SOY PROTEIN HYDROLYSATE; SOLUBILITY, THERMAL STABILITY, BIOACTIVITY, AND SENSORY ACCEPTABILITY IN A TEA BEVERAGE

A THESIS

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Abstract

Protein solubility affects the quality of many food products, especially high-protein beverages (>4.2% protein). Beverages formulated with soy protein at >4.2% protein content are currently unavailable in the market due to thermal challenges affecting the protein solubility and also due to flavor challenges. Enzyme hydrolysis of soy protein may lead to enhanced solubility and thermal stability. However, bitter taste caused by hydrolyzing soy protein is a limiting factor to using SPH in food applications. Therefore, controlled and limited hydrolysis of 2-8% is required to minimize the production of bitter peptides. Another advantage of controlled hydrolysis of soy protein is the release of bioactive peptides. Among the many physiological benefits of bioactive peptides, antihypertensive activity has gained much attention.

Therefore the objectives of this work were 1) to optimize hydrolysis conditions of soy protein for enhanced solubility, thermal stability and bioactivity, while maintaining low degree of hydrolysis (DH); and 2) to determine the sensory acceptability of lemon flavored iced tea beverages formulated with soy protein hydrolysate at various concentrations.

Soy protein isolate (SPI) was hydrolyzed by papain, bromelain, trypsin, and alcalase at various enzyme levels while keeping other hydrolysis conditions constant. Enzymatic activities were optimized based on DH measurements. Degree of hydrolysis (DH) was measured using O-phthaldialdehyde method. Samples were subjected to SDS-PAGE analysis to monitor enzyme selectivity. ACE inhibitory activity was measured using a standardized assay. For determination of solubility and thermal stability, solutions (5% w/v) of hydrolysates and SPI were subjected to heat treatment at 95°C for 60 min followed by centrifugation and determination of protein content in the supernatant using a nitrogen analyzer. Solubility and thermal stability at different protein concentrations (1%, 2.5%, 5%, and 7%) in a black tea beverage were also measured. Alcalase-hydrolyzed SPI was chosen for the sensory study as it had the highest solubility, thermal stability, and antihypertensive activity among the hydrolysates. Nine formulations were prepared, including a control which was a tea

with no added protein and the four different protein concentrations containing either SPI or alcalase-hydrolyzed SPI. One-hundred-one subjects rated overall liking, flavor, aroma, appearance, and mouthfeel on an 11-point hedonic scale and rated intensity of bitterness and off flavor on a line scale.

Hydrolysates produced using 2.654 GDU of bromelain, 0.012 AU-A of alcalase, 19,680 USP units of papain, and 235,000 U of trypsin had DH of 3.98%, 3.57%, 6.77%, and 2.60%, respectively, thus were selected for further experiments. Use of different enzymes resulted in distinctive differences in hydrolysis patterns. Among the four produced hydrolysates, alcalase-hydrolyzed SPI was highly soluble (75.25%) at relatively high protein concentrations, thermally stable, and possessed the most pronounced antihypertensive activity (IC_{50}=0.263 mg protein/ mL). Lemon flavored iced tea beverages fortified with alcalase-hydrolyzed SPI at various protein concentrations (1 up to 7 % protein) were found to be acceptable. The beverages formulated with the hydrolysate at 1%, 2.5%, and 5% protein content were liked as much as those without added protein. Beverages formulated with up to 5% hydrolysate also had significantly less bitter taste and off flavors than SPI beverages. These findings can be utilized in the development of high protein fortified beverages, which is acceptable with relatively low bitter taste and noted physiological benefits.

TABLE OF CONTENTS

Abstract……ii

List of Table……...…….ix

List of Figures…….……..…xxi

1. Literature review

1.1 Introduction……..……..1

1.2 Soybean……..…….4

1.3 Soy protein

1.3.1 Chemical composition…….…….....5

1.3.2 Functionality…….……..6

1.3.2.1 Solubility……..…….7

1.3.2.1.1 Thermal stability……..…….8

1.4 Soy protein ingredients

1.4.1 Soy flour……...……..10

1.4.2 Soy protein concentrate……...…….10

1.4.3 Soy protein isolate……...……..10

1.4.4 Soy protein hydrolysate……..……..10

1.4.4.1 Enzymatic hydrolysis……..……...11

1.5 Bioactive peptides…….……...13

1.5.1 Soy bioactive peptides……..…….13

1.5.1.1 Antihypertensive activity…….….14

1.6 Soy protein beverages…….…….……15

1.6.1 Sensory acceptability of soy protein beverages……..……16

1.6.1.1 Beany flavor……..….17

2. Soy protein hydrolysate; solubility, thermal stability, and bioactivity

2.1 Overview…….……...…...…...19

2.2 Introduction……...……...…20

2.3 Materials and methods

2.3.1 Materials……..……..24

2.3.2 Experimental design……....….24

2.3.3 Preparation of soy protein isolate (SPI) ……..…….26

2.3.4 Preparation of papain-, trypsin-, bromelain-, and alcalase-hydrolyzed SPI……...28

2.3.5 Degree of hydrolysis (DH) …….....28

2.3.6 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)…….…….....30

2.3.7 Solubility and thermal stability……..…….31

2.3.8 Measurement of ACE Inhibitory Activity……..……32

2.3.9 Dumas method……..……35

2.3.10 Micro BCA protein method……...…….....35

2.3.11 Turbidity……...…….36

2.3.12 Statistical analysis…….....36

2.4 Results and discussion

2.4.1 Degree of hydrolysis and hydrolysis pattern……..…36

2.4.2 ACE inhibitory activity of soy protein hydrolysates...……..39

2.4.3 Measurement of SPI and SPH solubility and thermal stability……...……41

2.4.4 Measurement of SPI and SPH solubility in black tea…...…44

2.4.5 Turbidity measurement……..…….47

2.5 Conclusion……..……..49

3. Sensory acceptability of a lemon flavored iced tea formulated with soy protein

3.1 Overview..……..……...51

3.2 Introduction……..……52

3.3 Materials and methods

3.3.1 Materials……..……..55

3.3.2 Preparation of SPI ……..…….55

3.3.3 Preparation of alcalase- hydrolyzed SPI……..……..55

3.3.4 Beverage preparation……..…….56

3.3.5 Products……...57

3.3.6 Subjects……..……57

3.3.7 Experimental procedure…….…….58

3.3.8 Data analysis…….……....58

3.4 Results

3.4.1 Overall liking……..…….59

3.4.2 Bitterness……..……61

3.4.3 Mouthfeel liking……..……..62

3.4.4 Flavor liking……..……63

3.4.5 Aroma liking..……..……65

3.4.6 Appearance liking……..……..66

3.4.7 Off flavor……..……..…68

3.4.8 Tea frequency……69

3.4.9 Protein beverage frequency…….……69

3.4.10 Gender effect……...70

3.5 Discussion……...……..71

3.6 Conclusion……...…….……73

4. Overall conclusions, implications, and recommendations……...…….…74

References……..……..75

Appendices

Appendix A: Hydrolysis produced at different enzyme activity level……...96

Appendix B: Hydrolysis patterns of heated and non-heated hydrolysates…....……97

Appendix C: Sensory study supporting documents……..…..……..101

Appendix D: Analysis of variance table for determining the effect of enzyme level on each hydrolysate…….……..106

Appendix E: Analysis of variance table for determining the effect of enzyme hydrolysis on ACE activity slope…..……107

Appedix F: Analysis of variance tables for determining the effect of heat treatment on solubility…….……..108

Appendix G: Analysis of variance tables for determining the effect of enzyme hydrolysis on solubility …….111

Appedix H: Analysis of variance table for determining the effect of protein concentration on solubility……..……..113

Appendix I: Analysis of variance tables for determining the effect of enzyme hydrolysis on turbidity…….……..114

Appendix J: Analysis of variance tables for determining the effect of heat treatment on turbidity…….……...115

Appendix K: Analysis of variance table for determining the effect of product and judge on liking ratings……...……116

Appendix L: SAS codes for determining the effect of tea drinking frequency, protein beverage drinking frequency, and gender on liking ratings……..……117

List of Tables

Table 1. Hydrolysis conditions for papain-, trypsin-, bromelain-, and alcalase-hydrolyzed SPI…….28

Table 2. Compostion of 15% acrylamide gel and 4.5% stacking gel for SDS-PAGE……...31

Table 3. Reaction mixture for ACE activity assay……..……....35

Table 4. Degree of hydrolysis of bromelain-, alcalase-, papain-, and trypsin-hydrolyzed SPI……..…….38

Table 5. Solubility measurement of SPI and SPH samples…….……...44

Table 6. Solubility measurement at 5% of SPI and SPH samples in black tea…..….46

Table 7. Solubility measurement of 1%, 2.5%, 5%, and 7% SPI and alcalase-hydrolyzed SPI in black tea ……47

Table 8. Turbidity of alcalase-, bromelain-, papain-, and trypsin-hydrolyzed sample…….49

Table 9. Formulations of soy protein beverages with lemon flavored iced tea ….....57

Table 10. Mean (n=101) overall liking scores……..……..61

Table 11. Mean (n=101) bitterness intensity scores……..…….62

Table 12. Mean (n=101) mouthfeel liking scores……..…….……63

Table 13. Mean (n=101) flavor liking scores……..…..……...65

Table 14. Mean (n=101) aroma liking scores……..…..……..66

Table 15. Mean (n=101) appearance liking scores……...……..67

Table 16. Mean (n=101) off flavor intensity scores……..…….…..……68

Table 17. Mean scores of people grouped by tea drinking frequency……..……69

Table 18. Mean scores of people grouped by protein beverages drinking frequency…….70

Table 19. Mean scores of men and women……..……..…..71

Table 20. Anaysis of variance on the effect of enzyme level on each hydrolysate…….106

Table 21. Analysis of variance of ACE activity slopes on the effect of enzyme hydrolysis by papain, bromelain, trypsin, and alcalase ……..…..…..…107

Table 22. Analysis of variance of heat treatment effect on solubility of each hydrolysate at 5% protein content in water …….…….108

Table 23. Analysis of variance of heat treatment effect on solubility at 5% protein content in a black tea solution……..…….109

Table 24. Analysis of variance on alcalase SPH solubility effect of heat treatment at each protein concentration in tea……..….109

Table 25. Analysis of variance on SPI solubility effect of heat treatment at each protein concentration in tea……..……110

Table 26. Analysis of variance on solubility effect of the different hydrolysate without heat treatment in water…….……111

Table 27. Analysis of variance on solubility effect of the different hydrolysate with heat treatment in water……..……111

Table 28. Analysis of variance on solubility effect of the different hydrolysate without heat treatment in tea……..……111

Table 29. Analysis of variance on solubility effect of the different hydrolysate with heat treatment in tea…….……..112

Table 30. Analysis of variance on SPI and alcalase SPH solubility effect of concentration with and without heat treatment……..……113

Table 31. Analysis of variance on turbidity effect of the different hydrolysate without heat treatment…….114

Table 32. Analysis of variance on turbidity effect of the different hydrolysate with heat treatment…….114

Table 33. Analysis of variance on the effect of heat treatment on tubidity of each hydrolysate……...……..…115

Table 34. Analysis of variance for determining the effect of product and judge on liking ratings…….……...……..116

List of Figures

Figure 1. Experimental design of the enzymatic hydrolysis using papain, trypsin, bromelain, and alcalase enzymes. ……..……..25

Figure 2. Experimental design of the heat treatment of selected hydrolysates……...26

Figure 3. Hydrolysis patterns of papain-, bromelain-, alcalase-, and trypsin hydrolyzed SPI with DH 2-8%.……...…39

Figure 4. ACE inhibitory activity of trypsin- (117500 U/ mL), bromelain- (1.327 GDU/ mL), papain- (9840 USP units/ mL), and alcalase-(0.006 AU-A/ mL) hydrolyzed SPI samples…..…….40

Figure 5. Dose-dependency inactivation of ACE by alcalase-hyrolyzed SPI (0.006 AU-A/ mL)……..……41

Figure 6. Mean (n=101) overall liking scores on an 11-point LAM scale……..…. 60

Figure 7. Mean (n=101) bitterness intensity scores on a Line scale……...61

Figure 8. Mean (n=101) mouthfeel liking scores on an 11-point LAM scale……....63

Figure 9. Mean (n=101) flavor liking scores on an 11-point LAM scale. ……...…..64

Figure 10. Mean (n=101) aroma liking scores on an 11-point LAM scale. ……..…66

Figure 11. Mean (n=101) appearance liking scores on an 11-point LAM scale…....67

Figure 12. Mean (n=101) off flavor intensity scores on a Line scale…….68

Figure 13. Hydrolysis patterns of papain-, bromelain-, alcalase-, and trypsin hydrolyzed SPI with different activities. ……96

Figure 14. Hydrolysis patterns of heat treated SPH with DH 2-8% and controls under (a) non-reducing condition and (b) reducing condition (β-mercaptoethanol). ……...97

Figure 15. Hydrolysis patterns of non-heated SPH with DH 2-8% and controls under (a) non-reducing condition and (b) reducing condition (β-mercaptoethanol)…….99

1. Literature review

1.1 Introduction and Objectives

Interest in soy protein consumption has increased since the Food and Drug Administration (FDA) released a statement that "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease". This statement was based on reviewing many long term studies on the effect of soy protein on cardiovascular diseases. Consumption of soy protein also has several other health benefits including, reduction in cholesterol level and body fat, prevention of osteoporosis, reduced incidences of cancers, and antihypertensive activity.

In addition to the health benefits, soy protein has many functional properties that can be utilized in various food applications. Functional properties of soy protein include solubility, water-holding capacity, viscosity, gelation, foaming, and emulsification. Soy proteins isolate (SPI) is the most refined among the different soy protein ingredients (soy flour and soy protein concentrate), have bland flavor and light color, the highest protein content, and the best functionality. Therefore, SPI is utilized in a wide range of food applications such as nutritional bars, meat analogs, infant formula, and beverages.

Solubility is one of the most important functional properties for high protein beverage applications. High protein beverages have gained attention immensely in recent years especially due to the associated physiological benefits. According to the FDA, protein content of the beverage should be greater than 4.2% in order to have a high protein claim. However, formulating high protein beverages (>4.2%) is one of the biggest challenges in beverage manufacturing because of protein solubility and thermal instability when present at relatively high concentrations. Given these challenges, and the potential health benefits of soy, it is worth while investigating soy protein solubility and ways to improve it for the potential use in high protein beverages.

Soy protein needs to go through thermal treatment in order to inactivate anti nutritive factors such as trypsin inhibitor, which can cause growth impairments or may also be lethal without proper inactivation. It is, therefore, necessary to heat at

95°C for 60 min to reduce trypsin inhibitor activity at least by 90% without disrupting essential amino acids, including lysine and methionine. The effect of such heat treatment on soy protein solubility and thermal stability is not well investigated, especially at high protein concentrations.

Researchers showed that limited hydrolysis of soy protein can result in enhanced functionality such as solubility, foaming, emulsification, and gelation. Several enzymes were used at various conditions, including pH, temperature, time, and enzyme to substrate ratio. Use of different enzymes at various conditions resulted in hydrolysates of distinct functionality. It is, therefore, important to control enzymatic conditions to result in a directed functionality enhancement, such as enhanced solubility.

Moreover, limited hydrolysis of soy protein has been shown to release bioactive peptides. Biologically active peptides are characterized as protein-derived amino acid sequences that may have regulatory and physiological effects on the human body beyond normal and adequate nutrition once released from the parent protein. Bioactive peptides can be liberated via enzymatic digestions, both in the human gastrointestinal tract and during food processing. While *in vivo* enzymatic digestion is random and thus may or may not result in the release of bioactive peptides, *in vitro* enzymatic hydrolysis can be controlled to result in the production of biologically active peptides. Bioactive peptides from soy protein have many physiological characteristics depending on the released amino acid sequences, including anticancerous, antioxidant, antimicrobial, antithrombotic, hypercholesterolemic, and antihypertensive.

Among the many physiological activities of soy bioactive peptides, antihypertensive activity has gained significant attention. Several antihypertensive peptides were identified in enzyme-hydrolyzed soy proteins. However, soy bioactive peptides are far less investigated compared to the bioactive peptides derived from milk proteins. To the best of our knowledge, products containing soy-derived antihypertensive peptides are not available in the US market. Additionally, soy protein hydrolysate with improved solubility and thermal stability, produced using

controlled enzymatic hydrolysis, has not been investigated for its potential antihypertensive activity. Improving the thermal stability and solubility of SPI in concert with the release of antihypertensive peptides is appealing for the developing of stable high protein beverages (>4.2% protein) with noted physiological benefit. However, developing soy protein beverages at high protein concentration causes flavor challenges. The presence of an unfavorable beany flavor and chalky mouthfeel of the natural soy plays a major role in deterring consumers from soy protein beverages especially when soy protein content is high. The removal of these unfavorable attributes appears to be difficult. Therefore, more work needs to be done to improve the acceptability of soy protein beverages.

The objectives of this study were:

1) To optimize hydrolysis conditions of soy protein for enhanced solubility, thermal stability and bioactivity, while maintaining low degree of hydrolysis (DH).
2) To determine the sensory acceptability of lemon flavored iced tea beverages formulated with soy protein hydrolysate at various concentrations.

Our hypotheses were:

1) Controlled enzymatic hydrolysis will enhance solubility, thermal stability, and result in antihypertensive activity.
    - 1.1 Different enzymes at various levels will result in distinct hydrolysis profile and will therefore result in different solubility and antihypertensive activity pattern.
    - 1.2 Limited degree of hydrolysis will enhance or maintain solubility and result in pronounced antihypertensive activity
2) Lemon flavored iced tea beverages formulated with soy protein subjected to limited and controlled hydrolysis will have good acceptability.

1.2 Soybean

Soybeans are among the most commonly consumed crops in the world and can be found in many types of foods. Particularly, soybean-based products have been consumed in Asian cultures over hundreds of years; whereas, in Western cultures, they have only been in use since the late 19th century, mainly for animal feed and oil extraction until the 1950s (Erdman and Fordyce, 1989; Quak and Tan, 1998). However, since soybeans are an excellent source of good quality protein, soy food consumption in the US has undergone tremendous growth. In addition to the nutritional quality, soybean is an economically and industrially feasible crop to plant (Messina and Messina, 1991; Riblett and others, 2001). Developing second-generation soy foods such as soy cheese, soy meat analogues, and soy frozen desserts has also stimulated the emergence of a substantial soy food market in the US during the past few decades, with soy food sales increasing from $1 billion in 1996 to $4.5 billion in 2009 (Soy foods Association of North America, 2010). This remarkable growth is predominantly attributed to the increasing consumer awareness of soybean-based foods as a natural, health-promoting product (Messina and Messina, 1991).

Soybean contains approximately 40% protein, 35% carbohydrate, 20% lipid, and 5% ash (USDA, 2010). Soy protein is a “complete protein” since it provides all of the essential amino acids for human nutrition such as lysine and methionine. According to the protein digestibility corrected amino acid score (PDCAAS), soy protein possesses a comparable biological value (0.96) to that of beef (0.92) and milk (1.0), indicating high quality protein based on the amino acid requirements of humans (FAO/WHO, 1991).

Interest in soy protein consumption increased considerably since the United States Food and Drug Administration approved the health claim of “25 g of soy protein in a day, as a part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease” (FDA, 1999). This statement was established by reviewing many long-term studies on the effect of soy protein on cardiovascular diseases (Kito and others, 1993; Sirtori and others, 1995; Anderson and others, 1995; Anthony and others, 1996). In addition to cardiovascular diseases, studies have shown that

consumption of soy protein has several other positive effects on health, including antihypertensive activity (De Leo and others, 2009), reduction in cholesterol level (Pipe and others, 2009) and body fat (Aoyama and others, 2000), prevention against osteoporosis (Bawa, 2010) and reduced incidences of cancers such as stomach (Nagata, 2000), colorectal (Yang and others, 2009), and breast (Messina, 1999; Lamartiniere, 2000; Messina and Loprinzi, 2001).

1.3 Soy protein

1.3.1 Chemical composition

Soy protein accounts for 40 to 45% of the whole soybean (USDA, 2010). Two main classes of storage proteins, glycinin and β-conglycinin, contribute about 65 to 80% of the total protein in a typical soybean seed. Soy protein also constitutes of many different enzymes such as lipoxygenase, chalconesynthase, catalase, and urease, however, they account for less than 1% of the total proteins. Based on their sedimentation coefficient, glycinin and β-conglycinin are categorized as 11S and 7S, respectively (Thanh and Shibasaki, 1976; Wang and De Mejia, 2005). Glycinin and β-conglycinin have considerably different physicochemical properties, including chemical composition and molecular configuration.

Glycinin is a hexamer consisting of six monomers with an average molecular weight of 320 to 375 kDa. Each monomer of glycinin is composed of a pair of polypeptide chains, an acidic amino acid chain at C-terminus and a basic chain at N-terminus, held together via a disulfide linkage. Three monomers are linked together to form a trimer via hydrophobic interactions and two trimers are stacked on top of each other to form a hexamer by electrostatic interactions and hydrogen bonding. Five major subunit types can exist in glycinin, G1, G2, G3, G4, and G5. Three subunits, G1, G2, and G3 possess about 90% of sequence homologies with higher molecular weight and sulfuric amino acid content than that of G4 and G5, which also have about 90% sequence homologies (Garcia and others, 1997; Wang and De Mejia, 2005).

β-conglycinin is a trimer with a molecular weight of around 180 kDa, composed of three subunits held by hydrophobic interactions. Four subunit types

have been found in β-conglycinin, including three major subunits α, α', and β and one minor subunit γ. The trimer can exist either as a homotrimer, which is made up of three identical subunit types, or a heterotrimer, which is made up with more than one different subunit type. Subunit shares about 53% sequence homologies (Garcia and others, 1997; Wang and De Mejia, 2005).

1.3.2 Functionality

The functional properties of soy protein are governed mainly by the major proteins, glycinn and β-conglycinin. Functional properties relevant to food application include solubility, water-holding capacity, viscosity, gelation, foaming, and emulsification (Kinsella, 1979; Garcia and others, 1997). These properties are determined by the physicochemical characteristics such as amino acid composition, molecular size, structural conformation of the protein, and inter- and intra- molecular interactions as well as environmental factors such as pH, temperature, and ionic strength (Kinsella, 1979; Lampart-Szczapa, 2001).

The dominant components of soy protein, glycinin and β-conglycinin, possess considerably different functional properties owing to differences in amino acid composition and molecular configuration (Utsumi and others, 1997). The larger molecular weight and high number of disulfide linkages in the glycinin subunit contribute to better gelation properties. Since glycinin contains acidic amino acids, glycinin contributes to the formation of hard and turbid gels. Turbidity of the gels increases as the amount of sulfhydryl groups and protein concentration increase (Utsumi and others, 1997). Main driving forces for a glycinin gel formation are disulfide bridges, hydrogen boding, and hydrophobic interactions. β-conglycinin, on the other hand, forms weak and transparent gels due to the absence of disulfide linkages (Maruyama and others, 1999; Maruyama and others, 2002). β-conglycinin has a smaller molecular weight and a balance between surface hydrophobicity and hydrophilicity that lead to desirable emulsifying and foaming properties. Hydrophobic pockets on the surface of β-conglycinin allow it to absorb and rearrange more rapidly at the oil/water interface (Utsumi and others, 1997).

1.3.2.1 Solubility

Solubility is one of the most valuable functional properties of food proteins in general. Highly soluble protein is required to obtain optimum functionalities. Greater protein solubility generally correlates with good gelling, foaming and emulsifying properties. Protein needs to be soluble to be able to form a gel. In order to effectively act at the interface in foam and emulsion systems, the protein should be soluble in the aqueous phase to allow it to migrate to, absorb at, unfold, and rearrange at the interfaces. Additionally, solubility is related to water holding capacity. In order to be easily incorporated into and retain water, protein should be soluble in a system. Viscosity of the protein is also significantly influenced by solubility. Soluble protein is necessary to allow protein to be able to absorb water and then thicken the protein dispersion (Kinsella, 1984; Nakai and Li-Chan, 1993).

Solubility is not only important in optimizing such functionalities but is also considered to be the most decisive criterion intended for formulating beverages with relatively high protein concentration (Kinsella, 1979; Phillips and others, 1994). Formulating high protein beverages is one of the big challenges in beverage manufacturing because solubility is highly affected by protein concentrations. As protein concentration increases, there is more chance for protein molecules to get closer to each other, thus promoting protein-protein interactions and lowering the solubility.

Similar to the other functional properties, solubility is significantly regulated by environmental conditions such as pH, ionic strength, and temperature. In general, soy protein solubility as a function of pH is a U-shaped curve with solubility being higher on either side of the isoelectric point of the soy proteins, which is around pH 4.5 (Tsukada and others, 2006; Jiang and others, 2010). Since the protein dispersion has no net charge at its isoelectric point, the protein molecules have minimum electrostatic repulsion and interact less with water, thus protein-protein association is mainly pronounced, leading to aggregation and precipitation. As the pH shifts from the isoelectric point, the protein molecules gain excess positive or negative charges, resulting in strong electrostatic repulsion between the molecules, and strong water-

protein interactions, leading to an increase in solubility (Van Megen, 1974; Phillips and others, 1994).

The influence of ionic strength on the solubility of soy protein has also been studied. Researchers compared soy protein solubility as a function of pH when ionic strength was at 0, 0.2, and 0.5. As salt concentration increased, the solubility increased between pH 4 and 5 and decreased at a pH lower than 3 and higher than 7 (Renkema and others, 2002). At pH around the isoelectric point, high salt concentration increased charge load on the protein, greatly enhancing protein solubility. At pH lower than 3 and higher than 7, on the other hand, high ionic strength caused shielding of charges, resulting in a lower electrostatic repulsion and lower solubility (Lakemond and others, 2000; Renkema and others, 2002).

1.3.2.1.1 Thermal stability

As with other environmental factors, thermal treatment also significantly affects protein solubility. The thermal effect is particularly important because soy protein needs to go through thermal treatment in real food systems. Therefore, it is necessary to investigate how solubility is altered upon heating.

During processing, food products containing soy protein are subjected to various thermal treatments to destroy anti-nutritive constituents such as trypsin inhibitors, phytic acids, tannin, and agglutinin (Bajpai and others, 2005; Ryan and others, 2008). Exposure to high concentrations of these anti-nutritive constituents can cause serious growth impairments or may be lethal without proper inactivation (Gumbmann and others, 1986).

In general, thermal treatment can cause protein denaturation, association, and aggregation. Thermal denaturation is a process in which proteins change their secondary and tertiary structure. Upon heating, proteins undergo reversible conformational changes. Some of the bonds that stabilize the protein secondary and tertiary structures, including hydrogen, electrostatic, and hydrophobic interactions, are broken and the interior hydrophobic groups get exposed. Hydrophobic intermolecular interactions can then take place leading to protein polymerization.

Additionally, intermolecular disulfide interchanges can take place leading to irreversible polymerization (Yamul and Lupano, 2005; Ryan and others, 2008). These newly formed disulfide linkages greatly contribute to protein aggregation and precipitation, significantly reducing the solubility (Nicorescu and others, 2009; Dissanayake and Vasiljevic, 2009). This process can augment during long term storage. Since protein solubility decreases during food processing and storage due to the thermal denaturation and aggregation, it is critical to investigate ways to improve the protein solubility, specifically for high protein beverage formulation.

Very few studies have investigated the effect of thermal treatment on the solubility of soy protein. Lakemond and others (2000) showed that the solubility of the glycinin subunits at 0.6% of protein concentration was not affected at neutral pH regardless of sodium concentrations when heated at 98°C for 30 min. However, this study was carried out at relatively low protein concentrations. The denatured proteins involved in aggregation may not reduce the solubility significantly upon heating at this low protein concentration. Increasing protein concentration yields more hydrophobic associations and disulfide linkages causing more protein aggregation and reduced solubility. Since solubility is significantly regulated by protein concentrations, solubility measured at higher concentrations is expected to be considerably different from the ones measured at low protein concentrations. To the best of our knowledge, there is no study investigating thermal stability of soy protein as affected by protein concentration greater than 4%. Moreover, no studies have investigated solubility affected by thermal treatment in a real food system such as high protein beverage formulation (>4%). Improving the thermal stability and solubility when protein content is high (> 4%) is desired for production of stable high protein beverages.

1. 4 Soy protein ingredients

1.4.1 Soy flour

Typically, soy flour contains a minimum of 50% protein, the least amount of protein compared to other soy protein ingredients. Soy flour is coarsely ground and

defatted soybean flakes. The intended use of soy flour is predominantly production of baked goods, sauces, and gravies (Lennon and others, 1971; Bookwalter, 1978; Lusas and Riaz, 1995) .

1.4.2 Soy protein concentrate

Soy protein concentrate possesses approximately 70~80% protein expressed on a dry weight basis. Manufacturing soy protein concentrate begins by defatting milled coarse grits and then extracting soluble protein by a moist heat treatment, or by mixing with organic solvents (McAnelly, 1964; Norris, 1964; Schweiger and Mullar, 1973; Lawhon and others, 1977). Soy protein concentrate has finer particles and less off flavor than soy flour and is utilized in food applications such as meat, meat substitutes, baked goods, and nutritional bars (Bookwalter, 1978; Lusas and Riaz, 1995; Mondor and others, 2004).

1.4.3 Soy protein isolate

The most purified form of soy protein commonly used in the food industry is soy protein isolate (SPI). Because of its high protein content (>90%), functional properties, and excellent sensory attributes - mainly bland flavor and light color, SPI is used in a wide range of food applications. Among these are nutritional beverages, infant formula, processed meat, nutritional bars, and dairy product replacements (Elias and others, 1984; Mateos-Aparicio and others, 2008). SPI is produced from defatted soy flakes followed by an alkaline extraction. The soluble protein is precipitated at pH 4.5 and centrifuged to separate the protein from soluble polysaccharides. The precipitate is dispersed in water, neutralized with an aqueous basic solution, and freeze dried (Bookwalter, 1978; Lusas and Riaz, 1995; Tsumura and others, 2004).

1.4.4 Soy protein hydrolysate

Soy protein can be enzymatically hydrolyzed to produce soy protein hydrolysate (SPH). A broad selection of proteases from animal, plant, and microbial sources have been used in the production of SPH, such as trypsin, chymotrypsin,

substilisin, pepsin, thermolysin, papain, bromelain, ficin, and alcalase (Barrett and others, 2004).

Soy protein was initially hydrolyzed to improve protein digestibility for individuals suffering from protein digestion disorders (Mahmoud and others, 1992). The hydrolyzed soy protein is easily absorbed in the small intestine due to the shortened amino acid chain (Ziegler and others, 1998). Due to the improved digestibility, SPH has become popular to incorporate in the production of infant formula as well as a replacement for people who are allergic to dairy based products (Terracciano and others, 2002; De Regil and De la Barca, 2004).

Furthermore, hydrolyzed soy protein has been utilized in various food products like nutritional bars and beverages because of the improved functionalities (Mietsch and others, 1989; Calderon de la Barca and others, 2000). Considering increased food applications of SPH, it is important to understand how enzymatic hydrolysis affects the functional properties.

1.4.4.1 Enzymatic hydrolysis

In general, proteins break down into and release smaller peptides at optimal hydrolysis conditions. Characteristics of the released peptides depend on the type of enzymes and hydrolysis conditions. Specifically, the nature of these released peptides can be affected by many factors, such as the origin of enzymes, enzyme activity and selectivity, enzyme to substrate ratio, and hydrolysis conditions including pH, temperature, and time (Barrett and others, 2004). The released peptides lead to enhanced functional properties depending on their physicochemical properties (Mietsch and others, 1989; Jung and others, 2005; Tsumura and others, 2005).

Gelation was improved when hydrophobic groups were exposed upon hydrolysis (Kuipers and others, 2005; Creusot and Gruppen, 2007; Kuipers and others, 2007). Additionally, the exposed hydrophobic groups facilitated interactions between protein and oil, considerably enhancing emulsification property (Qi and others, 1997; Wu and others, 1998). Similarly, increased surface hydrophobicity also

allows proteins to form more stable foams (Ortiz and Wagner, 2002; Ruiz-Henestrosa and others, 2007; Kuipers and others, 2007).

Furthermore, hydrolysis can induce changes in the balance of net charges that lead to enhanced solubility (Lamsal and others, 2007; Kuipers and others, 2007; Zorin and Baiarzhargal, 2009). These studies, however, were carried out at relatively low protein concentrations of 0.2~1% without any thermal treatment. Since protein solubility is governed by thermal stability and concentration, formulating a stable high protein beverage is one of the biggest challenges in food industry. Therefore, more work needs to be done to investigate how hydrolyzed protein solubility is affected by thermal stability and high protein content.

In addition to the enhanced functional properties, food protein-derived bioactive peptides can be released upon limited enzymatic hydrolysis. Biologically active peptides are characterized as protein-derived amino acid sequences that may have regulatory and physiological effects on the human body beyond normal and adequate nutrition (Korhonen and Pihlanto, 2003; Shahidi and Zhong, 2008). Since not all released peptides are bioactive, released peptides upon hydrolysis play an important role in protein functionality and bioactivity (Chen and others, 2002; Wang and others, 2008; De Leo and others, 2009).

Although hydrolyzed protein has improved functionality and digestibility and may result in bioactive peptide liberation overall, the bitter taste that can accompany hydrolysis limits the utilization of enzyme hydrolysates as food ingredients. Partially hydrolyzed soy protein isolate tends to have a bitter taste caused by the formation of low molecular weight peptides composed of mainly hydrophobic amino acids. The average hydrophobicity of a peptide and the position of the hydrophobic amino acids are highly related to the bitter taste (Matoba and Hata, 1972; Kim and others, 1992).

To minimize the adverse effect on the sensory quality while retaining the desired functionality and bioactivity, controlled and limited degree of hydrolysis (DH) at 2~8% is strongly recommended. Research on whey protein has shown that a low DH (2~8%) is sufficient to obtain enhanced functional properties and maximum liberation of bioactive peptides with minimal detection of bitter low molecular weight

peptides (Schlothauer and others, 2005). Therefore, it is important to maintain a low DH to achieve desired functional properties and bioactivity with reduced adverse effects on sensory quality.

1.5 Bioactive peptides

Various dietary proteins have been identified as sources of bioactive peptides including soy (Shin and others, 2001; Nagasawa and others, 2003; Wang and others, 2008), milk (Kilara and Panyam, 2003; Torres-Llanez Mde and others, 2005; Korhonen and Pihlanto, 2007), fish (Su, 2010), and eggs (Miguel and Aleixandre, 2006). Commonly, many bioactive peptides are relatively short length chains, composed of 2 to 9 amino acids (Kitts and Weiler, 2003). However, some of the peptides containing more than 20 amino acids can still have biological activity (Korhonen and Pihlanto, 2003). Bioactive peptides are naturally inactive within a sequence of the host protein and can be liberated via enzymatic digestions, both in the human gastrointestinal tract and during food processing. While *in vivo* enzymatic digestion is random and thus may or may not result in the release of bioactive peptides, *in vitro* enzymatic hydrolysis can be controlled to release peptides with distinctive bioactivity (Yamamoto and others, 2003; Kitts and Weiler, 2003; Shahidi and Zhong, 2008). Once liberated, these peptides appear to possess specific physiological characteristics depending on the released amino acid sequences, including antihypertensive (Inoue and others, 2009), anticancerous (Mateos-Aparicio and others, 2008), antioxidant (Sarmadi and Ismail, 2010), antimicrobial (Paul and Somkuti, 2010), antithrombotic (Gilani and others, 2008), immunomodulatory (Kanwar and others, 2009), opiate-like (Moller and others, 2008), hypercholesterolemic (Potter, 1995), and mineral binding capacity (Meisel and FitzGerald, 2003).

1.5.1 Soy bioactive peptides

Soy bioactive peptides are far less investigated compared to the bioactive peptides derived from milk and dairy products. As with other bioactive peptide

sources, soy protein derived bioactive peptides are mainly produced through enzymatic hydrolysis. Various selections of proteases, soy protein sources, and hydrolysis conditions result in unique bioactivity profiles of soy protein with different physiological activities such as antihypertensive (Matsui and others, 2010; Nakahara and others, 2010), antidiabetic (Kwon and others, 2010), anticancer (Zhou and others, 2003; Wang and others, 2008), antioxidant (Cassidy, 2003), antiobesity (Nagasawa and others, 2003; Jang and others, 2008), and hypercholesterolemic (Potter, 1995; Lovati and others, 1996). Among the many physiological activities of soy bioactive peptides, antihypertensive activity, which is based on inhibiting angiotensin converting enzyme (ACE), has gained significant attention.

1.5.1.1 Antihypertensive activity

Antihypertensive activity is fundamentally based on inhibiting angiotensin-converting enzyme (ACE) activity, which plays a crucial role in the regulation of blood pressure through the rennin-angiotensin system (RAS). RAS is a hormone-like system which regulates fluid balance and blood pressure in our body (Werner and others, 2008). When the body detects low blood pressure, the hormone angiotensinogen is secreted from liver and cleaved to angiotensin1, an inactive decapeptide, by rennin, which is secreted by the kidney. Angiotensin 1 is then further cleaved by ACE to angiotensin 2, which constricts the blood vessels, resulting in increased blood pressure. When the RAS is abnormally active, the blood pressure will rise too high, thereby, causing hypertension (Mondorf and others, 1998).

Hypertension can eventually lead to many other health problems, including coronary heart diseases, heart failure, stroke, and kidney failure (American Heart Association, 2010). Hypertension is the second leading cause of death and affects approximately 74.5 million individuals, one in every three adults in the US (Institute of Medicine, 2010). The associated medical cost is estimated to be around $74 billion per year (American Heart Association, 2010).

Captopril (D-3-mercapto-2-methylpropyl-L-prolin) is an oral medication that has been adopted for the treatment of hypertension, since it acts as an ACE inhibitor

(Carmona-Calero and others, 2005; Pechanova, 2007). To avoid the associated side effects of the drug such as dry cough, skin rashes, and angioedema, food-based bioactive peptides have received significant attention as a low-cost alternative and preventative treatment of hypertension (Tsevat and others, 1995; Kazerani and others, 2009).

Several antihypertensive peptides were identified in enzyme-hydrolyzed soy proteins (Kodera and Nio, 2002; Cha and Park, 2005; Chiang and others, 2006) and fermented soy products, being indigenous to Asian cuisines historically, including soy sauce (Okamoto and others, 1995), soybean paste (Shin and others, 2001), natto (Okamoto and others, 1995), and tempeh (Aoki and others, 2003). Contrasts to Asia, products containing soy-derived bioactive peptides with ACE inhibitory activity are not available for the US consumers. Therefore, delivering soy-derived antihypertensive peptides in food products that are regularly consumed in the US is a promising area of research. High protein beverages can be used to deliver these peptides.

1.6 Soy protein beverages

Given the potential health benefits of food protein, there is a growing interest in developing protein fortified beverages. Particularly, soy protein beverage sales in the US, according to Beverage Marketing Corporation of New York, have more than doubled since 2000 and will become more entrenched in the mainstream market in the near future (Beverage Marketing Corporation of New York, 2005).

Commonly, three categories of soy protein beverages are available in the US market, soymilk, powder or shake formula, and nutritional beverages. Soymilk contains ~3.5% protein and is considered the most popular drink made from soy. Soy protein powder or shake formula, on the other hand, has a relatively high protein content of ~6.5% to 10.4% when formulated into a beverage following label instructions. Due to the high protein content, protein powders and shakes have gained popularity with bodybuilders, athletes, and health conscious individuals. However, the protein powders and shakes are different from the ready-to-drink beverages. The

dried powder has to be mixed with and dispersed in water, fruit juices, or milk before serving because the proteins are not stable enough to be held in a solution at such high protein percentages, they fall out and precipitate during storage. Nutritional beverages, opaque protein suspensions that use a combination of soy and dairy proteins, contain roughly 3.07~6.5% protein. They are available and advertised in the market as a meal replacement ready-to-drink beverages because of their nutritional quality and convenience.

In 2008, FDA approved a protein concentration of at least 4.2 % to make a label claim of a "high protein beverage" (FDA, 2008). To the best of our knowledge, there is no protein beverage in the US market made with only soy protein at greater than 4.2% protein content. However, developing these soy protein beverages may cause flavor challenges apart from stability issues. Thus, it is crucial to investigate the sensory quality and acceptability of such beverages.

1.6.1 Sensory acceptability of soy protein beverages

A negative attitude towards soy flavor remained even when consumers were informed of the nutritional and health benefits of the products (Teh and others, 2007). The presence of an unfavorable beany flavor and chalky mouthfeel of the natural soy plays a major role in deterring consumers from soy protein beverages especially when soy protein content is high. Moreover, the removal of these unfavorable attributes appears to be difficult (MacLeod and Ames, 1988).

Only a few studies have been conducted on sensory acceptability of soy protein beverages. Potter and others (2007) reported that soy beverages formulated with 2.8% protein and wild blueberries received low acceptability ratings except for the one sweetened with juice concentrate, which was scored slightly below "like moderately" on a 9-point hedonic scale. The researchers also demonstrated that the consumer acceptability of the beverages decreased as the main sensory attributes associated with soy, including painty aroma, green grassy aroma, chalkiness, nutty taste, and bitterness, increased (Potter and others, 2007). A comparison between beverages containing whey and soy protein was addressed by Childs and others

(2007). Meal replacement beverages at 6.5% protein concentration containing either whey protein only or a mixture of whey and soy protein showed higher acceptability than the one containing only soy protein (Childs and others, 2007). Therefore, more work needs to be done to improve the acceptability of soy protein beverages.

To date, there has been no attempt to develop a high protein beverage at greater than 4.2% protein using hydrolyzed soy protein mainly due to the anticipated bitterness of some of the released peptides. Bitterness of soy peptides may become acceptable or masked when combining SPH with iced tea since tea bitterness is a key sensory attribute that is desired by tea consumers (Scharbert and Hofmann, 2005; Ahmed and others, 2010). However, beany flavor, the most offensive sensory characteristic associated with soy, can still be a challenge for beverages formulated with SPH.

1.6.1.1 Beany flavor

Beany flavor, an undesirable sensory characteristic of soybean, is caused by numerous volatile compounds including dimethyl trisulfide, methanethiol, hexanal, octanal, benzaldehyde, and many others (Boatright and Lei, 2000; Lei and Boatright, 2001). These compounds are generated by the conversion of polyunsaturated fatty acid chains to hydroperoxides by the enzyme lipoxygenase. Also, these compounds can be generated by the decomposition of linoleic 13- hydroperoxide in presence of hydroperoxide lyase (Rackis and others, 1979; Matoba and others, 1985; Matoba and others, 1985). Deodorization of these off-flavors has been attempted using enzymes such as aldehyde dehydrogenase (Takahashi and others, 1980; Matoba and others, 1985) and lipase (Trumbetas and others, 1993), microorganisms (Kobayashi and others, 1992), ion exchange resins (Nakamura and others, 1994), and ultra carbonic dioxide gas (Maheshwari and others, 1995). However, none of these methods have successfully eliminated the beany flavor-causing volatile compounds. A process that removes all oil-body-associated proteins from SPI has shown promise in diminishing the off-flavor of soy protein, however more studies are needed to confirm the feasibility of the method (Tzen and Huang, 1992; Samoto and others, 1998).

Because the beany flavor causing compounds cannot be eliminated completely, masking them with other flavors is the best choice to improve the acceptability of high protein beverages formulated with soy. We hypothesize that the roasted attribute of soy protein matches well with black tea and can cause the beany flavor to be less pronounced. Lemon flavor, commonly added to the iced tea, adds citrus flavor to black tea and may mask beany flavor at the same time. Therefore, soy beverages blended with lemon flavored iced tea may be a good combination and a convenient way to incorporate soy protein into a high protein beverage.

2. Soy protein hydrolysate; solubility, thermal stability, and bioactivity

2.1 Overview

Soy protein isolate (SPI) is the most refined soy protein ingredient utilized in many food applications for its nutritional quality and functional properties. Functional properties relevant to food application include solubility, water-holding capacity, viscosity, gelation, foaming, and emulsification. Particularly, solubility is one of the most valuable functional properties because it can affect the quality of many food products, especially nutritional beverages. Beverages formulated with soy protein (at >4% protein content) are currently unavailable in the market due to thermal challenges affecting the protein solubility and thermal stability. Limited and controlled hydrolysis (2-8%) may enhance protein solubility and thermal stability. Additionally, controlled hydrolysis of soy protein may release bioactive peptides. Among the many physiological activities of bioactive peptides, antihypertensive activity has gained significant attention.

Therefore, our objective was to optimize hydrolysis conditions of soy protein for enhanced solubility, thermal stability and bioactivity, while maintaining low degree of hydrolysis (DH).

Soy protein isolate (SPI) was hydrolyzed by papain, bromelain, trypsin, and alcalase at different activity levels while keeping other hydrolysis conditions constant. Hydrolysis conditions were optimized based on DH measurements. Degree of hydrolysis (DH) was measured using O-phthaldialdehyde method. Samples were subjected to SDS-PAGE analysis to monitor enzyme selectivity. ACE inhibitory activity was measured using a standardized assay. For determination of solubility and thermal stability, solutions (5% w/v) of samples and controls were subjected to heat treatment at 95°C for 60 min. Solubility and thermal stability at different protein concentrations (1%, 2.5%, 5%, 7%) in a black tea beverage were also monitored.

Controlled enzymatic hydrolysis conditions utilizing alcalase resulted in SPH that is soluble and thermally stable at relatively high protein concentrations (5% and 7%) and possesses pronounced antihypertensive activity. These findings can be

utilized in the production of high protein fortified beverages with noted physiological benefits.

2.2 Introduction

Soybeans are among the most commonly consumed crops in Asian countries for their excellent nutritional value, functional properties, and health benefits (Friedman and Brandon, 2001; Messina and Messina, 1991; Quak and Tan, 1998). Many studies have shown that consumption of soy protein has several positive effects on health, including antihypertensive activity (De Leo and others, 2009), reduction in cholesterol level (Pipe and others, 2009) and body fat (Aoyama and others, 2000), prevention against osteoporosis (Bawa, 2010) and reduced incidences of cancers such as stomach (Nagata, 2000), colorectal (Yang and others, 2009), and breast cancers (Messina, 1999; Lamartiniere, 2000; Messina and Loprinzi, 2001). In 1999, the United States Food and Drug Administration approved the health claim of "25 g of soy protein in a day, as a part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease" (FDA, 1999). As a result, soy protein-based foods have stimulated the emergence of a substantial soy food market in the US, with soy food sales increasing from $ 1 billion in 1996 to $ 4.5 billion in 2009 (Soy foods Association of North America, 2010).

Along with the health benefits, soy protein possesses many functional properties that can be utilized in various food applications. Functional properties of soy protein include solubility, water-holding capacity, viscosity, gelation, foaming, and emulsification. Soy protein isolate (SPI), which is the most refined among the different soy protein ingredients (soy protei isolate, soy flour and soy protein concentrate), has bland flavor, light color, the highest protein content, and the best functionality. Therefore, SPI is utilized in a wide range of food applications such as nutritional bars, meat analogs, infant formula, and beverages (Elias and others, 1984; Mateos-Aparicio and others, 2008).

Solubility is one of the most valuable functional properties for high protein beverage formulation (Kinsella, 1979; Phillips and others, 1994). High protein

beverages have gained increasing attention in recent years especially due to the associated physiological benefits. To claim a high protein beverage, protein content should be greater than 4.2% (FDA, 2001). However, formulating high protein beverages (>4.2%) is one of the biggest challenges in beverage manufacturing because of protein solubility (Kinsella, 1979; Phillips and others, 1994) and thermal instability (Dissanayake and Vasiljevic, 2009) when present at relatively high concentrations. Currently, whey protein is the most common choice for beverage application due to its relatively good solubility over a wide range of pH. However, challenges remain due to thermal instability and reduced shelf life at high protein concentration. Given these challenges, and the potential health benefits of soy, it is worthwhile investigating soy protein solubility and thermal stability for the potential use in high protein beverage.

Soy protein needs to go through thermal treatment in order to inactivate anti-nutritive factors such as trypsin inhibitor, which can cause growth impairments or may also be lethal without proper inactivation (Gumbmann and others, 1986). It is, therefore, necessary to heat at 95°C for 60 min to reduce trypsin inhibitor activity at least 90% without disrupting essential amino acids, including lysine and methionine (Kwok and others, 1993; Kwok and others, 2002). The effect of such heat treatment on soy protein solubility and thermal stability is not well investigated, especially at high protein concentrations.

Few studies have investigated the effect of thermal treatment on the solubility of soy protein. Thermal treatment at 98°C for 30 min (Renkema and others, 2000) and at 95°C for 1 hour (Renkema and others, 2002) did not cause significant reduction in solubility of SPI. However, these studies were carried out at relatively low protein concentrations, 1.2% and 1%, respectively. Protein aggregation is highly dependent on protein concentration. As the concentration of denatured protein increases in the solution, the chances of aggregation increase due to shorter distances between the protein molecules. Since protein concentration is one of the most important factors regulating protein solubility, solubility measured at higher concentrations is, therefore, expected to be considerably different from that measured

at low protein concentrations. To the best of our knowledge, there is no study that investigated thermal stability of soy protein as affected by protein concentration greater than 4%.

Researchers showed that limited hydrolysis of soy protein can result in enhanced functionality such as solubility (Lamsal and others, 2007; Kuipers and others, 2007; Zorin and Baiarzhargal, 2009), foaming (Ortiz and Wagner, 2002; Ruiz-Henestrosa and others, 2007), emulsification (Qi and others, 1997; Wu and others, 1998), and gelation (Kuipers and others, 2007; Creusot and Gruppen, 2007). Several enzymes including papain, pepsin, trypsin, bromelain, flavozyme, alcalase and many others were used at various conditions, including pH, temperature, time, and enzyme to substrate ratio. Use of different enzymes at various conditions resulted in hydrolysates of distinct functionality. It is, therefore, important to control enzymatic conditions to result in a directed functionality enhancement, such as enhanced solubility. Specifically, solubility of SPI was enhanced by 40% and 33% upon hydrolysis using bromelain and papain enzymes, respectively (Lamsal and others, 2007; Wu and others, 1998). However, these studies, again, were performed at relatively low protein contents, 1% and 0.2%, respectively, without thermal treatment, which is an essential food processing step for soy protein. The effect of limited hydrolysis of soy protein on its solubility and thermal stability at relatively high protein concentration is not documented.

On the other hand, limited hydrolysis of soy protein has been shown to release bioactive peptides (Chen and others, 2002; Wang and others, 2008; De Leo and others, 2009). Biologically active peptides are characterized as protein-derived amino acid sequences that may have regulatory and physiological effects on the human body beyond normal and adequate nutrition once released from the parent protein (Korhonen and Pihlanto, 2003; Shahidi and Zhong, 2008). Bioactive peptides can be liberated via enzymatic digestions, both in the human gastrointestinal tract and during food processing. While *in vivo* enzymatic digestion is random and thus may or may not result in the release of bioactive peptides, *in vitro* enzymatic hydrolysis can be controlled to result in the production of biologically active peptides (Yamamoto and

others, 2003; Kitts and Weiler, 2003; Shahidi and Zhong, 2008). Bioactive peptides from soy protein have many physiological characteristics depending on the released amino acid sequences, including anticancerous (Mateos-Aparicio and others, 2008), antioxidant (Sarmadi and Ismail, 2010), antimicrobial (Paul and Somkuti, 2010), antithrombotic (Gilani and others, 2008), immunomodulatory (Kanwar and others, 2009), opiate-like (Moller and others, 2008), hypercholesterolemic (Potter, 1995), and antihypertensive (Inoue and others, 2009).

Among the many physiological activities of soy bioactive peptides, antihypertensive activity has gained significant attention. Antihypertensive activity is fundamentally based on inhibiting angiotensin-converting enzyme (ACE) activity, which plays a crucial role in the regulation of blood pressure through the rennin-angiotensin system (RAS). RAS is a hormone-like system which regulates fluid balance and blood pressure in our body (Werner and others, 2008). When the RAS is abnormally active, the blood pressure will rise too high, thereby, causing hypertension (Mondorf and others, 1998). Hypertension can lead to many other diseases, such as coronary heart diseases, heart failure, stroke, and kidney failure (American Heart Association, 2010). Hypertension is the second leading cause of death in the US and affects approximately 74.5 million individuals, one in every three adults (Institute of Medicine, 2010). The associated medical cost is estimated to be around $74 billion per year (American Heart Association, 2010). Currently, drugs such as captopril are used to reduce ACE activity and thus lower blood pressure (Carmona-Calero and others, 2005; Pechanova, 2007). Due to the associated side effects of the drug such as dry cough, skin rashes, and angioedema, food-based bioactive peptides have received significant attention as a low-cost alternative and preventative treatment of hypertension (Tsevat and others, 1995; Kazerani and others, 2009).

Several antihypertensive peptides were identified in enzyme-hydrolyzed soy proteins (Kodera and Nio, 2002; Cha and Park, 2005; Chiang and others, 2006). However, Soy bioactive peptides are far less investigated compared to the bioactive peptides derived from milk proteins. To the best of our knowledge, products

containing soy-derived bioactive peptides with ACE inhibitory activity are not available in the US market. Additionally, soy protein hydrolysate with improved solubility and thermal stability, produced using controlled enzymatic hydrolysis, has not been investigated for its potential antihypertensive activity. Improving the thermal stability and solubility of SPI in concert with the release of antihypertensive peptides is appealing for the developing of stable high protein beverages (>4.2% protein) with noted physiological benefit. Therefore, the objective of this study was to optimize hydrolysis conditions to produce soy protein hydrolysates with relatively good solubility, thermal stability, and ACE inhibitory activity.

2.3 Materials and methods

2.3.1 Materials

Defatted soy flour (7B) and the food grade enzymes including papain, trypsin, bromelain, and alcalase were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL) and Novozymes (Omaha, NB), respectively. Angiotensin-converting enzyme (ACE, EC 3.4.16.1, A-6778, 5.5 units/mg) and N-[3-2-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAP-GG, F-7131) were purchased from sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit (23235) was purchased from Pierce (Rockford, IL). Pre-stained broad-range molecular weight (MW) standard (161-0318), Laemmli sample buffer (161-0737), 10X Tris/glycinin/SDS running buffer (161-0732), ammonium persulfate (161-0700), 40% Acrylamide/Bis solution, 37.5:1 (2.6% C)(161-0148), and N,N,N',N'-tetra-methyl-ethylemedimine (TEMED) (161-0800) were purchased from BioRad (Hercules, CA). Soy protein standards, glycinin and β-conglycinin, were supplied by EPL Bio Analytical Services (Niantic, IL). Analytical reagent grade chemicals were purchased from Fisher Scientific (Pittsburg, PA) and Sigma Chemical Co. Unsweetened and decaffeinated Lipton® instant tea (Englewood Cliffs, NJ) was purchased locally.

2.3.2 Experimental design

Two independent experiments were carried out. First, soy protein was

subjected to controlled and limited hydrolysis using papain, trypsin, bromelain, and alcalase. To obtain a degree of hydrolysis (DH) ranging between 2 and 8%, various enzyme concentrations were tested for each enzyme while maintaining other hydrolysis conditions, pH, temperature, and reaction time constant (**Figure 1, Table 1**). Limited hydrolysis (DH 2-8%) is required to have enhanced functional properties and maximum liberation of bioactive peptides, with minimal detection of low molecular weight bitter peptides (Schlothauer and others, 2005). All hydrolysis was carried out in triplicate. Hydrolysates with DH 2-8% were selected and subjected to further analysis. Selected hydrolysates were subjected to heat treatment to test solubility and thermal stability at 5% protein concentration (**Figure 2**).

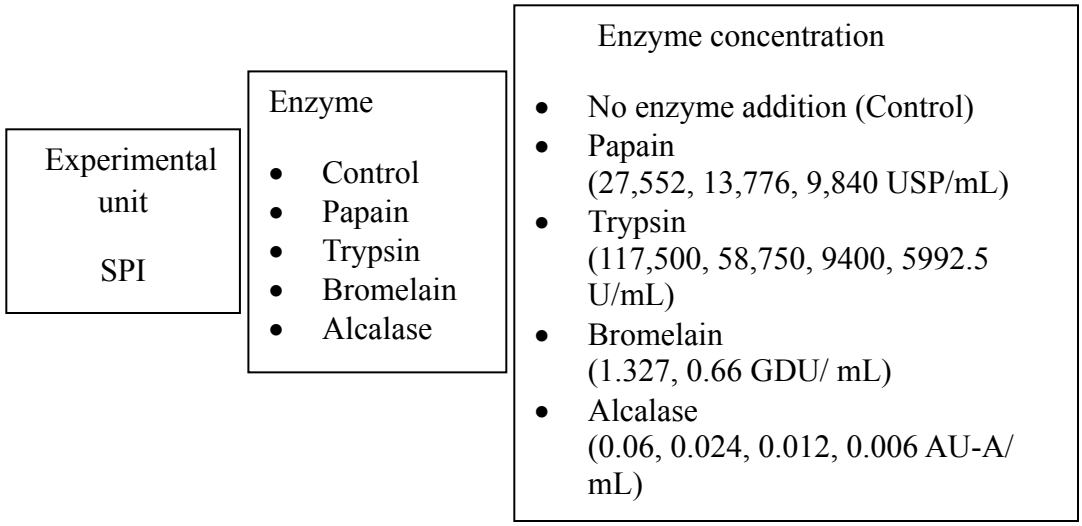

Figure 1. Experimental design of the enzymatic hydrolysis using papain, trypsin, bromelain, and alcalase enzymes. ANOVA was done separately for each enzyme, with enzyme level as a single factor.

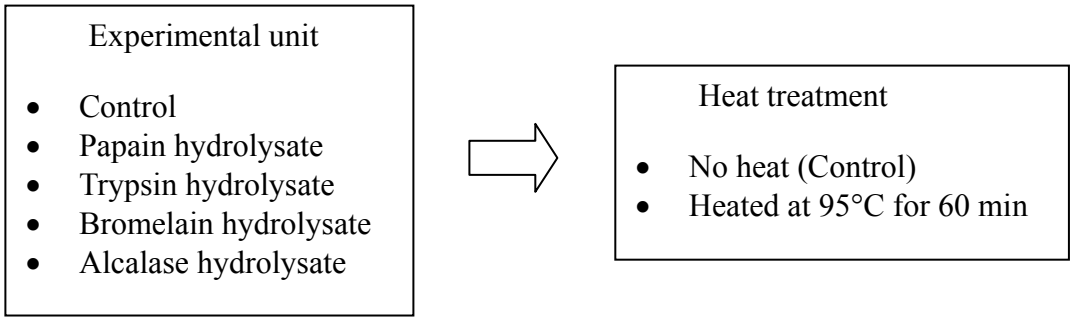

Figure 2. Experimental design of the heat treatment of selected hydrolysates. Two-factor factorial design was followed with hydrolysate type and treatment as factors.

2.3.3 Preparation of soy protein isolate (SPI)

Minimally heat-treated and defatted soy flour was used to prepare SPI following the method described by Tsumura and others (2005). Soy flour was dispersed in de-ionized distilled water (DDW) (1:10 w/w) and adjusted to pH 7.0 with 2 N NaOH, stabilized by stirring at room temperature for 1 hour. The dispersion was then centrifuged (Beckman J2-MC, Fullerton, CA) at 7500 rpm for 30 min to remove the insoluble material. The supernatant was adjusted to pH 4.5 with 2 N HCl and centrifuged (Beckman J2-MC, Fullerton, CA) at 7500 rpm for 10 min to precipitate the protein. The precipitate was resolubilized (1:4 w/w) in DDW, neutralized with 2 N NaOH, and freeze-dried. Protein content of the samples was determined following Dumas method (AOAC method 992.15, 2005) outlined in section 2.3.9. The freeze-dried samples were kept at -20 °C until further analysis.

2.3.4 Preparation of papain-, trypsin-, bromelain-, and alcalase-hydrolyzed SPI

Dispersions (5g/ 100mL) of SPI in DDW were prepared in 250mL beakers and subjected to enzymatic hydrolysis, in triplicate, by papain, trypsin, bromelain, and alcalase. The pH of the SPI dispersions was adjusted with 2N NaOH to the desired pH (**Table 1**). Beakers were covered with aluminum foil and placed in a pre-heated water bath (heated to a desired temperature, **Table 1**) for 10 min prior to the

addition of 2mL enzyme solution (**Table 1**), and then incubated for 45 min. Control samples, for all the enzyme treatments, were prepared and incubated under the same conditions without the addition of the enzyme. The hydrolysates and controls were subjected to boiling for 5 min to inactivate the enzyme, and then were freeze-dried. Protein content of the samples was determined following Dumas method (AOAC method 992.15, 2005) as outlined in section 2.3.9. The freeze-dried samples were kept at -20 °C until further analysis.

Table 1. Hydrolysis conditions for papain-, trypsin-, bromelain-, and alcalase-hydrolyzed SPI

Enzyme*	Environmental factor	Condition
Papain	pH	7
	Temperature	70°C
	Enzyme activity (USP units/ mL)	27,552
		13,776
		9,840
Trypsin	pH	8
	Temperature	37°C
	Enzyme activity (U/ mL)	117,500
		58,750
		9,400
		5,992.5
Bromelain	pH	7
	Temperature	50°C
	Enzyme activity (GDU/ mL)	1.327
		0.66
Alcalase	pH	7.5
	Temperature	55°C
	Enzyme activity (AU-A/ mL)	0.06
		0.024
		0.012
		0.006

* 2mL of enzyme solution was added to SPI-water suspension.

2.3.5 Degree of hydrolysis (DH)

Degree of hydrolysis (DH) was measured in triplicate. Each control and

hydrolyzed sample was dispersed (0.01g/ 10mL) in DDW and centrifuged (Eppendorf 5415D, Westbury, NY) at 13,000 rpm for 10 min. The protein content of the collected supernatant was determined following a micro BCA kit as outlined in section 2.3.10. The DH was measured following the O-phthaldialdehyde (OPA) method as outlined by Nielsen and others (2001), using DDW and 0.1% L-serine (w/v) as blank and standard, respectively. Triplicate of blank, standard, and samples (400 µL) were added to 3 mL of OPA reagent (3.81% disodium tetraborate decahydrate (w/v), 0.1% sodium dodecyl sulfate (w/v), 0.08% o-phthaldialdehyde (w/v), 2% ethanol (v/v), and 0.088% dithiothreitol (w/v)), vortexed for 5 sec, and let react for 2 min. The absorbance then was read at 340 nm using a UV/Vis spectrophotometer (DU 640 B, Beckman Coulter, Fullerton, CA). The %DH was calculated following the equations reported by Adler-Nissen (1984) (Equation 1-3).

Equation 1

$$\text{Serine NH}_2 = \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times 0.9516 \times 0.01 \times \frac{100}{X \times P}$$

Equation 2

$$h = \frac{(\text{Serine NH}_2 - \beta)}{\alpha}$$

Equation 3

$$DH = \frac{h}{htot} \times 100$$

Where:

h = number of hydrolyzed bonds

htot = total number of peptide bonds per protein equivalent (7.8 specific to soy protein)

β = 0.342 (specific for soy protein)

α = 0.970 (specific for soy protein)

ODsample = absorbance of sample at 340 nm

ODblank = absorbance of water at 340 nm

ODstandard = absorbance of L-serine at 340 nm

X = amount of sample (g)

P = protein (%) in sample

Sample calculation

%DH of one of the samples with 45.61% protein

$$\text{Serine } NH_2 = \frac{0.3031 - 0.0626}{0.7325 - 0.0626} \times 0.9516 \times 0.01 \times \frac{100}{0.1 \times 45.61} = 0.7409$$

$$h = \frac{(0.7489 - 0.342)}{0.97} = 0.4195$$

$$DH = \frac{0.4195}{7.5} \times 100 = 2.65\%$$

2.3.6 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

All hydrolysates regardless of their DH were subjected to SDS-PAGE analysis. SDS-PAGE was performed based on the method reported by Laemmli (Laemmli, 1970) to monitor the protein hydrolysis pattern. Each sample was dissolved in DDW (11.25 mg/mL, -10.60 mg protein/mL), an aliquot (100 µL) was mixed with Laemmli buffer (95 µL) and β-mercaptoethanol (5 µL), for reducing condition, or mixed 1:1 (v/v) with Laemmli buffer, for non-reducing condition, and then boiled for 5 min. Pre-stained MW standard, soy protein standards (glycinin and β-conglycinin), control and hydrolyzed samples (5 µL) were loaded into 18-well hand-cast 15% acrylamide and 4.5% stacking gels (**Table 2**). Electrophoresis was carried out with 10X tris/glycine as running buffer (diluted 1:10 (v/v) with DDW) at a constant voltage of

200 V for approximately 1 hour. Gels were stained using Coomassie blue (50% methanol (v/v), 10% glacial acetic acid (v/v), 0.25% Coomassie brilliant blue G-250 (w/v) for another hour followed by destaining overnight in a solution of 10% glacial acetic acid (v/v) and 5% methanol (v/v). The gels were scanned using White/ UV scanner (BioRad, Hercules, CA).

Table 2. Compostion of 15% acrylamide gel and 4.5% stacking gel for SDS-PAGE

Ingredients	Resolving gel (15%)	Stacking gel (4.5%)
DDW	6 mL	9 mL
Lower buffer[a]	4 mL	---
Upper buffer[b]	--	3.75 mL
Acrylamide	4.8 mL	1.35 mL
Glycerol[c]	1.2 mL	---
TEMED	10 µL	9 µL
Ammonium persulfate[d]	60 µL	53 µL
Total	16 mL	14.1 mL

[a] 1.5 M Tris-HCl buffer, pH 8.8, 0.4% SDS

[b] 0.5 M Tris-HCl buffer, pH 6.8, 0.4% SDS

[c] 87% solution prepared in DDW

[d] 10% solution prepared in DDW (prepared fresh)

2.3.7 Solubility and thermal stability

Only the soy protein hydrolysate (SPH) samples with DH between 2-8% were used to determine solubility and thermal stability. Because a sensory study was planned to be carried out with soy protein fortified lemon flavored iced tea beverages, solubility of a control SPI and the hydrolysates were also measured when dispersed in a black tea solution. Unsweetened and decaffeinated Lipton® instant tea (Englewood Cliffs, NJ) was dispersed in DDW (0.7g/ 240mL) following the manufacturer's

instruction.

The SPI and SPH(s), in triplicates, were weighed in 1.2 mL screw cap tubes (0.05g/ 1mL), dispersed in DDW or a black tea solution, and heated at 95°C for 65 min in a pre-heated water bath. This condition was chosen in order to inactivate trypsin inhibitor (Kwok and others, 1993; Kwok and others, 2002; Guerrero-Beltran and others, 2009). For controls, SPI and SPH samples were prepared as described above and left without heat treatment. After heat treatment, samples were cooled for 30 min at room temperature and both heated samples and unheated controls were centrifuged at 13,000 rpm for 10 min. Protein content of the supernatant was determined following Dumas method (AOAC method 992.15, 2005) as outlined section 2.3.9. Solubility was calculated as protein content in supernatant over total protein content in the initial dispersion (Equation 4).

Equation 4

$$\% \text{ Solubility} = \frac{\text{protein content in supernatant}}{\text{total protein content in the initial dispersion}} \times 100\%$$

Sample calculation

Solubility of SPI sample without heat treatment

$$\% \text{ Solubility} = \frac{4.309\ \%}{5\%} \times 100\% = 86.18\%$$

2.3.8 Measurement of ACE Inhibitory Activity

Only the SPHs with DH between 2-8% were subjected to ACE inhibitory activity measurement. Measurement of ACE inhibitory activities of the SPH(s) was carried out in triplicate, adopting the assay outlined by Otte and others (2007) and Shalaby and others (2006), using FA-PGG as a substrate and sodium borate buffer (0.1 M borate, 0.3 M chlorine ion, pH 8.3) instead of 50 mM-tris-HCl buffer (pH 7.5).

Higher enzyme activity was expressed when sodium borate buffer was used at pH 8.3 rather than Tris buffer (Bunning et al., 1983; Holmquist et al., 1976). Inhibitory solutions were prepared by diluting samples (0.1g/ 10mL) in DDW, centrifuging at 13,000 rpm for 10 min and filtering through 0.45 µm syringe filters. The filtered solutions were then further diluted to various concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 mg/mL that are equivalent to 0.133, 0.200, 0.268, 0.335, and 0.399 mg protein/mL content in order to determine IC_{50} value. IC_{50} value is the inhibitor concentration that causes 50% ACE inhibition. ACE was dissolved in DDW to reach a final concentration of 0.288 units/ mL. The reaction mixture (**Table 3**) was prepared in a 96-well microplate, which was pre-heated at 37 ºC and placed in a microplate reader. While incubated at 37 ºC, absorbance at 340 nm was recorded every 30 sec for 30 min. The ACE activity was expressed as the slope of the decrease in absorbance at 340 nm taken from 10-25 min of incubation time. The %ACE inhibition by inhibitory solutions was calculated using Equations 5 & 6. IC_{50} values were determined from plots of %ACE inhibition versus inhibitor concentration using equation of the line (Equation 7). IC_{50} value was calculated based on the protein content present in the filtered supernatant. The protein content of the collected supernatant was determined using a micro BCA kit as outlined in section 2.3.10.

Equation 5

$$\text{Amount of protein in the well} = \frac{\text{sample concentration(mg/mL)}}{170\mu L} \times \text{protein(\%)}$$

Equation 6

$$\%\text{ACE inhibition} = \frac{(\text{slope control} - \text{slope inhibitor})}{\text{slope control}} \times 100\%$$

Equation 7

$$IC_{50}\ \text{value} = y = mx + b$$

Where:

m= slope of the line

b= intercept of the line

y= % inhibition; y= 50 to determine concentration of inhibitor needed to result in 50% inhibition

Sample calculation

% ACE inhibition of 5 mg/mL sample/mL alcalase-hydrolyzed SPI with 45.29%

$$\text{Amount of protein in the well} = \frac{5(\text{mg/mL}) \times 10\mu\text{L}}{170\mu\text{L}} \times 45.29\% = 0.133 \text{ mg protein/mL}$$

$$\%\text{ACE inhibition} = \frac{-0.00693 - (0.00557)}{-0.00693} \times 100\% = 19.62\%$$

$$IC_{50} \text{ value} = \frac{(0.133 \times 50\%)}{19.62\%} = 0.339 \text{ mg protein/mL}$$

%ACE inhibition of 10 mg sample /mL alcalase-hydrolyzed SPI with 45.56% protein

$$\text{Amount of protein in the well} = \frac{10(\text{mg/mL}) \times 10\mu\text{L}}{170\mu\text{L}} \times 45.56\% = 0.268 \text{ mg protein/mL}$$

$$\%\text{ACE inhibition} = \frac{-0.00693 - (0.00327)}{-0.00693} \times 100\% = 52.81\%$$

$$IC_{50} \text{ value} = 50 = 229.5082x - 10.4184\,; \; x = 0.263 \text{ mg protein/mL}$$

Table 3. Reaction mixture for ACE activity assay

	Na-Borate Buffer [a] (μL)	FA-PGG [b] (μL)	Hydrolysate [c] (μL)	ACE [d] (μL)
Control Blank	160	---	---	10
Control	10	150	---	10
Hydrolysate Blank	150	---	10	10
Hydrolysate Sample	---	150	10	10

[a] Na-borate buffer: 0.1M borate, 0.3 M Chlorine ion, pH 8.3

[b] 0.88 nM FA-PGG in Na-borate buffer

[c] 5.0, 7.5, 10.0, 12.5, and 15.0 mg/mL trypsin-, papin-, bromelain-, or acalase-hydrolyzed SPI in DDW

[d] 0.228 units/mL ACE in DDW

2.3.9 Dumas method

Protein content of the samples was determined following Dumas method (AOAC method 992.15, 2005) using a Leco TruSpec^{TM} N Nitrogen Analyzer (Leco Coporation. St. Joseph, MI). Tin foils were used to place the samples. Ethylenediaminetetraacetic acid (EDTA) and glycine solution were used as standards for powder samples and liquid samples, respectively. The nitrogen percentage was converted into protein percentage by using 6.25 as a factor following Equation 8.

Equation 8

$$\% \text{ Protein} = \text{Nitrogen } \% \times 6.25$$

2.3.10 Micro BCA protein method

The protein content of the collected supernatant was determined using a micro BCA protein kit following manufacturer's instructions. Nine different concentrations (0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 μg/mL) of bovine serum albumin

were used to formulate a standard curve. Standards and samples (20 µL), in triplicate, were mixed with BCA reagent (200 µL) in a 96-well microplate. The plate was incubated for 30 min at 37°C and absorbance of samples was recorded at 562 nm using Biotek Synergy HT microplate reader (Winooski, VT). The percent of soluble protein in the sample was calculated based on the standard curve.

2.3.11 Turbidity

Solutions prepared with SPH samples (DH between 2-8%) and SPI were subjected to turbidity measurement. The SPI and SPH(s), in triplicates, were weighed in 1.2 mL screw cap tubes (0.05g/ 1mL), dispersed in DDW or a black tea solution, and heated at 95°C for 65 min in a water bath. Unheated SPI and hydrolyzed samples were also prepared as controls. After heat treatment, samples were cooled for 30 min at room temperature, and then 1 mL of heated samples and unheated controls were placed in cuvettes. The turbidity of the solution was determined at 600 nm using a UV/Vis spectrophotometer (DU 640 B, Beckman Coulter, Fullerton, CA) as outlined by Jiang and others (2010). DDW was used as the reference.

2.3.12 Statistical analysis

Analysis of variance (ANOVA) was carried out utilizing SPSS 15 for Windows. When a factor effect or an interaction was found significant, indicated by a significant F test ($P \leq 0.05$), differences between the respective means (if more than 2 means) were determined ($P \leq 0.05$) using Tukey-Kramer multiple means comparison test.

2.4 Results and discussion

2.4.1 Degree of hydrolysis and hydrolysis pattern

Excessive hydrolysis of soy protein (normally more than 8%) results in the release of bitter peptides (Matoba and Hata, 1972). In order to avoid producing noticeable amount of bitter peptides, protein hydrolysis using different enzymes at various activity levels was optimized to reach a desired level of hydrolysis (DH 2-

8%). Functionality improvement has been reported for protein hydrolysates obtained after limited hydrolysis, with DH of 2-8% (Tsumura, 2005; Ruiz-Henestrosa and others, 2007; Lamsal and others, 2007). The DH values obtained after hydrolysis of SPI using different enzymes at various activity levels are summarized in **Table 4**. Specifically, hydrolysates produced using 2.654 GDU of bromelain, 0.012 AU-A of alcalase, 19,680 USP units of papain, and 235,000 U of trypsin had DH of 3.98%, 3.57%, 6.77%, and 2.60%, respectively, thus were selected for further experiments.

Use of different enzymes resulted in distinctive differences in hydrolysis patterns (**Figure 13, Appendix A**; hydrolysate produced at different enzyme activity levels). Papain (lane 6, **Figure 3**) and bromelain (lane 8, **Figure 3**) hydrolyzed both glycinin and β-conglycinin subunits, indicating similar hydrolysis pattern. Alcalase is known as an aggressive enzyme and thus (**Figure 13, Appendix A**) was capable of hydrolyzing both gycinin and β-conglycinin subunits reaching DH of 21.71% at activity level of 0.12 AU-A/ assay. When enzyme activity was reduced by 10 fold, DH reached the desired range (3.57%) and mainly β-conglycinin subunits were hydrolyzed as indicated by their faint bands as compared to glycinin bands (compare lane 12 to lane 11, **Figure 3**). Trypsin did not cause significant change in both DH (**Table 4**) and hydrolysis pattern (lane 10, **Figure 3**). Soy protein resisted trypsin hydrolysis, most likely due to the presence of trypsin inhibitor in the non-heated SPI used. Our data shows that the produced hydrolysates have different DH and hydrolysis patterns, thus will most likely have different solubility, thermal stability, and bioactivity.

Table 4. Degree of hydrolysis of bromelain-, alcalase-, papain-, and trypsin-hydrolyzed SPI

Enzyme	Enzyme Activity/ Assay	Degree of hydrolysis (%)
Bromelain	1.32 GDU	1.60[b]
	2.654 GDU	**3.98[a]**
Alcalase	0.12 AU-A	21.71[a]
	0.048 AU-A	9.40[b]
	0.024 AU-A	8.67[c]
	0.012 AU-A	**3.57[d]**
Papain	55104 USP units	15.51[a]
	27552 USP units	9.42[b]
	19680 USP units	**6.77[c]**
Trypsin	11985 U	1.02[c]
	18800 U	0.87[c]
	117500 U	2.12[b]
	235000U	**2.60[a]**

Different lowercase letters indicate significant differences in among the enzyme activities within each enzyme according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

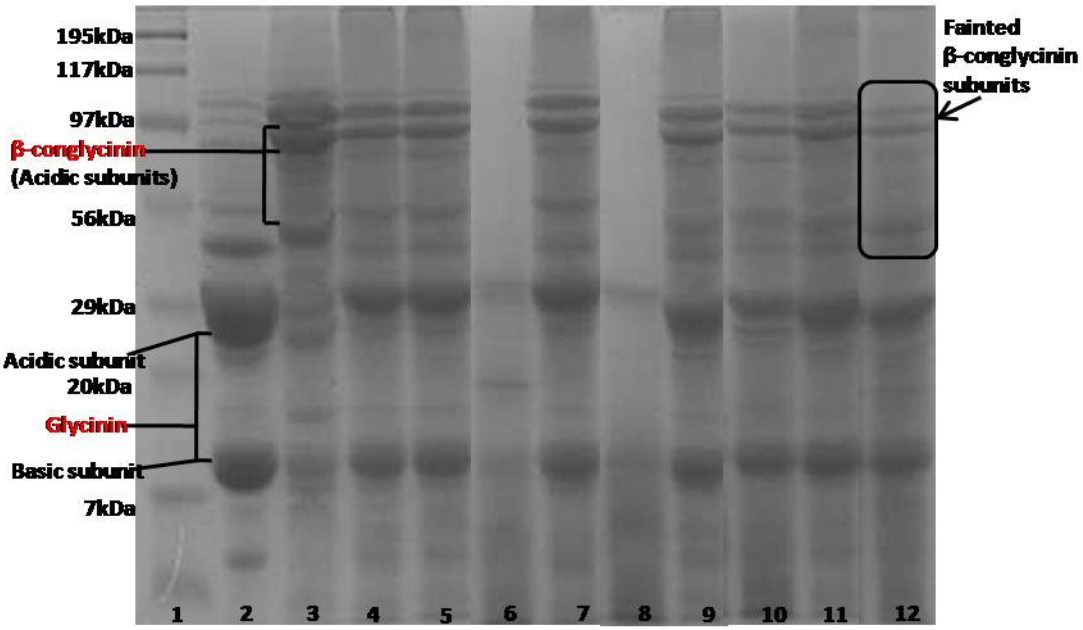

Figure 3. Hydrolysis patterns of papain-, bromelain-, alcalase-, and trypsin hydrolyzed SPI with DH 2-8%. Lanes are as follows: 1: molecular weight marker; 2: glycinin standard; 3: β-conglycinin standard; 4: SPI; 5: papain control; 6: papain-hydrolyzed SPI (9840 USP units/ mL); 7: bromelain control; 8: bromelain-hydrolyzed SPI (1.327 GDU/ mL); 9: trypsin control; 10: trypsin-hydrolyzed SPI (117500 U/ mL); 11: alcalase control; 12: alcalase-hydrolyzed SPI (0.006 AU-A/ mL)

2.4.2 ACE inhibitory activity of soy protein hydrolysates

ACE inhibitory activity of the hydrolysates and control was measured at 340 nm using FA-PGG, the substrate (Shalaby and others, 2006; Otte and others, 2007). The absorbance decreases due to conversion of substrates to products over time. Therefore, the steeper the slope the higher is the ACE activity, and the flatter the slope the higher is the ACE inhibitory activity. The assay with no inhibitor, which is the control, has the steepest slope (**Figure 4**). The assay with SPI showed no significant inhibition, since the slope is not significantly different from that of the control (**Table 21, Appedix E**). Slight reduction in ACE activity was observed for trypsin-, bromelain-, and papain- hydrolyzed samples (**Figure 4**). However, a significant drop in ACE activity, as indicated by the significant drop in slope ($P <$

0.001) (**Table 21, Appedix E**), was observed when alcalase-hydrolyzed SPI was used, indicating a greater potential for ACE inhibition (**Figure 4**)

For further characterization of ACE inhibition by alcalase-hydrolyzed SPI, IC_{50} (**Figure 5)** was determined from plots of % ACE inhibition versus inhibitor concentration. Higher IC_{50} value indicates lower ACE inhibitory activity since more protein is required to achieve 50% inhibition. The calculated IC_{50} of alcalase-hydrolyzed SPI was 0.263 mg protein/ mL (**Figure 5**). This value falls within the reported range of IC_{50} values for peptides with ACE inhibitory activity (0.046 to 0.930 mg protein/ mL) (Lo and Li-Chan, 2005; Cha and Park, 2005; Chiang and others, 2006). The bioavailability of the released peptides needs to be confirmed *in vivo* before launching such soy protein ingredients in the market. Isolation and characterization of the released bioactive peptides is a natural follow up to this work.

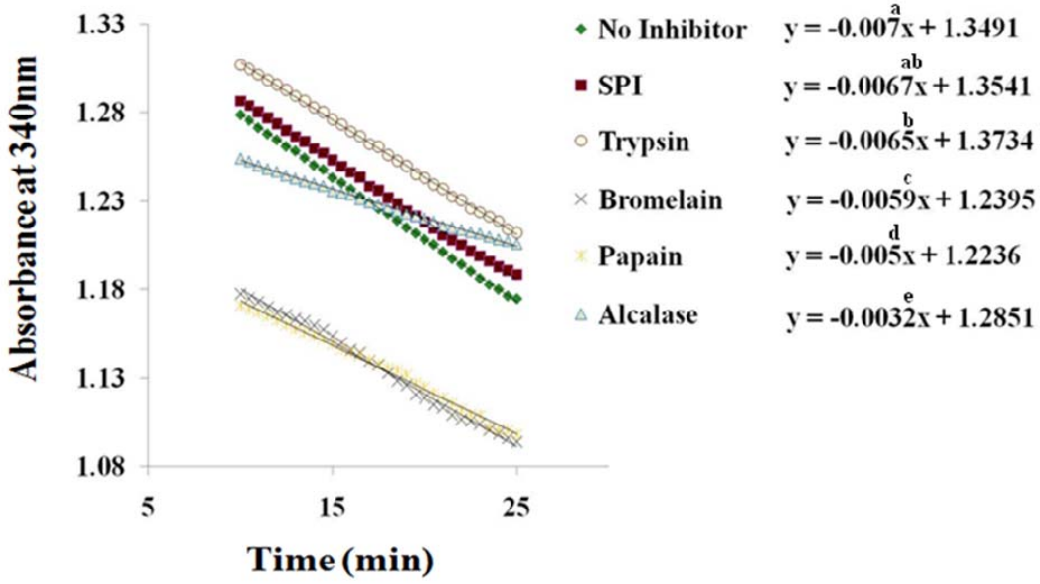

Figure 4. ACE inhibitory activity of trypsin- (117500 U/ mL), bromelain- (1.327 GDU/ mL), papain- (9840 USP units/ mL), and alcalase-(0.006 AU-A/ mL) hydrolyzed SPI samples. ACE activity as determined by the decrease in absorbance of the FA-PGG substrate over time. Decrease in the steepness of the slope indicates inhibition of the ACE. Different lowercase letters indicate significant differences among the slopes according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

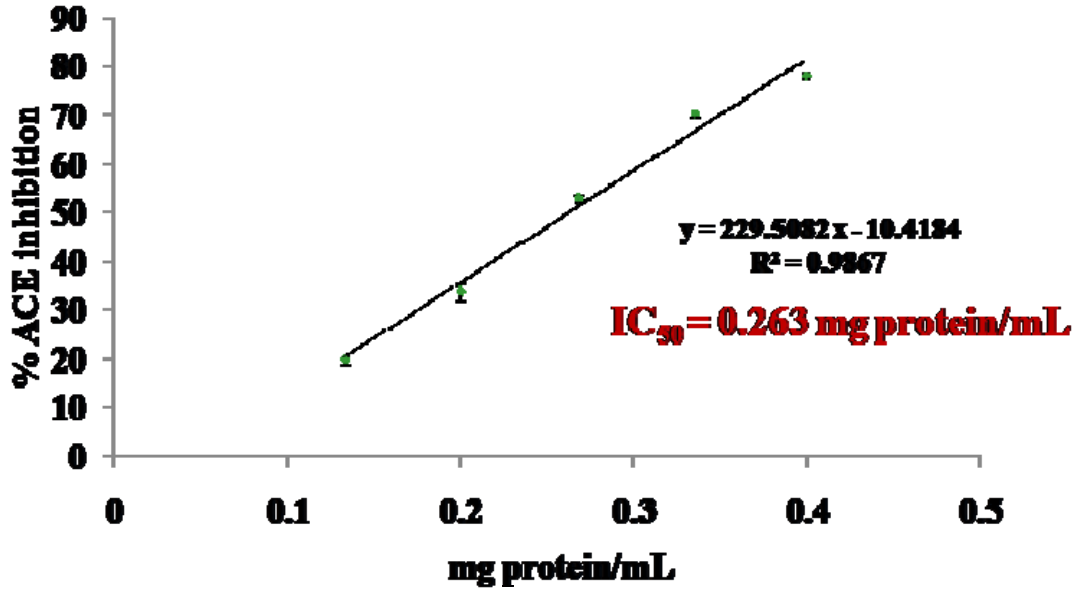

Figure 5. Dose-dependency inactivation of ACE by alcalase-hyrolyzed SPI (0.006 AU-A/ mL). Values are mean of three determinations with error bars representing standard deviations.

2.4.3 Measurement of SPI and SPH solubility and thermal stability

The solubility of SPI was higher than all of the hydrolyzed samples regardless of heat treatment (**Table 5**). Reported studies have shown that a significant increase in solubility was achieved by bromelain (Ortiz and Wagner, 2002; Lamsal and others, 2007), trypsin (Maria and others, 2008), and papain hydrolysis (Wu and others, 1998). However, our observation contradicts the findings of these studies. This can be attributed to several factors, one being the post heat treatment done in the present study in order to terminate the enzymatic reaction. The heat treatment, boiling for 5 min, may have contributed to loss in solubility due to enhanced protein-protein interactions. The mentioned studies, however, terminated enzyme reactions with slightly different methods such as freezing (Lamsal and others, 2007) and using trichloroacetic acid solution (Ortiz and Wagner, 2002). Wu and others (1998) and Maria and others (2008) used heat treatments at 95°C for 5 min and 87°C for 5 min, respectively. These heating conditions are similar to what we have done, however, the solubility of these hydrolysates was measured at 0.2% and 0.25% protein

concentrations which were relatively low. Increasing protein concentration yields more protein-protein interactions and thus reduced solubility. Since solubility is greatly influenced by protein concentrations, solubility measured at higher concentrations is expected to be considerably different from the ones measured at low protein concentrations, as confirmed by our results.

In addition to the post heat treatment, hydrolysis conditions, pH, time, enzyme activity, and temperature, can be another reason why the solubility obtained in present study is in disagreement with the mentioned studies. Wagner and others (2002), for instance, used bromelain to hydrolyze soy protein as we have done in present study. While the hydrolysis was carried out at pH 7 and 50°C for 45 min in the present study, Wagner and others performed the hydrolysis at pH 8 and 40°C for 2 hours up to 12 hours. With these different hydrolysis conditions, the released peptides in the present study will differ from the ones released from the other conditions. Therefore, solubility may increase or decrease depending on these released peptides. Difference in DH can also cause the solubility discrepancy. While the present study aimed to limit DH 2-8%, studies mentioned above did not control DH.

Among the hydrolysates, alcalase-hydrolyzed SPI showed the highest solubility (75.27%) followed by bromelain-, papain- and trypsin-hydrolyzed SPI. As confirmed by our results, utilization of various enzymes with controlled hydrolysis conditions result in different DH and hydrolysis patterns (Section 2.4.1). The resulting hydrolysates thus demonstrated different protein solubility profile. When more hydrophobic groups get exposed by hydrolysis, less protein will be soluble in a solution. On the flip side, hydrolysis of protein releases charged amino and carboxyl groups that can interact with water and potentially enhance solubility. Depending on the released peptides and the resulting conformation, hydrophobic interaction may be more or less influential than electrostatic repulsion, thus the solubility of the hydrolysates may or may not decrease. Solubility is, therefore, dependent on the balance between the hydrophobic peptide-peptide interaction, electrostatic repulsion, and peptide-water interaction. Among all the hydrolysates, alcalase-hydrolyzed SPI

seemed to have a better balance of interaction and repulsion, leading to relatively good solubility. Therefore, alcalase-hydrolyzed SPI, the most soluble hydrolysates among the hydrolyzed samples, will be a great soy protein ingredient for beverage application.

Extensive heat treatment at 95°C for 60 min caused a reduction in the solubility of both SPI and SPH samples (**Table 5**). Proteins undergo reversible conformational changes due to heat treatment. Some of the bonds that stabilize the protein secondary and tertiary structures, including hydrogen and electrostatic interactions can be broken and the interior hydrophobic groups can be exposed. Hydrophobic intermolecular interactions can then take place leading to protein polymerization. Additionally, intermolecular disulfide interchanges can take place leading to irreversible polymerization (Yamul and Lupano, 2005; Ryan and others, 2008). These newly formed disulfide linkages greatly contribute to protein aggregation and precipitation, significantly reducing the solubility (Nicorescu and others, 2009; Dissanayake and Vasiljevic, 2009). However, while the solubility differences between heated and non-heated samples are statistically significant, they are relatively minor (**Table 22, Appendix F**).

To monitor the nature of polymerization induced by heat, heated SPI and hydrolyzed samples were run on SDS-PAGE gels under reducing and non-reducing conditions (**Figure 14 and Figure 15, Appendix B)** High molecular weight bands were observed on top of the gels for both heated and non-heated samples under non-reducing condition. However, no major difference in high molecular bands was observed between the heated and non-heated samples on the gels. Glycinin subunits are naturally linked via disulfide linkages and so those high molecular weight linkages (> 300 kDa) cannot run through the gels. While disulfide linkages require reducing agents to reduce the linkages, hydrophobic interactions are disrupted by SDS. The aggregation induced by heat or aggregation, therefore, is most likely induced by hydrophobic interactions rather than disulfide linkages.

Table 5. Solubility measurement of SPI and SPH samples

Samples	Solubility without heat treatment (%)	Solubility with heat treatment at 95°c for 60 min (%)
SPI	86.40^{aA}	84.08^{aB}
Alcalase-hydrolyzed SPI (0.006 AU-A/ mL)	75.27^{bA}	73.43^{bB}
Bromelain-hyrolyzed SPI (1.327 GDU/ mL)	52.85^{cA}	47.97^{cB}
Papain-hydrolyzed SPI (9840 USP units/ mL)	44.08^{dA}	41.19^{dB}
Trypsin-hydrolyzed SPI (117500 U/ mL)	36.93^{eA}	34.95^{eB}

Different lowercase letters indicate significant differences among different hydrolysates according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$). Different capital letters indicate significant differences between heat treated and non heated samples within each hydrolysate sample according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

2.4.4 Measurement of SPI and SPH solubility in black tea

Similar to what was observed in water system, SPI had greater solubility (85.69%) than all the hydrolysates. Alcalase-hydrolyzed SPI had the highest solubility (75.19%) in black tea compared to the other hydrolysates, both with heat and without heat treatment (**Table 6**). Solubility of bromelain- and papain-hydrolyzed SPI was not significantly affected by heat treatment (**Table 23, Appendix F**).

Solubility of SPI and alcalase-hydrolyzed SPI decreased significantly ($P < 0.001$) as protein concentration increased (**Table 7**) (**Table 30, Appendix H**). Heat treatment did not cause significant differences in solubility of both SPI (**Table 25, Appendix F**) and alcalase-hydrolyzed SPI (**Table 24, Appendix F**) at low protein concentration levels (1% and 2.5%). As protein concentration increased, however, a significant difference was observed at relatively high protein concentrations between heated SPI and alcalase-hydrolyzed SPI samples. Solubility of SPI after heat treatment showed a significant drop at 5% and more so at 7% protein content (**Table 7**) (**Table 25, Appendix F**). At 5% and 7% protein concentrations, effect of heating on solubility was less for alcalase-hydrolyzed sample as compared to SPI (**Table 7**). Solubility of alcalase-hydrolyzed SPI was not reduced upon heat at 5% and 7% protein content (**Table 7**). These observations indicate that alcalase-hydrolyzed SPI shows better thermal stability than SPI, especially at relatively high protein concentrations.

Very few studies have investigated the effect of thermal treatment on the solubility of soy protein. Renkema and others (2000) showed that the solubility of SPI at 1.2 % of protein concentration did not cause a significant change in solubility when heated at 98°C for 30 min. Another study showed that heating at 95°C for 1 hour did not affect the solubility of SPI at 1% protein content (Renkema and others, 2002). Lakemond and others (2000) showed that the solubility of the glycinin subunits at 0.6% of protein content was not affected at neutral pH irrespective of sodium concentrations when heated at 98°C for 30 min. However, these studies were carried out at relatively low protein concentrations. The denatured proteins involved in aggregation may not reduce the solubility significantly upon heating at this low protein concentration. As discussed in previous section, protein solubility is highly governed by protein concentration. Moreover, no studies have investigated solubility as affected by thermal treatment in a real food system such as high protein beverage formulation (>4%). Our observation confirmed that the controlled enzymatic conditions utilizing alcalase resulted in SPH that is soluble and thermally stable in

high protein concentration such as 5% and 7% solutions. These findings can be utilized in the development of high protein fortified beverages.

Table 6. Solubility measurement at 5% of SPI and SPH samples in black tea

Samples	Solubility without heat treatment (%)	Solubility with heat treatment at 95°c for 60 min (%)
SPI	85.69^{aA}	83.92^{aB}
Alcalase-hydrolyzed SPI (0.006 AU-A/ mL)	75.19^{bA}	76.54^{bB}
Bromelain-hyrolyzed SPI (1.327 GDU/ mL)	55.37^{cA}	56.18^{cA}
Papain-hydrolyzed SPI (9840 USP units/ mL)	44.63^{dA}	44.06^{dA}
Trypsin-hydrolyzed SPI (117500 U/ mL)	43.55^{eA}	39.62^{eB}

Different lowercase letters indicate significant differences among different hydrolysates according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$). Different capital letters indicate significant differences between heat treated and non heated samples within each hydrolysate sample according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Table 7. Solubility measurement of 1%, 2.5%, 5%, and 7% SPI and alcalase-hydrolyzed SPI in black tea

Protein concentration	SPI solubility (%)		Alcalase-hydrolyzed SPI solubility (%)	
	Non-heated	Heated*	Non-heated	Heated*
1%	92.91^{aA}	93.12^{aA}	84.04^{aA}	87.20^{aB}
2.5%	88.76^{bA}	89.67^{bA}	79.85^{bA}	80.27^{bA}
5%	86.14^{cA}	84.21^{cB}	75.19^{cA}	76.54^{cB}
7%	80.07^{dA}	76.75^{dB}	71.88^{dA}	71.48^{dA}

* Samples were heated at 95°C for 60 min.

Different lowercase letters indicate significant differences among the samples with different concentrations according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$). Different capital letters indicate significant differences between heat treated and non heated samples within each concentration according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

2.4.5 Turbidity measurement

The turbidity of SPI and SPHs were measured in water. The turbidity of SPI was lower in both heated and non-heated samples than that of the hydrolysates (**Table 8**). It seems that minimally heat treated SPI was resistant to aggregation and this is in agreement with results reported by Lee and others (2003) and Jiang and others (2010). Among the hydrolyzed samples, alcalase-hyrolyzed SPI had the lowest turbidity followed by bromelain-, trypsin- and papain- hydrolyzed SPI. Turbidity of SPI and hydrolysates significantly increased with heat treatment except for trypsin-hydrolyzed SPI, which was already high to start with (**Table 8**) (**Table 33, Appendix J**). Again, this is due to the protein aggregation and precipitation caused by thermal treatment.

These findings are very significant for developing translucent beverages. Clarity of protein fortified beverages is another challenge especially when protein content is high. Although a high percentage of proteins is soluble in a solution, they are usually opaque and thus negatively affect the sensory acceptability. Moreover, clarity of protein fortified beverages is adversely affected when protein undergoes thermal treatment. Our observation confirmed that alcalase-hydrolyzed SPI, which has the lowest turbidity out of all SPHs, will be a great ingredient to incorporate into protein fortified beverages in order to make relatively translucent beverages.

Table 8. Turbidity of alcalase-, bromelain-, papain-, and trypsin-hydrolyzed samples

Samples	Turbidity without heat treatment	Turbidity with heat treatment at 95°c for 60 min
SPI	0.4983^{aA}	0.6356^{aB}
Alcalase-hydrolyzed SPI (0.006 AU-A/ mL)	0.9313^{bA}	1.1721^{bB}
Bromelain-hyrolyzed SPI (1.327 GDU/ mL)	2.1654^{cA}	2.4616^{cB}
Papain-hydrolyzed SPI (9840 USP units/ mL)	2.1287^{dA}	2.3874^{dB}
Trypsin-hydrolyzed SPI (117500 U/ mL)	2.2094^{eA}	2.1467^{eA}

Different lowercase letters indicate significant differences among different hydrolysates according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$). Different capital letters indicate significant differences between heat treated and non heated samples within each hydrolysate sample according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

2.5 Conclusion

To the best of our knowledge, this is the first study that evaluates both soy protein functionality and bioactivity of an enzymatically modified soy protein for beverage application. Moreover, soy protein hydrolysates with proven ACE inhibitory activity are not available in the US market. Overall, our observation confirmed that controlled enzymatic hydrolysis conditions utilizing alcalase resulted

in SPH that is soluble and thermally stable at relatively high protein concentrations (> 4%) and possesses pronounced antihypertensive activity. These findings can be utilized in the development of high protein fortified beverages with physiological benefits. However, developing these soy protein beverages may cause flavor challenges because of beany flavor and bitterness mainly when soy protein content is high and when hydrolysates are utilized. A study showed that a negative attitude towards soy flavor remained even when consumers were informed of the nutritional and health benefits of the products (Teh and others, 2007). Therefore, sensory quality and acceptability of the soy protein fortified beverages have to be investigated.

3. Sensory acceptability of a lemon flavored iced tea formulated with soy protein

3.1 Overview

Soy protein beverages have gained popularity due to their potential health benefits relevant to the consumption of soy proteins. However, beany flavor and chalky mouthfeel of soy play a major role in deterring consumers from soy protein beverages. Solubility adds to the challenge of formulating high soy protein beverages (>4.2% protein). Enzymatic hydrolysis of soy proteins could potentially improve the solubility of the proteins and result in the production of bioactive peptide, but may cause bitterness, which is an additional flavor defect to the utilization of soy protein. The bitterness, however can be controlled by limited degree of hydrolysis (DH) 2~8%.

The objectives of this study were to develop a lemon flavored iced tea formulated with soy protein hydrolysate (SPH) and determine the sensory acceptability of the beverages.

Nine formulations were prepared, including a control which was a tea with no added protein and four different protein concentrations (1%, 2.5%, 5%, and 7%) containing either soy protein isolate (SPI) or soy protein hydrolysates (SPH) prepared using alcalase enzyme. One-hundred-one subjects rated overall liking, flavor, aroma, appearance, and mouthfeel on an 11-point hedonic scale and rated intensity of bitterness and off flavor on a line scale.

SPH beverages up to 5% protein content were liked as much as the control. SPH beverages up to 5% also had significantly less bitter taste and off flavors than SPI beverages. The beverages formulated with 7% protein were rated lower in appearance liking, mouthfeel liking, flavor liking, and aroma liking. Bitterness and off flavors increased as protein content increased. Based on our observations, alcalase-hydrolyzed SPI may be an effective ingredient for producing acceptable high-protein beverages.

3.2 Introduction

Soy protein-based food has gained popularity due to its excellent nutritional value and functional properties (Messina and Messina, 1991; Quak and Tan, 1998; Friedman and Brandon, 2001). Epidemiological and clinical studies have proven that consumption of soy protein has several positive effects on health, including antihypertensive activity (De Leo and others, 2009), reduction in cholesterol level (Pipe and others, 2009) and body fat (Aoyama and others, 2000), prevention against osteoporosis (Bawa, 2010) and reduced incidences of cancers such as stomach (Nagata, 2000), colorectal (Yang and others, 2009), and breast (Messina, 1999; Lamartiniere, 2000; Messina and Loprinzi, 2001). In 1999, the United States Food and Drug Administration approved the health claim of "25 g of soy protein in a day, as a part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease" (FDA, 1999). As a result, soy protein-based foods has stimulated the emergence of a substantial soy food market in US, with soy food sales increasing from $1 billion in 1996 to $4.5 billion in 2009 (Soy foods Association of North America, 2010).

In addition to the positive health effects, soy protein possesses many functional properties. Functional properties relevant to food applications include solubility, visicosity, gelation, and emulsification. Soy protein is utilized in four different types: soy protein flour, soy protein concentrate, soy protein isolate (SPI), and soy protein hydrolysate (SPH). Their uses depend upon the needed functionality as well as nutritional profile. Soy protein was initially hydrolyzed to improve protein digestibility for individuals suffering from protein digestion disorders. Additionally, hydrolysis of soy protein enhances other functional properties, such as foaming, hydration, solubility, emulsification, and gelation. Furthermore, soy protein derived-bioactive peptides that may possess physiological benefits such as antihypertensive activity can be released upon limited enzymatic hydrolysis.

Among various soy protein-based foods, protein fortified beverages is one of the fasted growing categories in food industry. Soy protein beverage sales in the US, according to Beverage Marketing Corporation of New York, showed a significant

increase since 2000 and will become more entrenched in the mainstream market in the near future (Beverage Marketing Corporation of New York, 2005).

Commonly, three categories of soy protein beverages are available in the US market. Soymilk, powder or shake formula, and nutritional beverages. Soymilk contains ~3.5% protein and is considered the most popular drink made from soy. Soy protein powder or shake formula, on the other hand, has a relatively high protein content of ~6.5% to 10.4% when formulated into a beverage following label instructions. Due to the high protein content, protein powders and shakes have gained popularity with bodybuilders, athletes, and health conscious individuals. However, the protein powders and shakes are different from the ready-to-drink beverages. The dried powder has to be mixed with and dispersed in water, fruit juices, or milk before serving. This is because the proteins are not stable enough to be held in a solution at such high protein percentages. They fall out and precipitate during storage. Nutritional beverages, opaque protein suspensions that use a combination of soy and dairy proteins, contain roughly 3~6.5% protein. They are available and advertised in the market as a meal replacement ready-to-drink beverage because of their nutritional quality and convenience.

In 2008, FDA approved a protein concentration of at least 4.2 % to make a label claim of a “high protein beverage” (FDA, 2008). To the best of our knowledge, there is no protein beverage in the US market made with only soy protein at greater than 4.2% protein content. However, developing these soy protein beverages may cause flavor challenges apart from stability issues. Thus, it is crucial to investigate the sensory quality and acceptability of such beverages.

A negative attitude towards soy flavor remained even when consumers were informed of the nutritional and health benefits of the products (Teh and others, 2007). The presence of an unfavorable beany flavor and chalky mouthfeel of the natural soy plays a major role in deterring consumers from soy protein beverages especially when soy protein content is high. Moreover, the removal of these unfavorable attributes appears to be difficult (MacLeod and Ames, 1988).

Only a few studies have been conducted on sensory acceptability of soy protein beverages. Potter and others (2007) reported that soy beverages formulated with 2.8% protein and wild blueberries received low acceptability ratings except for the one sweetened with juice concentrate, which was scored slightly below "like moderately" on a 9-point hedonic scale. The researchers also demonstrated that the consumer acceptability of the beverages decreased as the main sensory attributes associated with soy, including painty aroma, green grassy aroma, chalkiness, nutty taste, and bitterness, increased (Potter and others, 2007). A comparison between beverages containing whey and soy protein was addressed by Childs and others (2007). Meal replacement beverages at 6.5% protein concentration containing either whey protein only or a mixture of whey and soy protein showed higher acceptability than the one containing only soy protein (Childs and others, 2007).

To date, there has been no attempt to develop a high protein beverage at greater than 4.2% protein using hydrolyzed soy protein mainly due to the anticipated bitterness of some of the released peptides. Bitterness of soy peptides may become acceptable or masked when combining soy protein hydrolysate (SPH) with iced tea since tea bitterness is a key sensory attribute that is desired by tea consumers (Scharbert and Hofmann, 2005; Ahmed and others, 2010). However, beany flavor, the most offensive sensory characteristic associated with soy, can still be a challenge for beverages formulated with SPH.

Beany flavor is caused by numerous volatile compounds including dimethyl trisulfide, methanethiol, hexanal, octanal, benzaldehyde, and many others (Boatright and Lei, 2000; Lei and Boatright, 2001). Some efforts have been made to remove these off-flavors using enzymes such as aldehyde dehydrogenase (Takahashi and others, 1980; Matoba and others, 1985), lipase (Trumbetas and others, 1993), microorganisms (Kobayashi and others, 1992), ion exchange resins (Nakamura and others, 1994), and ultra carbonic dioxide gas (Maheshwari and others, 1995). However, none of these methods has successfully eliminated the beany flavor-causing volatile compounds and more studies need to confirm the feasibility of the methods.

Because the beany flavor-causing compounds cannot be eliminated completely, masking them with other flavors may be the best choice to improve the acceptability of high protein beverages formulated with soy. We hypothesize that the roasted attribute of soy protein matches well with black tea and can cause the beany flavor to be less pronounced. Lemon flavor, commonly added to the iced tea, adds citrus flavor to black tea and may mask the beany flavor at the same time. Soy beverages blended with lemon flavored iced tea may be a good combination and a convenient way to incorporate soy protein into a high protein beverage. Therefore, the objectives of this study were to develop a lemon flavored iced tea formulated with SPH and determine the sensory acceptability of the beverages.

3.3 Materials and methods

3.3.1 Materials

Defatted soy flour (7B) and food-grade alcalase were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL) and Novozymes (Omaha, NB), respectively. Unsweetened and decaffeinated Lipton® instant tea (Englewood Cliffs, NJ) and Market Pantry® granulated sugar (Target Corp, Minneapolis, MN) were purchased locally. Lemon flavor MWNI (28-85-4752) was supplied by Weber Flavor (Wheeling, IL) and Oringer 354Q Caramel Liquid color were supplied by Concord Foods (Brockton, MA). Food grade hydrochloric acid and sodium hydroxide were purchased from Sigma Chemical Co (St. Louis, MO). Food grade utensils available in the pilot plant were used.

3.3.2 Preparation of SPI

SPI was prepared from minimally heat-treated and defatted soy flour following the method outlined in section 1.2.

3.3.3 Preparation of alcalase- hydrolyzed SPI

Preparation of alcalase-hydrolyzed SPI was done following the method outlined in section 1.3. Alcalase-hydrolyzed SPI was chosen as it had the highest solubility and antihypertensive bioactivity when compared to papain-, trypsin-, and

bromelain-hydrolyzed SPI (see section 2.3.8).

3.3.4 Beverage preparation

Beverage preparation was conducted in the University of Minnesota Pilot Plant. Nine formulations (**Table 9**) were prepared, including a control, which was a lemon flavored iced tea without soy proteins. Beverages were made in 2400-mL batches. Soy proteins were weighed and slowly added to distilled water while being stirred with a whisk. The mixture was then stabilized by stirring at room temperature for 1 hour. When the soy proteins were dispersed into the water completely, the protein mixture was transferred to two 1500-mL glass beakers and covered with aluminum foil. The mixture was heated at 95°C for 1 hour in a pre-heated water bath while being stirred continuously using stir bars. After cooling to room temperature, black tea powder was added to the mixture while stirring with a whisk. Lemon flavor and caramel colorant were added at the end and the beverages were kept refrigerated until tested. More caramel colorant was added to the beverages containg more protein because beverages appeared to be cloudier as more protein was present.

Table 9. Formulations of soy protein beverages with lemon flavored iced tea

Formulation	SPI (%)	SPH (%)	Sugar (%)	Lemon Flavor (%)	Caramel Colorant (%)	Tea Powder (%)	Distilled Water (%)
Control	0	0	8	0.5	0	2.08	89.42
1% SPI	1	0	8	0.5	0	2.08	88.42
2.5% SPI	2.5	0	8	0.5	0.03	2.08	87.89
5% SPI	5	0	8	0.7	0.03	2.08	85.39
7% SPI	7	0	8	0.7	0.05	2.08	83.37
1% SPH	0	1	8	0.5	0	2.08	88.42
2.5% SPH	0	2.5	8	0.5	0.03	2.08	87.89
5% SPH	0	5	8	0.7	0.03	2.08	85.39
7% SPH	0	7	8	0.7	0.05	2.08	83.37

3.3.5 Products

Eight soy protein-fortified iced teas and the control were formulated as listed in section 3.3.4. Soy protein isolate (SPI) and soy protein hydrolysate (SPH) hydrolyzed by alcalase were used at four different protein concentration levels 1%, 2.5%, 5%, and 7%. Details are listed in **Table 9**.

3.3.6 Subjects

One hundred and one subjects (74 females, 24 males) were recruited from the University of Minnesota through e-mail listservs and fliers and paid a cash incentive for their participation. Only sweetened tea drinkers were selected for the study. People who were allergic to any foods were excluded from the study (**Email-listserv, Appendix C**). Age range of the recruited subjects was 18 to 64. Median of the age was 27. The study was held at the Sensory Center located in McNeal Hall on the St. Paul campus of the University. The study was approved by the University Institutional Review Board (IRB) and consent forms were provided (**Consent form, Appendix C**).

3.3.7 Experimental procedure

Nine 30mL samples were placed in clear 2-ounce sample cups with lids and were served at refrigeration temperature (10°C). The samples were assigned 3-digit codes, and the order of presentation was balanced among the participations using a Latin square design. Distilled water, a spit cup, and napkin were provided with the samples. Participants scored all samples for appearance liking, aroma liking, flavor liking, mouthfeel liking, and overall liking on a Labeled Affective Magnitude (LAM) where the liking scores ranged from 0 (greatest imaginable dislike) to 120 (greatest imaginable like). Intermediate scale anchors and their distances (in parenthesis) included dislike extremely (13), dislike very much (25), dislike moderately (39.5), dislike slightly (53), neutral (60), like slightly (67), like moderately (81), like very much (93), and like extremely (104) (**Scales, Appendix C**). Intensity of bitterness and off-flavor were scored using a Line scale with two anchors indicating no sensation (0) and strongest imaginable sensation of any kind (150) (**Scales, Appendix C**). A series of questions listed below were asked after tasting all the beverages (**List of questions, Appendix C**).

- How frequently do you drink tea?
- Which type of tea do you drink?
- How frequently do you drink protein fortified beverages?

3.3.8 Data analysis

An Analysis of Variance (ANOVA) in XLSTAT® 2009 (Addinsoft Inc., Deutschland, Germany) was used to determine if the beverages differed from each other in liking. The product type and judges were set as independent variables and ratings were dependent variables. Fisher's LSD test was used to make multiple mean comparisons among the different beverage formulations ($\alpha = 0.05$). People who drank tea more than once a week were sorted into high frequency tea drinkers. For high protein fortified beverages, people drinking the beverages more than once a month were categorized by high frequency drinkers. An analysis of variance (Proc GLM in

SAS® v.9.2 (SAS Institute Inc., Cary, NC)) was used to compare liking ratings from the high frequency and low frequency groups. The ratings=samples, tea drinking frequency (protein beverage drinking frequency), judges nested in tea drinking frequency (protein beverage drinking frequency), and sample * tea drinking frequency (protein beverage drinking frequency) were set as independent variables and ratings were dependent variables.

3.4 Results

3.4.1 Overall liking

The beverages with 1% (w/v), 2.5%, and 5% SPH were liked the most and were not significantly different in liking from the control (**Figure 6**) (**Table 34, Appendix K**). The beverages tended to be less liked as protein concentration increased except the one containing 7% SPI. Overall liking of beverages formulated with SPHs was significantly greater than the ones with SPIs (**Table 10**) (**Table 34, Appendix K**).

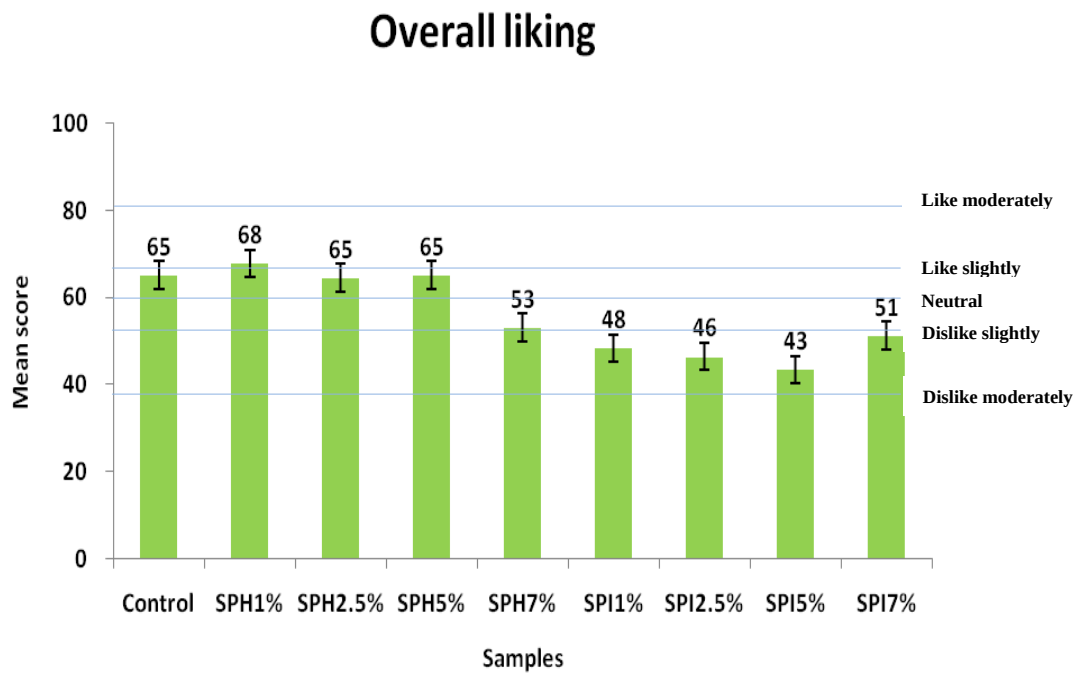

Figure 6. Mean (n=101) overall liking scores on an 11-point LAM scale. Error bars represent standard errors. A mean score of 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. The end of the scale (120) was labeled greatest liking imaginable.

Table 10. Mean (n=101) overall liking scores. A mean score 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	65^{a}	68^{a}	65^{a}	65^{a}	53^{b}	48^{cd}	46^{de}	43^{e}	51^{bc}

3.4.2 Bitterness

The beverages formulated with SPHs were significantly less bitter than the beverages with SPIs (**Figure 7, Table 11**) (**Table 34, Appendix K**).

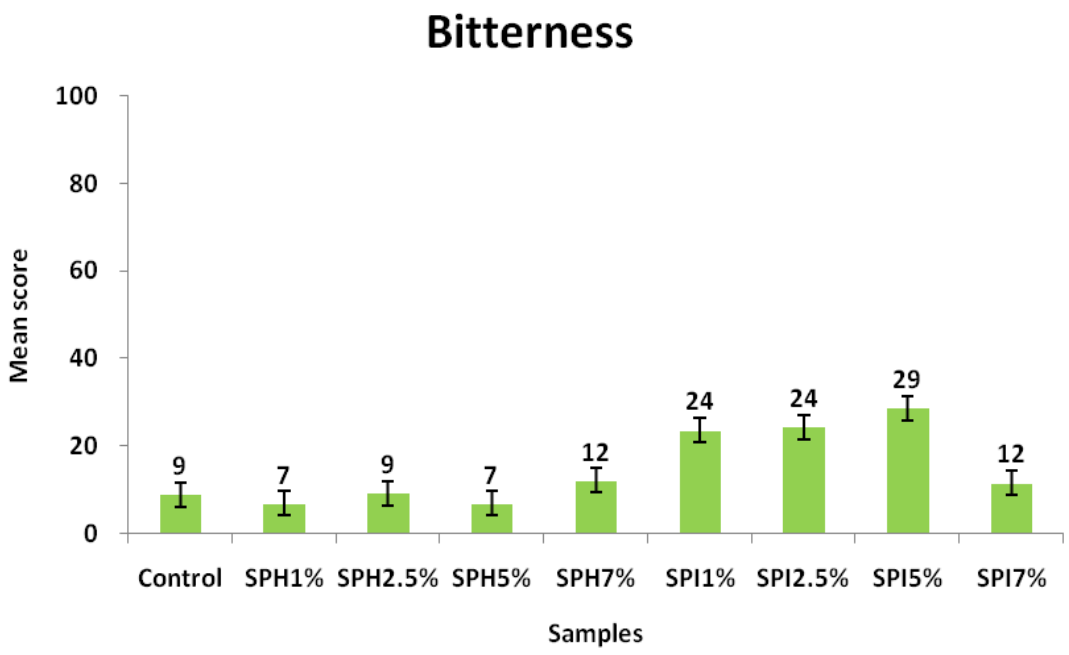

Figure 7. Mean (n=101) bitterness intensity scores on a Line scale. Error bars represent standard errors. A score of 0 corresponds to no sensation; a score of 150 corresponds to strongest imaginable sensation of any kind.

Table 11. Mean (n=101) bitterness intensity scores. A score of 0 corresponds to no sensation; a score of 150 corresponds to strongest imaginable sensation of any kind. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	9^{cd}	7^{d}	9^{cd}	7^{d}	12^{c}	24^{b}	24^{b}	29^{a}	12^{c}

3.4.3 Mouthfeel liking

The mouthfeel of the beverages formulated with SPHs was more liked than the mouthfeel of the beverages formulated with SPIs (**Figure 8**). Specifically, mouthfeel of the formulations containing 1%, 2.5%, and5% SPHs and the control did not show significant differences in mouthfeel liking (**Table 12**) (**Table 34, Appendix K**). Mouthfeel liking tended to decrease with increasing protein concentration with the exception of the sample formulated with 7% SPI (**Figure 8**).

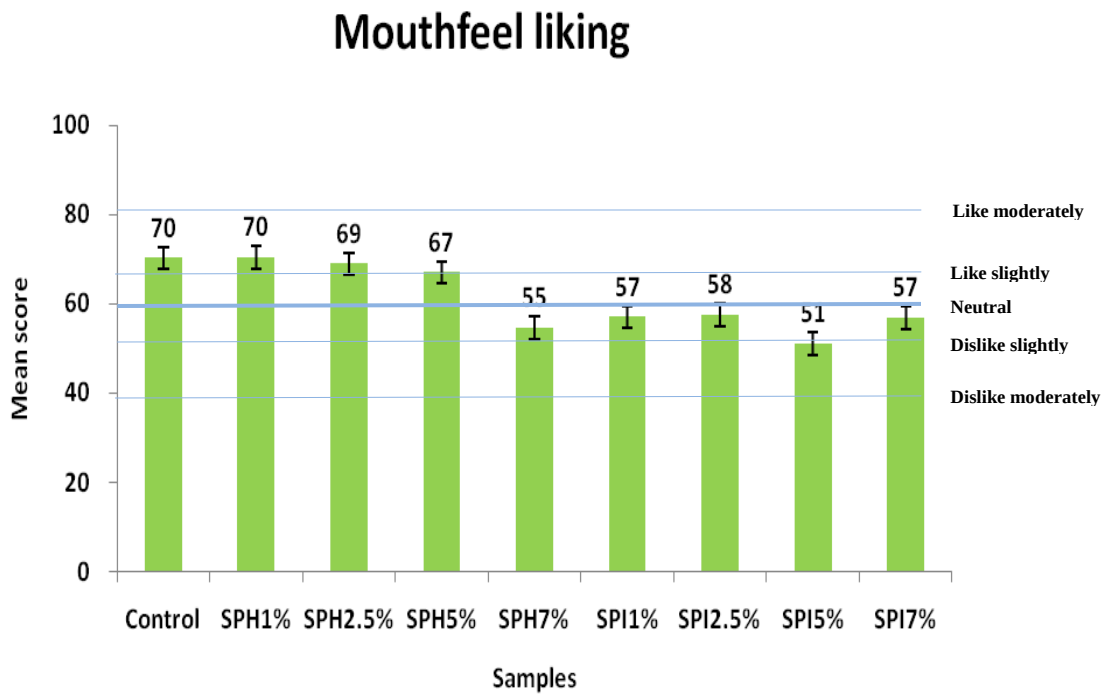

Figure 8. Mean (n=101) mouthfeel liking scores on an 11-point LAM scale. Error bars represent standard errors. A mean score of 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. The end of the scale (120) was labeled greatest liking imaginable.

Table 12. Mean (n=101) mouthfeel liking scores. A mean score 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	70^{a}	70^{a}	69^{a}	67^{a}	55^{bc}	57^{b}	58^{b}	51^{c}	57^{b}

3.4.4 Flavor liking

Similar to what was observed in overall liking, flavors of the beverages formulated with 1%, 2.5%, and 5% SPH were liked the most (**Figure 9**). They were not significantly different from the control (**Table 13**) (**Table 34, Appendix K**). The flavors of the beverages with SPH were more liked than the ones made with SPI.

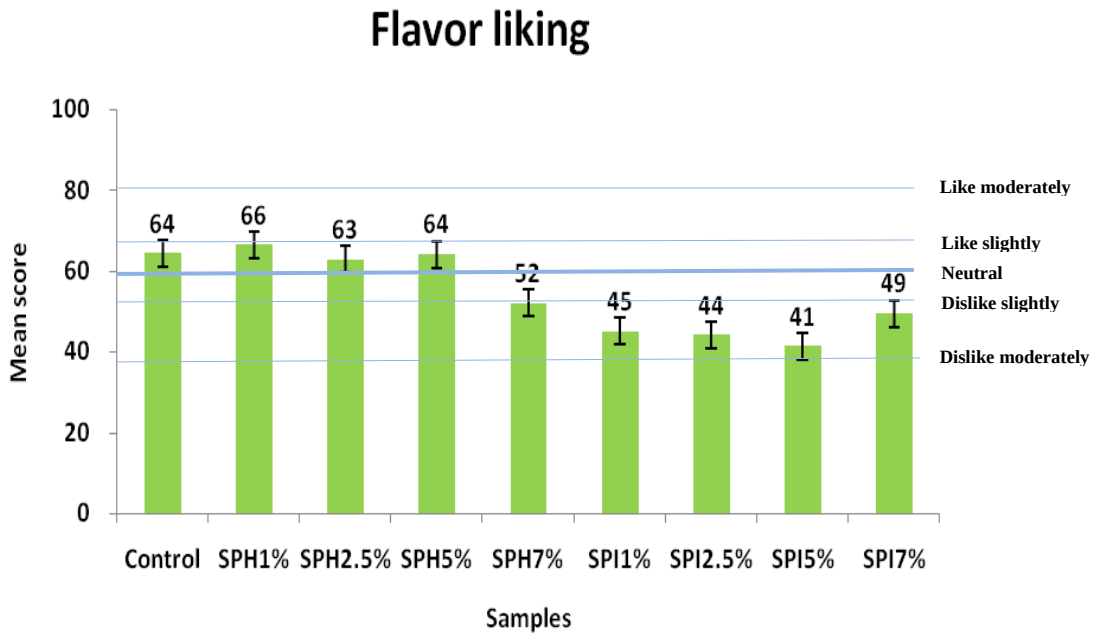

Figure 9. Mean (n=101) flavor liking scores on an 11-point LAM scale. Error bars represent standard errors. A mean score of 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. The end of the scale (120) was labeled greatest liking imaginable.

Table 13. Mean (n=101) flavor liking scores. A mean score 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	65^{a}	66^{a}	63^{a}	64^{a}	52^{b}	45^{cd}	44^{d}	41^{d}	49^{bc}

3.4.5 Aroma liking

Aroma liking of the beverages increased with decreasing protein content (**Figure 10**). The 7% protein samples had a noticeably lower aroma liking rating. The beverages made with 1% SPI, 2.5% SPI, 1% SPH, and 2.5% SPH did not show a significant difference in aroma liking ($P \leq 0.05$) (**Table 14**) (**Table 34, Appendix K**). The aroma of the beverages with SPI was liked as much as ones formulated with SPH.

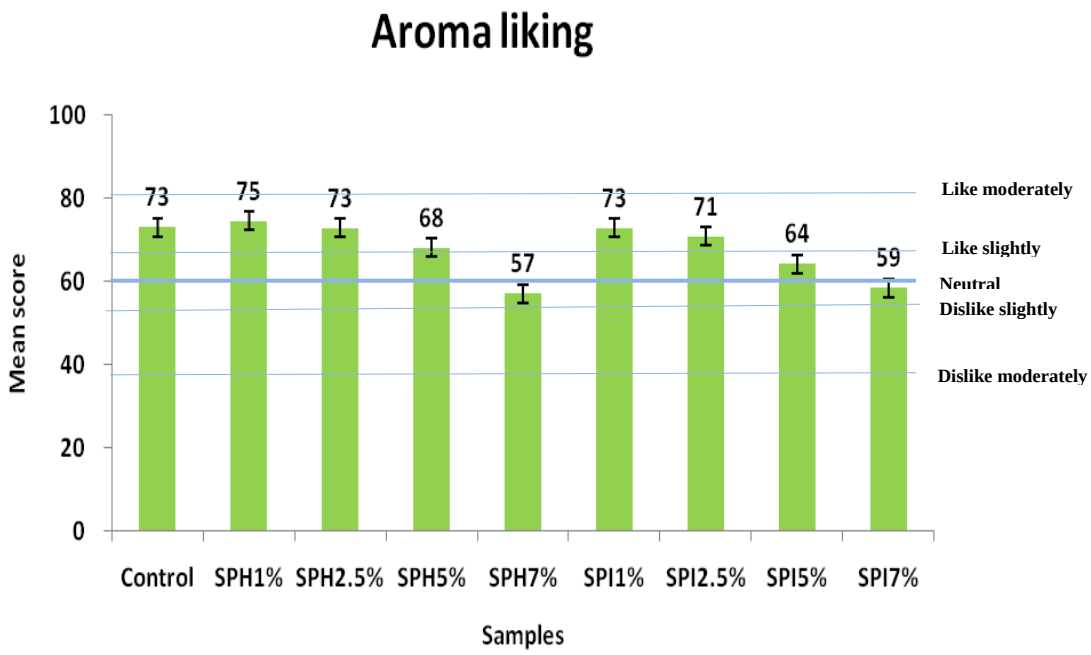

Figure 10. Mean (n=101) aroma liking scores on an 11-point LAM scale. Error bars represent standard errors. A mean score of 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. The end of the scale (120) was labeled greatest liking imaginable.

Table 14. Mean (n=101) aroma liking scores. A mean score 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	73^{a}	75^{a}	73^{a}	68^{bc}	57^{d}	73^{a}	71^{ab}	64^{c}	59^{d}

3.4.6 Appearance liking

Liking of appearance decreased when more protein was present in the beverage (**Figure 11**). The beverages formulated at 1% and 2.5% SPH were rated the highest in appearance liking (**Table 15**).

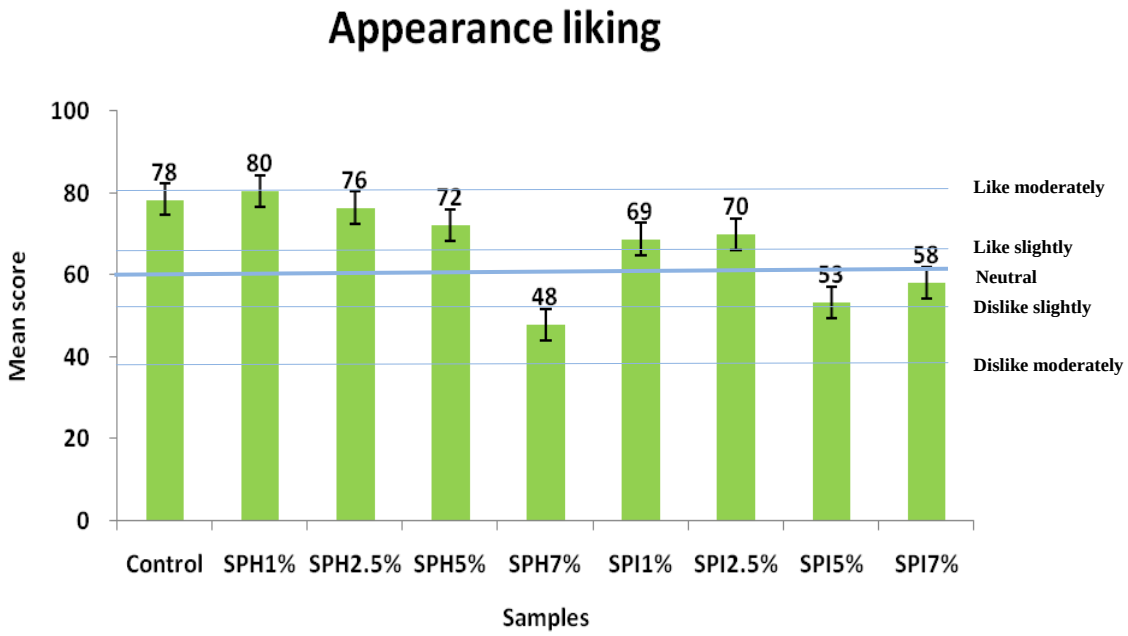

Figure 11. Mean (n=101) appearance liking scores on an 11-point LAM scale. Error bars represent standard errors. A mean score of 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. The end of the scale (120) was labeled greatest liking imaginable.

Table 15. Mean (n=101) appearance liking scores. A mean score 60 corresponds to the neutral point on the scale. A score of 53 corresponds to dislike slightly; a score of 67 corresponds to like slightly; a score of 81 corresponds to like moderately. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	78^{a}	80^{a}	76^{a}	72^{b}	48^{e}	69^{b}	70^{b}	53^{d}	58^{c}

3.4.7 Off flavor

The beverages formulated with 1%, 2.5%, and 5% SPH showed the least amount of off flavor (**Figure 12**). The off flavor of these three samples was significantly lower than all SPI samples (**Table 16**) (**Table 34, Appendix K**).

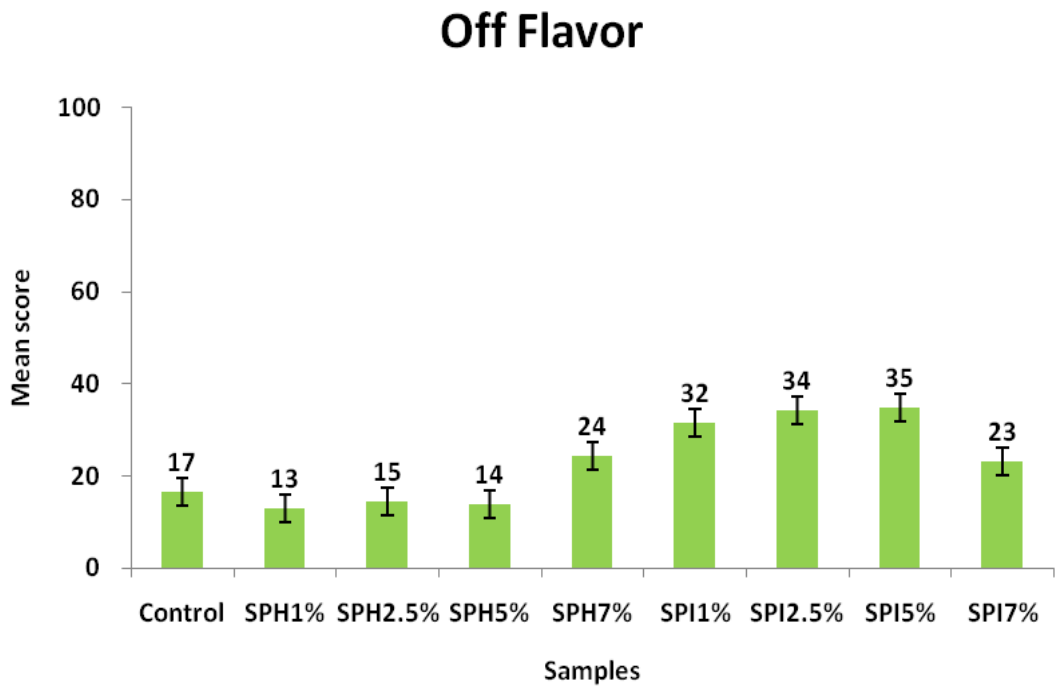

Figure 12. Mean (n=101) off flavor intensity scores on a Line scale. Error bars represent standard errors. A score of 0 corresponds to no sensation; a score of 150 corresponds to strongest imaginable sensation of any kind.

Table 16. Mean (n=101) off flavor intensity scores. A score of 0 corresponds to no sensation; a score of 150 corresponds to strongest imaginable sensation of any kind. Means with the same letters are not significantly different ($p > 0.05$) as determined by a Fisher's LSD test.

	Control	SPH1%	SPH2.5%	SPH5%	SPH7%	SPI1%	SPI2.5%	SPI5%	SPI7%
Mean score	17^{c}	13^{c}	15^{c}	14^{c}	24^{b}	32^{a}	34^{a}	35^{a}	23^{b}

3.4.8 Tea frequency

High frequency tea drinkers rated the beverages significantly lower in overall liking, appearance liking, flavor liking, and mouthfeel liking than low frequency tea drinkers (**Table 17**). High frequency tea drinkers perceived the beverages as significantly more bitter and as having more off flavor. SAS code is provided (**Appendix L**).

Table 17. Mean scores of people grouped by tea drinking frequency. People drinking tea more than once a week were sorted into high frequency tea drinkers. Liking scores could range from 0 (greatest imaginable dislike) to 120 (greatest imaginable like). Intermediate scale anchors and their distances (in parenthesis) included dislike extremely (13), dislike very much (25), dislike moderately (39.5), dislike slightly (53), neutral (60), like slightly (67), like moderately (81), like very much (93), and like extremely (104).

Attributes	Tea drinking frequency		F-value	P-value
	High	Low		
Aroma liking	68	68	0	0.68
Overall liking	55	58	9	0.002
Appearance liking	66	70	20	<0.001
Flavor liking	53	57	9	0.002
Mouthfeel liking	59	65	35	<0.001
Bitterness	17	11	35	<0.001
Off flavor	26	18	47	<0.001

3.4.9 Protein beverage frequency

People drinking protein beverages more frequently rated the products significantly lower in mouthfeel liking (**Table 18**). SAS code is provided (**Appendix L**).

Table 18. Mean scores of people grouped by protein beverages drinking frequency. People drinking the beverages more than once a month were categorized by high frequency drinkers. Liking scores could range from 0 (greatest imaginable dislike) to 120 (greatest imaginable like). Intermediate scale anchors and their distances (in parenthesis) included dislike extremely (13), dislike very much (25), dislike moderately (39.5), dislike slightly (53), neutral (60), like slightly (67), like moderately (81), like very much (93), and like extremely (104).

	Protein beverages drinking frequency			
Attributes	High	Low	F-value	P-value
Aroma liking	67	69	3	0.11
Overall liking	57	56	0	0.54
Appearance liking	67	68	0	0.50
Flavor liking	55	54	0	0.62
Mouthfeel liking	59	62	5	0.03
Bitterness	15	15	0	96
Off flavor	23	23	0	0.99

3.4.10 Gender effect

Women rated the products higher for aroma liking than men (**Table 19**). Men rated the products higher in overall liking and flavor liking than women. Men

perceived the beverages as more bitter and as having more off flavor. SAS code is provided (**Appendix L**).

Table 19. Mean scores of men and women. Liking scores could range from 0 (greatest imaginable dislike) to 120 (greatest imaginable like). Intermediate scale anchors and their distances (in parenthesis) included dislike extremely (13), dislike very much (25), dislike moderately (39.5), dislike slightly (53), neutral (60), like slightly (67), like moderately (81), like very much (93), and like extremely (104).

	Gender			
Attributes	Men	Women	F-value	P-value
Aroma liking	64	70	26	<0.001
Overall liking	58	55	6	0.02
Appearance liking	68	66	1	0.23
Flavor liking	58	53	12	0.001
Mouthfeel liking	62	62	0	0.91
Bitterness	15	13	4	0.05
Off flavor	25	17	47	<0.001

3.5 Discussion

While soy protein hydrolysate has been believed to be accompanied by an anticipated bitter taste that limits utilization in food applications, our study showed that SPH was less bitter than SPI in the tea beverage application. This can be explained by two reasons. First, hydrolyzing soy protein using alcalase might be effective to reduce the amount of bitter peptides. Research has shown that alcalase caused strong reduction in bitterness of fish protein hydrolysate (Hoyle and others, 1994; Kristinsson and others, 2000). Alcalase is an endopeptidase that cleaves much

smaller number of free amino acids and so generally is capable of producing less bitter hydrolysates than exopeptidases (Skanderby and others, 1994). Additionally, limited hydrolysis at DH 3.57% is enough to result in functional changes with the minimum release small molecular weight bitter peptides. A sensory study evaluating six different hydrolysates, including flavourzyme-, alcalase-, neutrase-, protamex-, papain-, and bromelain-hydrolyzed SPI, showed that bitterness intensity of the SPH solutions increased as DH of the hydrolysates increased up to about 40% (Seo and others, 2008). Limited hydrolysis 2-8% is strongly suggested to minimize the production of detectable bitter peptides (Matoba and others, 1972). Therefore, limited enzymatic hydrolysis 2-8% using alacalse, as our observation confirmed, is a good choice to reduce bitterness associated with the hydrolysis of soy protein. Moreover, high protein beverages (> 4.2%) formulated only with SPH are not available in the US market, thus our observation will be a good starting point for the production of high protein beverages with SPH at relatively low bitterness level.

Clarity of the beverages may affect the appearance ratings. The more protein in the beverages the higher are the chances for protein aggregation, which results in undesired opaque brown color. Turbidity of soy protein (Inouye and others, 2002) and whey protein (LaClair and Etzel, 2009) solutions increased as protein concentration increased. Increasing protein concentration results in shorter distances between protein molecules, more competition for water and higher chances of protein-protein interactions, thus increasing the protein aggregation and turbidity (Nicorescu and others, 2009). Since iced tea is expected to be a clear brown, loss of clarity by protein addition may have had a negative influence on appearance rating.

Flavor of the SPH beverages was more liked than the SPI beverages because hydrolyzing soy protein may have caused a reduction in beany flavor. A couple of studies showed that hydrolyzing soy protein with aldehyde dehydrogenase (Takahashi and others, 1980; Matoba and others, 1985) and lipase (Trumbetas and others, 1993) resulted in a reduction in the amount of the beany flavor compounds presumably by cleaving them. However, reduction of beany flavor by utilization of alcalase needs to be investigated. The role of proteins in the perception of a beany

flavor has not been examined. Since proteins are capable of binding flavor compounds through hydrophobic interactions, upon hydrolysis of the protein some of the associated beany flavor compounds might get dissociated and released. Although we did not measure the intensity of beany flavor in this study, the beany flavor intensity of the beverages made from the hydrolysates should be compared with the beany flavor intensity of beverages made with SPI. The added sweet (sugar), tea, and lemon flavors should have suppressed the beany flavor of the added protein. Even though we increased the amount of added lemon flavor in the higher protein samples that added flavor was insufficient to overcome the undesirable flavor contribution of the protein.

Frequent tea drinkers might have noticed easily that the formulated beverages were different from the regular tea that they usually drink. The frequent drinkers are most likely used to the sensory characteristics of the regular tea, therefore, formulated beverages with soy protein received lower liking ratings. Also the frequent drinkers liked less the beverages because they might not have met their expectations as a tea. Studies have shown that foods are less liked when temperature (Zellner and others, 1988), appearance (Hurling and Shepherd, 2003), and processing methods (Cardello, 2003) of food were not as the way the consumers expected. Frequent protein beverage drinkers, on the other hand, seem to be more tolerant of the differences in the beverages.

3.6 Conclusion

Lemon flavored iced tea beverages formulated with SPH up to 5% protein content were liked as much as those without added protein. SPH beverages up to 5% also had significantly less bitter taste and off flavors than SPI beverages. As protein concentration increased, the beverages were rated lower in appearance liking, mouthfeel liking, flavor liking, and aroma liking. Perception of bitterness and off flavors increased at higher protein concentration (7%). Although some bitterness and off flavors were observed in the beverages with SPH, high protein fortified beverages

with hydrolyzed soy protein were found to be acceptable when formulated in lemon flavored iced tea.

4. Overall conclusions, implications, and recommendations

Hydrolysis conditions using minimally heat treated SPI were optimized in order to produce SPH with relatively good solubility, thermal stability, and ACE inhibitory activity. Among the four produced hydrolysates, including papain, bromelain, trypsin, and alcalase SPH, alcalase SPH was the most soluble (75.25%) and thermally stable at relatively high protein concentrations such as 5%. Previously, researchers have investigated solubility and thermal stability of soy protein hydrolysates at relatively low protein concentrations, which is a limiting factor for food applications such as high-protein beverages. Therefore, this work provided for the first time solubility data of heated soy protein hydrolysate solutions at concentration greater than 4%. Additionally, limited hydrolysis with alcalase (DH=3.57%) resulted in pronounced antihypertensive activity. Soy protein hydrolysate with improved solubility and thermal stability was not previously investigated for its potential antihypertensive activity (IC_{50}=0.263 mg protein/ mL). Our results indicated that soy protein hydrolyzed using alcalase under controlled hydrolysis conditions has a great potential to be used as an ingredient for a stable high-protein beverage (>4.2% protein) that contributes to pronounced physiological benefit.

However, more work needs to be done to modify hydrolysis conditions to enhance solubility of alcalase SPH and optimize production of bioactive peptides, such as modifying hydrolysis conditions other than enzyme activity. It is necessary to investigate ways to terminate enzyme reaction other than boiling to avoid decrease in solubility. Approaches may include use of enzyme inhibitors, immobilized enzymes, and pH adjustment. More research involving isolation and characterization of the bioactive peptides released upon alcalase hydrolysis is needed. Monitoring physico-chemical properties of the released peptides would be a natural complementary follow up. Additionally, *in vivo* bioavailability of the bioactive peptides has to be

determined before a generalized health statement is made for a high-protein beverage fortified with alcalase-hydrolyzed soy protein. Storage studies to examine the shelf life of the formulated beverages are also needed.

Lemon flavored iced tea beverages formulated with alcalase-hydrolyzed SPI (>4.2%) were found to be acceptable. The beverages formulated with SPH at 1%, 2.5%, and 5% protein content were liked as much as those without added protein. SPH beverages up to 5% also had significantly less bitter taste and off flavors than SPI beverages. Many studies have reported that bitter taste caused by hydrolyzing food proteins limits the utilization of the hydrolysates in food applications. Previously, studies have reported that alcalase is an endopeptidase that releases peptides that are not bitter. The results of our work showed that alcalase-hydrolyzed soy protein isolate resulted in an acceptable beverage with relatively low bitter taste when formulated into lemon flavored iced tea. These findings are remarkable especially for high protein fortified beverage market, since there is no beverage formulated with only SPH available in US market.

However, more studies need to confirm if utilization of alcalase is effective to reduce beany flavor compounds and bitter peptides. Beverage formulation with other flavors would be worthwhile to try in order to have better acceptability.

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Appendices

Appendix A: Hydrolysis produced at different enzyme activity level

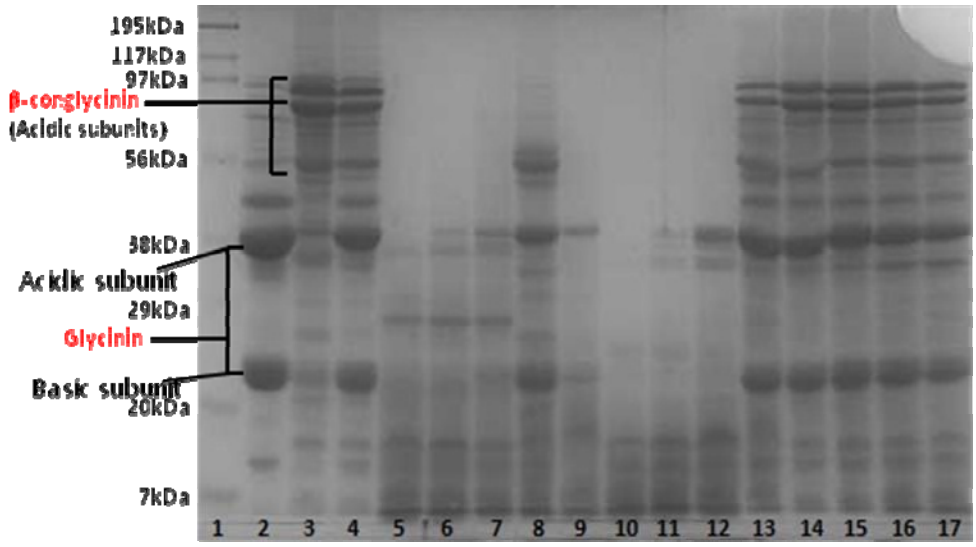
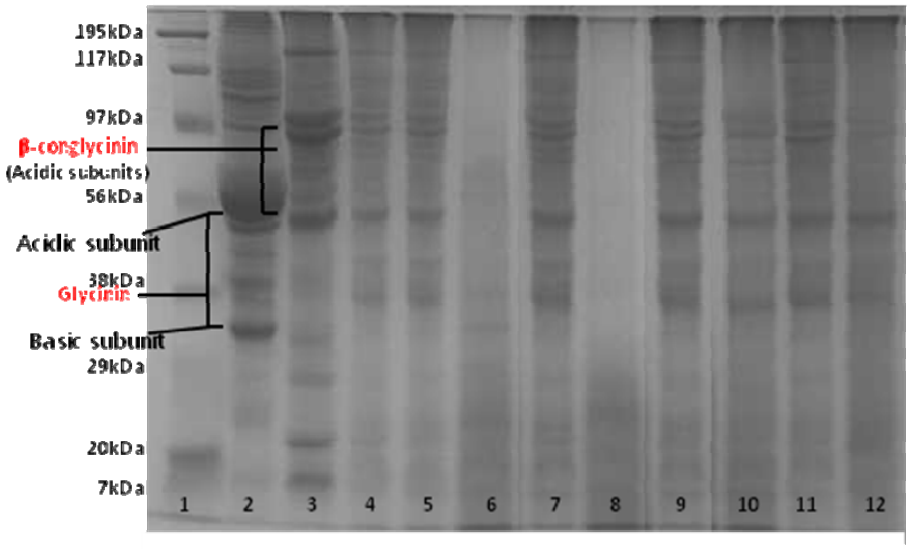


Figure 13. Hydrolysis patterns of papain-, bromelain-, alcalase-, and trypsin hydrolyzed SPI with different activities. Lanes are as follows: 1: molecular weight marker; 2: glycinin standard; 3: β-conglycinin standard; 4: SPI; 5: papain-hydrolyzed SPI (27552 USP units/ mL); 6: papain-hydrolyzed SPI (13776 USP units/ mL); 7: papain-hydrolyzed SPI (9840 USP units/ mL); 8: bromelain-hydrolyzed SPI (0.66 GDU/ mL); 9: bromelain-hydrolyzed SPI (1.327 GDU/ mL); 10: alcalase-hydrolyzed SPI 0.06 (AU-A/ mL); 11: alcalase-hydrolyzed SPI (0.024 AU-A/ mL); 12: alcalase-hydrolyzed SPI (0.012 AU-A/ mL); 13: alcalase-hydrolyzed SPI (0.006 AU-A/ mL); 13: trypsin-hydrolyzed SPI (5992.5 U/ mL); 14: trypsin-hydrolyzed SPI (9400 U/ mL); 15: trypsin-hydrolyzed SPI (58750 U/ mL); 16: trypsin-hydrolyzed SPI (117500 U/ mL)

Appendix B: Hydrolysis patterns of heated and non-heated hydrolysates

a.



b.

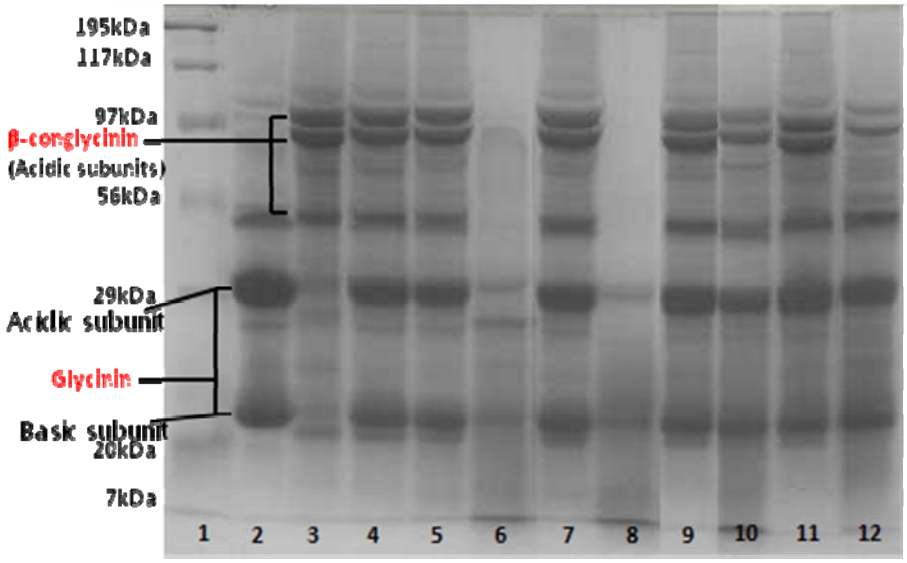
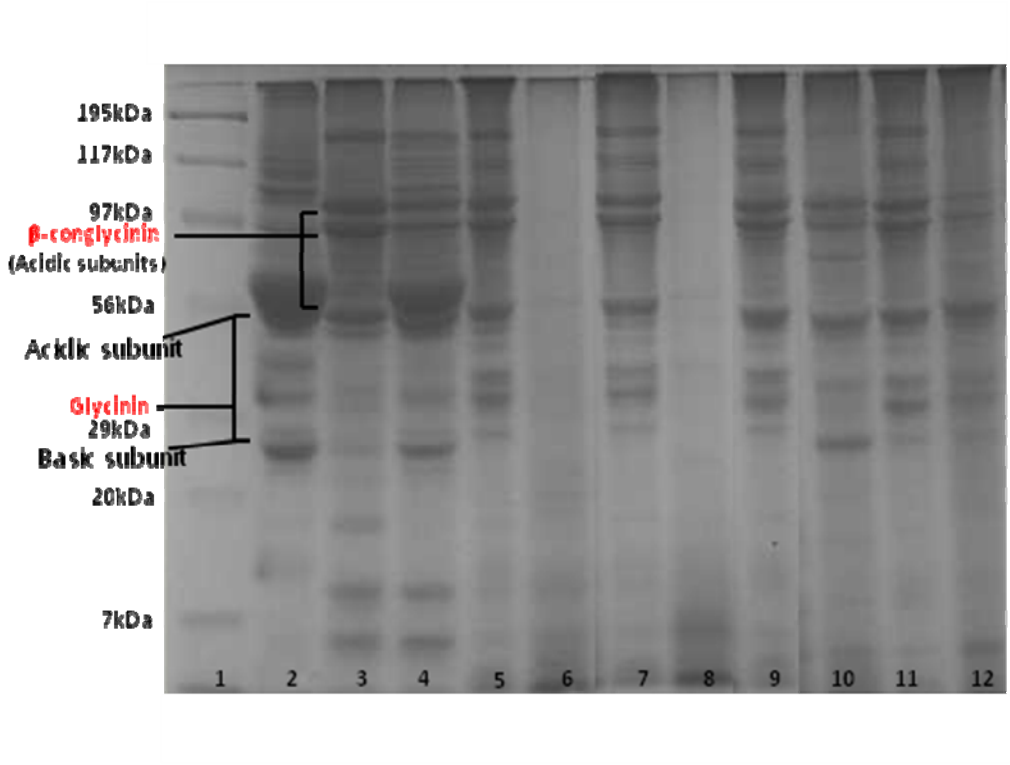


Figure 14. Hydrolysis patterns of heat treated SPH with DH 2-8% and controls under (a) non-reducing condition and (b) reducing condition (β-mercaptoethanol). Lanes for both (a) and (b) are as follows: 1: molecular weight marker; 2: glycinin standard; 3: β-conglycinin standard; 4: SPI; 5: papain control; 6: papain-hydrolyzed SPI (9840 USP units/ mL); 7: bromelain control; 8: bromelain-hydrolyzed SPI (1.327 GDU/ mL); 9: trypsin control; 10: trypsin-hydrolyzed SPI (117500 U/ mL); 11: alcalase control; 12: alcalase-hydrolyzed SPI (0.006 AU-A/ mL)

a.


195kDa
117kDa
97kDa
β-conglycinin
(Acidic subunits)
56kDa
Acidic subunit
Glycinin
29kDa
Basic subunit
20kDa
7kDa
1
2
3
4
5
6
7
8
9
10
11
12

b.

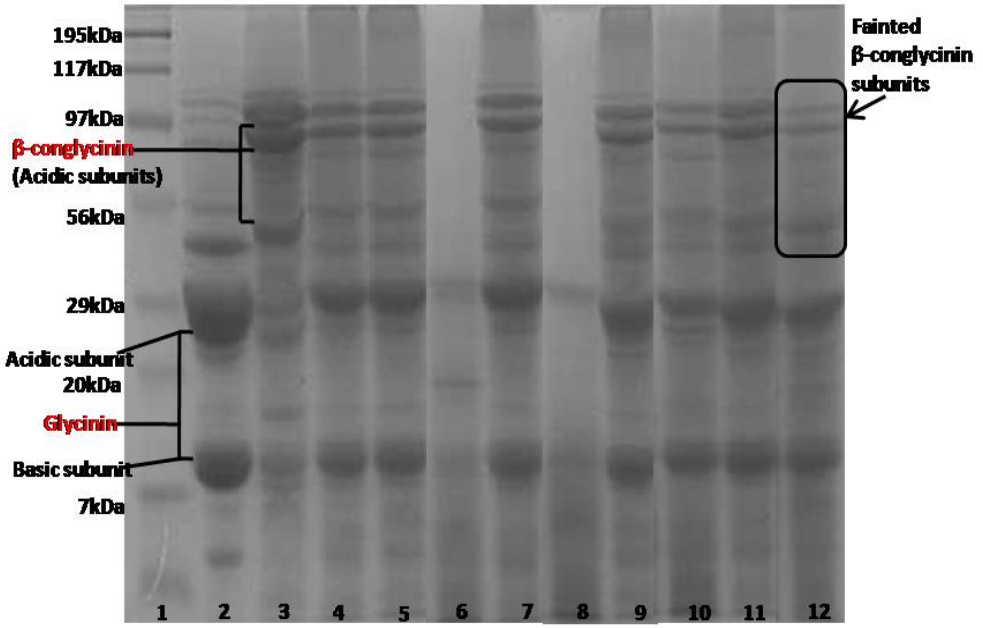


Figure 15. Hydrolysis patterns of non heated SPH with DH 2-8% and controls under (a) non-reducing condition and (b) reducing condition (β-mercaptoethanol).Lanes for both (a) and (b) are as follows: 1: molecular weight marker; 2: glycinin standard; 3: β-conglycinin standard; 4: SPI; 5: papain control; 6: papain-hydrolyzed SPI (9840 USP units/ mL); 7: bromelain control; 8: bromelain-hydrolyzed SPI (1.327 GDU/ mL); 9: trypsin control; 10: trypsin-hydrolyzed SPI (117500 U/ mL); 11: alcalase control; 12: alcalase-hydrolyzed SPI (0.006 AU-A/ mL)

Appendix C: Sensory study supporting documents

CONSENT FORM

You are invited to be in a research study of high protein beverages. You were selected as a possible participant because of your availability and willingness. We ask that you read this form and ask any questions you may have before agreeing to be in the study.

This study is being conducted by Jookyeong Lee from the Department of Food Science and Nutrition.

Procedures:
If you agree to be in this study, we would ask you to do the following things: Taste high protein beverages and answer questions relevant to the beverages you have tasted. Approximate time commitment 10-15 min.

Confidentiality:
The records of this study will be kept private. In any sort of report we might publish, we will not include any information that will make it possible to identify a subject. Research records will be stored securely and only researchers and the course instructor will have access to the records.

Voluntary Nature of the Study:
Participation in this study is voluntary. Your decision whether or not to participate will not affect your current or future relations with the University of Minnesota. If you decide to participate, you are free to not answer any question or withdraw at any time without affecting those relationships.

Contacts and Questions:

The researcher conducting this study is Jookyeong Lee. You may ask any questions you have now. If you have questions later, you are encouraged to contact leex3750@umn.edu for Jookyeong. You may also contact the academic advisor, Zata Vickers, FScN 225, 612 624 2257, zvickers@umn.edu.

If you have any questions or concerns regarding this study and would like to talk to someone other than the researcher(s), you are encouraged to contact the Research Subjects' Advocate Line, D528 Mayo, 420 Delaware St. Southeast, Minneapolis, Minnesota 55455; (612) 625-1650.

You will be given a copy of this information to keep for your records.

Email- listserv

Hello!

The Sensory Center at the University of Minnesota is recruiting panelists to participate in a study on protein fortified lemon iced tea on Dec. 13th. The test will be held in McNeal Hall on the Saint Paul Campus and will be scheduled based on your availability.

To participate in the study, we would ask you to do the following things:

You would attend 1 session (approximately 10 minutes long). During the session you will be asked to indicate your liking of several samples of protein fortified iced tea. Each participant will be compensated $5 at the end of evaluation.

***To be eligible for the study, please provide the following information ***

1) Do you have any food allergy?

___ Yes

___ No

2) Do you drink sweetened iced tea?

___ Yes

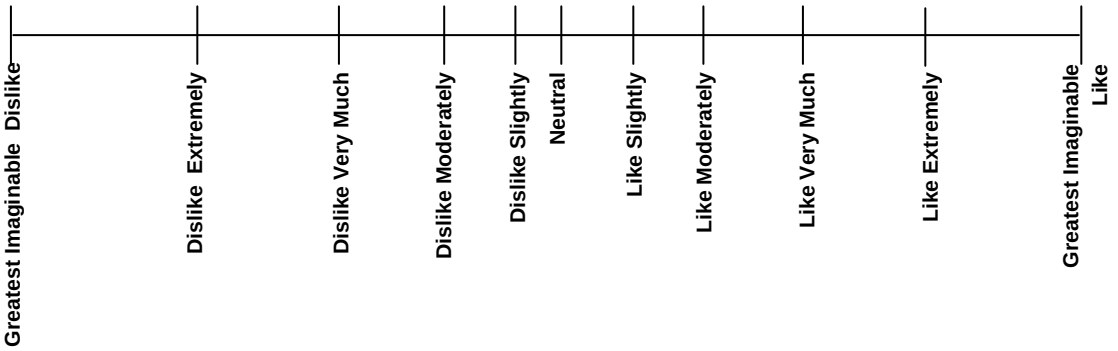
___ No

If you are interested in participating, please fill out the screener above and return by e-mail to leex3750@umn.edu. Your responses will be evaluated to see if you qualify to be part of the study. Your responses to all questions on this form will be kept confidential. If you qualify, we will contact you to schedule your participation.

Thanks,

Scales

Labeled affective magnitude scale (used to rate liking/disliking):



Line scale (used to rate intensity):

List of questions

1) What is your gender? ___ Female ___ Male

2) What is your age?

3) How frequently do you drink tea?
   ___ More than once a day
   ___ Less than once a day bout one or more times a week
   ___ Less than once a week, but one or more times a month
   ___ Less than once a month but more than once a year
   ___ Less than once a year

4) Which type of tea do you drink? (check all that apply)
   ___ Black tea
   ___ Green tea
   ___ Herbal tea
   ___ Others

5) How frequently do you drink protein fortified beverages?
   ___ More than once a day
   ___ Less than once a day bout one or more times a week
   ___ Less than once a week, but one or more times a month
   ___ Less than once a month but more than once a year
   ___ Less than once a year

Appendix D: Analysis of variance table determining the effect of enzyme level on each hydrolysate

Table 20. Anaysis of variance on the effect of enzyme level on each hydrolysate

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Bromelain SPH DH	Enzyme level	1	8.65	<0.001
	Error	4	0.002	
Papain SPH DH	Enzyme level	2	59.64	<0.001
	Error	6	0.002	
Trypsin SPH DH	Enzyme level	3	2.204	<0.001
	Error	8	0.008	
Alcalase SPH DH	Enzyme level	3	175.47	<0.001
	Error	8	0.042	

Appendix E: Analysis of variance table for determining the effect of enzyme hydrolysis on ACE activity slope

Table 21. Analysis of variance of ACE activity slopes on the effect of enzyme hydrolysis by papain, bromelain, trypsin, and alcalase

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
ACE activity slope	Enzyme	5	5.87E-6	<0.001
	Error	12	8.33E-9	

Appedix F: Analysis of variance tables for determining the effect of heat treatment on solubility

Table 22. Analysis of variance of heat treatment effect on solubility of each hydrolysate at 5% protein content in water

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Bromelain SPH	Heat treatment	1	35.81	0.001
	Error	4	0.002	
Papain SPH	Heat treatment	1	12.57	0.011
	Error	4	0.62	
Trypsin SPH	Heat treatment	1	6.27	0.002
	Error	4	0.005	
Alcalase SPH	Heat treatment	1	5.06	0.017
	Error	4	0.325	
SPI	Heat treatment	1	8.60	0.006
	Error	4	0.307	

Table 23. Analysis of variance of heat treatment effect on solubility at 5% protein content in a black tea solution

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Bromelain SPH	Heat treatment	1	0.99	0.124
	Error	4	0.263	
Papain SPH	Heat treatment	1	1.25	0.11
	Error	4	0.052	
Trypsin SPH	Heat treatment	1	23.11	<0.001
	Error	4	0.167	
Alcalase SPH	Heat treatment	1	2.73	0.049
	Error	4	0.355	
SPI	Heat treatment	1	4.73	0.004
	Error	4	0.14	

Table 24. Analysis of variance on alcalase SPH solubility effect of heat treatment at each protein concentration in tea

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
1% SPH	Heat treatment	1	14.96	0.005
	Error	4	0.46	
2.5% SPH	Heat treatment	1	0.26	0.396
	Error	4	0.289	
5% SPH	Heat treatment	1	2.734	0.05
	Error	4	0.335	
7% SPH	Heat treatment	1	0.245	0.137
	Error	4	0.071	

Table 25. Analysis of variance on SPI solubility effect of heat treatment at each protein concentration in tea

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
1% SPI	Heat treatment	1	0.07	0.07
	Error	4	0.251	
2.5% SPI	Heat treatment	1	1.241	0.396
	Error	4	0.205	
5% SPI	Heat treatment	1	5.568	0.025
	Error	4	0.454	
7% SPI	Heat treatment	1	16.52	<0.001
	Error	4	0.118	

Appendix G: Analysis of variance tables for determining the effect of enzyme hydrolysis on solubility

Table 26. Analysis of variance on solubility effect of the different hydrolysate without heat treatment in water

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Solubility	Enzyme	4	1322.12	<0.001
	Error	10	0.408	

Table 27. Analysis of variance on solubility effect of the different hydrolysate with heat treatment in water

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Solubility	Enzyme	4	1361.29	<0.001
	Error	10	0.455	

Table 28. Analysis of variance on solubility effect of the different hydrolysate without heat treatment in tea

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Solubility	Enzyme	4	1061.36	<0.001
	Error	10	0.057	

Table 29. Analysis of variance on solubility effect of the different hydrolysate with heat treatment in tea

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Solubility	Enzyme	4	1147.14	<0.001
	Error	10	0.334	

Appedix H: Analysis of variance table for determining the effect of protein concentration on solubility

Table 30. Analysis of variance on SPI and alcalase SPH solubility effect of concentration with and without heat treatment

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Alcalase SPH (Non-heated)	Concentration	3	85.1	<0.001
	Error	8	0.183	
Alcalase SPH (Heated)	Concentration	3	131.55	<0.001
	Error	8	0.404	
SPI (Non-heated)	Concentration	3	86.7	<0.001
	Error	8	0.229	
SPI (Heated)	Concentration	3	152.83	<0.001
	Error	8	0.285	

Appendix I: Analysis of variance tables for determining the effect of enzyme hydrolysis on turbidity

Table 31. Analysis of variance on turbidity effect of the different hydrolysate without heat treatment

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Turbidity	Enzyme	4	1.824	<0.001
	Error	10	5.9E-5	

Table 32. Analysis of variance on turbidity effect of the different hydrolysate with heat treatment

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Turbidity	Enzyme	4	1.984	<0.001
	Error	10	9.56E-5	

Appendix J: Analysis of variance tables for determining the effect of heat treatment on turbidity

Table 33. Analysis of variance on the effect of heat treatment on tubidity of each hydrolysate

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Bromelain SPH	Heat treatment	1	0.132	<0.001
	Error	4	2.69E-5	
Papain SPH	Heat treatment	1	0.1	<0.001
	Error	4	1.1E-5	
Trypsin SPH	Heat treatment	1	0.028	<0.001
	Error	4	3.48E-6	
Alcalase SPH	Heat treatment	1	0.087	<0.001
	Error	4	3.17E-6	

Appendix K: Analysis of variance table for determining the effect of product and judge on liking ratings

Table 34. Analysis of variance for determining the effect of product and judge on liking ratings

Sample analysis	Source of variation	Degree of freedom	Mean sqaure	Significance
Overall liking	Product, judge	108	2251	<0.001
	Error	800	206	
Bitterness	Product, judge	108	1774	<0.001
	Error	800	221	
Mouthfeel liking	Product, judge	108	1916	<0.001
	Error	800	227	
Flavor liking	Product, judge	108	2869	<0.001
	Error	800	312	
Aroma liking	Product, judge	108	1388	<0.001
	Error	800	256	
Mouthfeel liking	Product, judge	108	2251	<0.001
	Error	800	206	
Off flavor	Product, judge	108	4097	<0.001
	Error	800	287	

Appendix L: SAS codes for determining the effect of tea drinking frequency, protein beverage drinking frequency, and gender on liking ratings

```
proc glm data = xxx.tracy;
classes judge sample gender    teafreq Black_tea      Green_tea
Herbal_tea     teatype___Other
       proteinfreq;
model aromalikeoverlike       appearancelike flavorlike
       mouthfeel      bitterness     flavoroff
=  teafreq judge(teafreq) sample teafreq*sample;
means teafreq sample/snk;
run;


proc glm data = xxx.tracy;
classes judge sample gender    teafreq Black_tea      Green_tea
Herbal_tea     teatype___Other
       proteinfreq;
model aromalikeoverlike       appearancelike flavorlike
       mouthfeel      bitterness     flavoroff
=  proteinfreq judge(proteinfreq) sample proteinfreq*sample;
means proteinfreq sample/snk;
run;


proc glm data = xxx.tracy;
classes judge sample gender    teafreq Black_tea      Green_tea
Herbal_tea     teatype___Other
       proteinfreq;
model aromalikeoverlike       appearancelike flavorlike
       mouthfeel      bitterness     flavoroff
=  gender judge(gender) sample gender*sample;
means gender sample/snk;
run;
```