine closely resemble the bitterness in MN soy hydrolysate. Although it has been suggested that bitterness is caused in part by hydrophobicity, the hydrophobic free amino acids of leucine and phenylalanine did not show the same bitterness as soy

hydrolysate. These findings suggest that free hydrophobic amino acids are not be responsible for the bitterness in soy hydrolysates and are not an ideal standard to use in training a sensory panel for hydrolysate bitterness. Synthetic bitter peptides may elucidate a similar bitterness as in soy hydrolysate, and would be an interesting compound to use in future sensory research.

REFERENCES

Cho M, Unklesbay N, Hsieh F, Clarke A. 2004. Hydrophobicity of bitter peptides from soy protein hydrolysates. J Agric Food Chem 52:5895-901.

Cornelis M, El-Sohemy A, Campos H. Genetic polymorphism of the adenosine A2A receptor is associated with habitual caffeine consumption. Am J Clin Nutr 86(1):240-4.

Kodera T, Asano M, Noriki N. 2006. Characteristic property of low bitterness in protein hydrolysates by a novel soybean protease D3. J Food Sci 72(9):609-13.

Li L, Yang Z, Yang X, Zhang G, Tang S, Chen F. 2008. Debittering effect of *Actinomucor elegans* peptidases on soybean protein hydrolysates. J Ind Microbiol Biotechnol 35:41-7.

Lock S. 2007. Flavor characteristics of soy products modified by proteases and alpha-galactosidase. [MSc thesis]. Ames, IA.: Iowa State University. 95 p. Available from: Iowa State Park Library, Ames, IA.

Seo W, Lee H, Baek H. 2008. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. J Food Sci 73:41-6.

APPENDIX A. RECRUITMENT EMAIL TO PANELISTS

You are invited to participate in a sensory evaluation test of soy protein hydrolysates. The evaluation will require that you be trained to evaluate bitterness of soy protein hydrolysate samples in water. Training will require about 6-10 hours.

Training sessions will be held from 2:30-3:30 on Monday, Wednesdays, and Fridays. Training will begin Oct. 27 and evaluation should finish before Thanksgiving Break. During evaluation, you will taste 5-6 samples of soy protein hydrolysate in water and evaluate bitterness for each sample. All volunteers must be 18 years of age or above and have no soy protein allergies.

Your reward will be a food snack, a contribution to soy research, and financial compensation for your time. Please reply to me if you are interested in participating.

Thank you,
Heidi Geisenhoff
Graduate Research Assistant
Food Science & Human Nutrition
2312 Food Sciences Building
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APPENDIX B. INFORMED CONSENT DOCUMENT

Sensory Evaluation of Bitterness in Soy Protein Hydrolysates 2008-9

This research is being conducted by Dr. Cheryll Reitmeier and Heidi Geisenhoff, Department of Food Science and Human Nutrition, Iowa State University. **Participation in this study is voluntary.** The evaluation involves the sensory evaluation of soy protein hydrolysates. Soy protein isolate was treated with food grade enzymes in a food grade laboratory in the Food Sciences Building, ISU.

You must be 18 years of age or older to participate. Volunteers will sample various bitter food items in training, which may include tea, coffee, chocolate, and dilute solutions of caffeine or quinine. Taste evaluations will be done on samples of soy protein isolate and soy protein hydrolysates in water. You will be asked to evaluate 5-6 samples (1-oz portions). Participants may skip any questions you do not feel comfortable answering. Sensory evaluation will be conducted in the CDFIN sensory facility, 1121 Human Nutritional Sciences Bldg., ISU.

Responses to the sensory evaluation will be used only in coded statistical analysis without reference to the respondent. There is risk associated with the evaluation of soy protein hydrolysates if you have an allergy to soy. If you are allergic to soy or any of the bitter food items used in training, you should not participate in this study. Benefits include a reward of food at each session, a significant contribution to soy research, and $50 compensation. Dr. Reitmeier (294-4325, creitmei@iastate.edu) will be available throughout the study to answer questions associated with the evaluation.

Emergency treatment of any injuries that may occur as a direct result of participation in this research is available at the Iowa State University Student Health Services, and/or referred to Mary Greeley Hospital or another physician. Compensation for any injuries will be paid if it is determined under the Iowa Tort Claims Act, Chapter 669 Iowa Code. Claims for compensation should be submitted on approved forms to the State Appeals Board and are available from the ISU Office of Risk Management and Insurance.

If you have any questions about the rights of research subjects or research-related injury, please contact **IRB Administrator**, **(515) 294-4566, IRB@iastate.edu, or Director, Office of Research Assurances, (515) 294-3115, 1138 Pearson Hall, Ames, IA 50011.**

***** I understand the research being conducted and agree to evaluate soy protein hydrolysate. *****

_____ NAME

_____Birth date

_____ DATE

APPENDIX C. 3-ALTERNATIVE FORCED CHOICE TEST

Name _____
Panelist Number_____

Please rinse your mouth with water before starting the test. Your will receive 8 sample sets. Each set has 3 samples for you to evaluate. Taste the coded samples in each set in the order presented, from left to right. Within each group of three, circle the code number of the sample that is odd. Expectorate all samples. Rinse your mouth with water and each some cracker between sets. Do not go back once you have completed a set.

1	040	906	568
2	229	799	697
3	658	256	510
4	839	282	208
5	722	007	540
6	555	234	101
7	143	062	376
8	332	223	461

APPENDIX D. BITTER/ NOT BITTER TEST

Date_____ Name_____

There are two soy protein isolate “blanks” and a sample with a soy protein hydrolysate . After tasting, select the odd sample and then check whether it is bitter or different because of some other sensory attribute. Please taste the sample in the order they are presented (left to right) and circle the odd sample after following the tasting procedure:

1. Rinse mouth with water.
2. Place two drops of the first sample in the set on the back of your tongue and push up to the roof of your mouth.
3. Wait at least 15 seconds while deciding whether you can detect any bitterness.
4. Eat a portion of cracker and drink/rinse with water.
5. Rinse eyedropper with water at least twice to cleanse.
6. Wait at least 15 more seconds before repeating procedure steps 2-6 with the second and third sample in the set.
7. After completing a set of three samples, wait one minute before continuing on to next set.

**Shake each sample vigorously or use eyedropper to disperse protein before tasting.

1st Set: 115 976 672

Check one: ___ odd sample is bitter
___ odd sample is different due to another sensory attribute

2nd Set: 086 147 926

Check one: ___ odd sample is bitter
___ odd sample is different due to another sensory attribute

3rd Set: 822 358 105

Check one: ___ odd sample is bitter
___ odd sample is different due to another sensory attribute

4th Set: 542 740 210

Check one: ___ odd sample is bitter
___ odd sample is different due to another sensory attribute

APPENDIX E: INDIVIDUAL PANELIST RESPONSES TO HYDROLYSATE FRACTIONS[a]

Panelist	F1			F2			F3			F4		
	Odd	Bitter	Comments	Odd	Bitter	Comments	Odd	Bitter	Comments	Odd	Bitter	Comments
1	Y	N	Not sure	Y	N	Sour	Y	Y	Very bitter	Y	N	Extremely salty
2	Y	N	Hard to describe, some off-flavor	N	/		N	/		Y	N	Strongly salty
3	Y	Y		N	/		Y	Y		Y	Y	Salty
4	Y	N	Salty	Y	N	Salty	Y	N	Soapy	Y	N	Salty
5	Y	N	Salty	Y	N	Salty	Y	Y		Y	N	Salty
6	Y	Y		Y	N	Difference subtle, hard to pinpoint	Y	Y		Y	N	Salty
7	Y	Y		Y	N	Slight hint of salt	Y	Y		Y	N	Salty
8	N	/		N	/		Y	Y		Y	N	Salty
9	N	/		Y	N	Mildly salty	Y	Y		Y	Y	Salty
10	Y	Y		Y	N	Cardboard	Y	N	Cardboard	Y	N	Slightly bitter, very salty
11	Y	N	Couldn't distinguish difference	Y	N	Couldn't distinguish difference	Y	N	Soapy	Y	N	Salty
12	Y	N	Salty	Y	N	Slightly salty	Y	Y		Y	N	Salty
13	Y	N		Y	N		Y	N		Y	N	Salty
14	Y	N	Soapy	Y	N	Soapy, grassy	Y	Y		Y	N	Salty and meaty
15	Y	N	Slightly sweet, not much difference	Y	N	Heavier mouth feel	Y	Y	Slightly bitter, mostly chalky and astringent	Y	Y	Very salty, slight bitterness

[a]A '/' indicates an irrelevant bitter response because the panelist did not select the correct sample as odd

APPENDIX F: INDIVIDUAL PANELIST RESPONSES TO UNFRACTIONATED HYDROLYSATE*

Unfractionated hydrolysate			
Panelist	Odd?	Bitter?	Comments
1	Y	N	mushrooms
2	Y	N	salty, oxidized nuts flavor
3	Y	Y	salty
4	Y	N	salty, doesn't taste good
5	Y	N	salty
6	Y	Y	heavy on tongue, tinge of bitterness
7	Y	N	absolutely gross, slightly salty, very unpleasant, not really bitter
8	Y	N	salty
9	Y	Y	bitter and salty
10	Y	Y	very bitter and salty
11	Y	Y	kind of bitter, tastes burnt
12	Y	N	salty
13	Y	Y	
14	Y	Y	
15	Y	Y	initially very salty and bitter, unpleasant

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