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ANALYSIS OF PROTEIN IN SOY FLOUR AND IN TAPIOCA BASED SNACK CHIPS

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Degree

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ABSTRACT

Two experiments were conducted: E1 to evaluate a rapid analysis method for the proteins in PDI 20 and PDI 85 defatted soy flours (SF) and E2 to investigate the interactions of soy proteins during production of tapioca based snack chips. In E1 total proteins were extracted from each SF by a 0.03M Tris buffer (pH 8.0) with and without 10Mm 2-mercaptoethanol (ME) and were separated into 2S, 7S and 11S fractions. Total protein samples and each fraction were analyzed by high performance liquid chromatography (HPLC) on a TSK 3000-SW size exclusion column. E2 consisted of three parts. In the first part, soy proteins from each production stage: flour, batter, gel and cooked (baked), of tapioca based snack chips containing each SF were analyzed by HPLC. A second part consisted of measurement of percent protein extractable from each stage by buffer alone or with 0.5M NaCl, 6M urea, 0.2M ME, or 20% propylene glycol (PG) to determine specific protein interaction forces. In the last part, the percent moisture and protein of each chip production stage containing each SF and of baked and fried chips were measured, and the hardness and expansion during cooking of chips were determined. Two replications were completed for E1 and for each part of E2.

A total of 11 peaks were found in the chromatograms of the total soy proteins and among the chromatograms of the

fractions of each SF in E1. Time for HPLC analysis was less than 45 min. Tentative identity (ID) of the peaks in the 2S chromatogram included a small amount of beta-conglycinin, lipoxygenase and monomers and aggregates of the two trypsin inhibitors (TI). One 2S peak eluting at 23.9 min was positively identified as an aggregate of the Kunitz TI. Major peaks in the 7S chromatogram were identified as an aggregate of beta-conglycinin with a molecular weight (MW) of over 500,000 Daltons and glycinin. The major peak in the 11S chromatogram was glycinin with a MW of 325,000 Daltons. The chromatogram of PDI 85 SF had better peak resolution and sharper peaks than the chromatogram of PDI 20 SF.

Heating to produce the gel stage of the chip resulted in lower ($P<0.05$) levels of extractable protein and a decrease (($P<0.05$) in the combined peak areas of the beta-conglycinin protomer and aggregate and the glycinin in the chromatogram. Baking did not further reduce protein solubility. Data from the protein interaction study showed that disulfide bonds, hydrogen bonds and hydrophobic interactions were involved in the heat denaturation and reduced solubility of the proteins from the SF of different PDIs and at the gel and cooked stages of the chips. Baked and fried chips from either SF contained, respectively, 17.5% and 12.2% protein and 5.0 and 3.8% moisture. Baked chips containing PDI 20 SF were the hardest and had the least expansion during cooking while fried chips

from PDI 85 SF were the easiest fractured with next to the least expansion during cooking.

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LIST OF ABBREVIATIONS

ANS	1-anilino-8-naphthalene sulfonate
BBTI	Bowman-Birk trypsin inhibitor
CPA	cis-parinaric acid
df	Degree of freedom
DPH	1,6-diphenyl hexatriene
HPLC	High performance liquid chromatography
ME	2-Mercaptoethanol
MS	Mean squares
MW	Molecular weight
NSI	Nitrogen Solubility Index
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein Dispersibility Index
PG	Propylene glycol
SDS	Sodium dodecyl sulfate
SEC	Size Exclusion Chromatography
SNK	Student Newman Keuls multiple range test
SS	Sum of squares
TI	Trypsin inhibitor

CHAPTER 1

INTRODUCTION

Today, widespread hunger and malnutrition due primarily to a deficiency in total calorie and protein intake are still a major problem in the developing countries. The daily supplies of protein and calories in Africa and Far East Asia, where 20 to 23% of the population are undernourished, is only approximately 50 to 65% that of the developed countries such as the U.S.A. and Canada (FAO, 1987; Forbes and Merrill, 1986; Wolf, 1988). Protein fortification of carbohydrate based foods appears to be one way to promote better nutrition in these regions.

In order to provide better nutrition at a reasonable cost, many common food commodities such as baby formula and bread have been fortified with soy protein (Khan et al., 1975; Mermelstein, 1983). However, snack foods can also be a suitable form for mass-feeding and distribution because of their nature of a snack (Warren et al., 1983). According to one definition, a snack is a small ready to eat item (as-is or after minimal preparation) with an adequate shelf life which is eaten between, during or in place of meals (Blenford, 1982).

For this study, a protein fortified carbohydrate (tapioca) based snack chip was produced modeled after the fish flavored chips common in Far East Asia, but substituting the

fish content with defatted soy flour entirely. This particular snack food was chosen because protein fortification of a food in order to improve nutrition is more likely to succeed if the fortified food item is already part of the diet among the targeted population, and if that item maintains the traditional quality and appeal (Sellers et al., 1988). The acceptability (color, flavor, texture and overall acceptability) of similar fish flavored snack chips which were developed by Anderson (1988), Siegel and Lineback (1976) and Wahid (1983) using fish, shrimp, sesame and soybean were equal to (or better than in some cases) either commercially available products or the "home made" variety product produced following a traditional recipe. Defatted soy flour has been previously incorporated into carbohydrate based snack chips at University of Tennessee (Anderson, 1988; Mancuso, 1987). Other traditional foods items from various regions including Africa and West Pakistan have also been successfully fortified with various high-protein flours including soy flour (Lindell and Walker, 1984; Reber et al., 1983).

Defatted soy flour and soy meal which contain high levels of protein are frequently chosen as a protein source for fortification because of low cost, ready availability and production efficiency. Defatted soy meal (or flour) has a considerable economic advantage and a stability of supply since it is generated as a by-product of edible oil production

and is mainly utilized as animal feed at the present time (Wolf, 1988). In addition, utilization of soy meal is efficient since the protein yield per acre of soy is larger than that of most cereal grains including wheat and maize, or animal proteins such as beef (Seal, 1980).

In addition to the nutritional benefits, soy protein is also added to many foods to take advantage of its low cost and functional properties, especially in the developed countries. Examples of such food items include: baked goods such as breads and doughnuts, meat products such as sausages and salisbury steak, and dairy products such as infant formula and whipped toppings (Snyder and Kwon, 1987). It has been reported that emulsification, foaming, fat and water absorption, and browning are improved by addition of soy protein (Snyder and Kwon, 1987).

Total substitution of the fish component in the snack chip with defatted soy flour would not only increase the level of protein due to high protein content of the soy flour, but is also likely to change the physical and functional properties of the chips due to the difference in the protein types (e.g. fibrous fish protein vs. globular soy protein). Changes in these properties, which may possibly affect the acceptance of the final product, may require modifications in the formula and/or processing method. Changes in the chip properties could be due to the protein interactions with other

ingredients. Analysis of the soy protein, both quantitative and qualitative, and study of the interaction forces at different processing stages could provide explanations for any changes in the chemical, physical and functional properties of the chips.

In order to study the soy protein and to monitor the production process in "real-time" with a minimum amount of time elapsed from the time of sampling to the results of the analysis, a simple and rapid analytical method would be desirable. However, commonly used methods for protein analysis including size exclusion chromatography (SEC) and gel electrophoresis require a considerable length of time (Obara and Kimura, 1967; Hasegawa et al., 1963). Even though High Performance Liquid Chromatography (HPLC) which is a simple, rapid and highly sensitive technique has been applied in investigations of other proteins including wheat, potato and milk, this technique, specifically the Size Exclusion HPLC (SEC-HPLC), has not been utilized in analysis of soy protein (Kruger, 1984; Liedl et al., 1987; Okumura et al., 1990). Therefore, the objectives of this study were twofold:

1. To evaluate a simple and rapid soy protein analysis method using SEC-HPLC and

2. To study soy proteins and their interactions in a carbohydrate (tapioca) matrix and their relationship to physical and chemical properties of the tapioca chip at the various production stages.

CHAPTER 2

LITERATURE REVIEW

2.1. Snack Foods and Their Ingredients

According to Blenford (1982), a snack food may be sweet or savory, liquid or solid, crunchy or chewy. This covers a wide range of products including: cookies, crackers, cakes, pies, pastries, potato chips, corn chips, pretzels, popped corn, nut meat, meat snack and frozen pizza. These snack foods are in turn made from a variety of ingredients, which include: nuts, fruit, seed, meat, fish, daily products, cereals such as wheat, maize and oat, legumes such as soybeans and starches such as potato, rice and tapioca. These ingredients are processed and cooked by a variety of methods to produce different types of snack foods. Frying, roasting, extrusion, baking and fermentation are a few examples of the cooking methods used for preparation of snack foods (Blenford, 1982).

Among the wide variety of snack items available, chips made from potato and corn such as potato chips and tortilla chips are very popular (Blenford, 1986; Stauffer, 1983). Another type of snack chip which is commonly available in Far East Asia is fish (or shrimp) flavored carbohydrate based chips (Siegel and Lineback, 1976). According to a native of Thailand, this "fish chip" is typically prepared at home from

a starchy flour such as rice or tapioca, and flavored with any fish (or shrimp) available at the time of production. Following a traditional recipe, the ingredients are mixed with water and spices, and shaped into a log. After gelatinizing by immersion in boiling water, and cooling, this gelatinized log is sliced thinly and then fried in oil to yield puffed crunchy snack chips. These "fish chips" are eaten with, between or in place of meals, just like we eat potato chips in the United States (Hasin, 1987).

2.2. Physical and Chemical Characteristics of the Ingredients for Soy Fortified Tapioca Based Snack Chips

2.2.1. Cassava (Tapioca)

Knowledge of the physical and chemical characteristics of individual ingredients is essential to understanding their interactions. One of the tapioca based snack chip ingredients, tapioca flour is produced from the cassava plant which is cultivated mainly in Asia, Latin America and Africa (Balogopalan et al., 1988). According to Leung et al. (1972), tapioca flour is high in carbohydrates (88.2%) and low in both protein (1.1%) and fat (0.5%).

The majority of the carbohydrate in tapioca flour is starch (approximately 80%) which exists as granules averaging 20 microns in size (Balogopalan et al., 1988; Heckman, 1977). The tapioca starch is further classified into two components:

amylose which is composed of linear chains of D-glucose through alpha-1,4 linkages, and amylopectin which is composed of branched chains, having alpha-1,6 linkages at the branching points in addition to the alpha-1,4 linkages (DeMan, 1980). Only 16-18% of tapioca starch is amylose, and the rest is amylopectin (Balogopalan et al., 1988; Heckman, 1977). Both amylose and amylopectin are extremely large molecules; the molecular weights can reach as high as 2,000,000 g/mol for amylose and 400,000,000 g/mol for amylopectin (Ring and Orford, 1986).

One of the unique characteristics of tapioca is its gelling property. A starch gel is formed when a water suspension of tapioca is heated above the gelation temperature (initiated at 49.0 to 58.5°C and completed at 64.5 to 70.0°C, depending upon the type), and then cooled rapidly (Heckman, 1977). Upon heating, the granules increase in size and release amylose which is responsible for the gel network formation (Heckman, 1977; Leach, 1965; Oakenfull, 1987). When this hot liquid is cooled rapidly, the remaining porous granules consisting mainly of amylopectin become connected with amylose which is in a double helical formation, thus forming a gel (Dea, 1982). If the granules are sufficiently swelled, the bulk of the water is absorbed in the granules, and a relatively small amount of amylose is required to form a continuous gel network. If the swelling is limited, on the

other hand, amylose entraps the water which was not absorbed by the granules, and more amylose is then needed to form a gel network (Otto and Hester, 1965).

The swelling power and solubility of the starch depends upon the type and strength of interaction forces present in starch granules, and varies widely among different starch types (Balogopalan et al., 1988). The interaction forces are influenced by many factors including: the ratio of amylose to amylopectin, molecular weights and their distribution, degree of branching and length of outer branches, conformation and other compounds present (Balogopalan et al., 1988; Leach, 1965). For example, the swelling power of normal corn, rice and sorghum starches are less than their waxy (or glutinous) counterparts which consist mainly of amylopectin (Leach, 1965; Schoch, 1969) The swelling of tapioca is higher than that of other starches including rice, sorghum and corn, and takes place in a single stage process instead of the two stages which are more common with cereal starches. The solubility of tapioca starch is also higher, reflecting the low strength of attractive forces in the granules, even though the gel produced from tapioca starch is more stable than those of most cereal starches (Balogopalan et al., 1988).

The gelatinization process of starch is also influenced by the presence of other compounds including salt,

sugar and protein. In the presence of 0.001N sodium chloride, a slight reduction in the viscosity of tapioca starch is observed (Balogopalan et al., 1988). Addition of a small amount of sucrose, on the other hand, increases the peak viscosity of starch, whereas addition of a large amount of sucrose (i.e., above 20%) decreases the peak viscosity (Balogopalan et al., 1988). A similar nonlinear relation is found in the peak viscosity of increasing quantities of tapioca with addition of defatted soy flour (Anderson, 1988).

2.2.2. Soybeans

In addition to the Orient, where soybeans have long been a traditional food commodity, North America is one of the largest producers of soybeans. Of the total soybeans produced in the world during 1988 (9.2×10^7 metric tons), more than half come from either Asia (1.5×10^7 metric tons) or North America (4.3×10^7 metric tons) (FAO, 1988).

Compared with cassava which is mainly starch, soybeans are a good source of both oil and protein, containing approximately 20% oil, 40% protein and 25% carbohydrate (Snyder and Kwon, 1987). Most of the soy protein in the bean (60-70% of the total protein) is located within protein bodies (also known as aleurone grains) which are 2 to 20 microns in diameter (Snyder and Kwon, 1987; Wolf, 1978).

In addition to their use as a raw material for traditional oriental foods including Tofu and Soy sauce,

soybeans are also grown for edible oil and feed meal production especially in North America because of their high content of oil and protein. Oil from soy beans is obtained mainly by mechanical expulsion or solvent extraction, though the latter is the predominant method in the U.S.A. and in Western Europe (Snyder and Kwon, 1987). After the solvent extraction process, the methods used to remove the residual solvent from the soybean have a significant effect on the protein dispersibility and other functional properties. For example, treatment by a desolventizer-toaster produces a fully toasted defatted meal with a low protein dispersibility index (PDI) of 10 to 30, whereas a flash desolventizer-deodorizer produces a meal with a high PDI of 70 to 90 (Snyder and Kwon, 1987; Wolf, 1975).

The PDI or NSI (nitrogen solubility index) is often used as an indicator of the functional properties of soy protein in determining its applications in the food industry. For example, soy flour with a very high solubility (PDI 75 or greater) is used to bleach wheat flour, since it is normally enzyme active. Soy flour with a medium to low PDI (or NSI) is incorporated into bakery items such as cookies and waffles because of many beneficial properties including improved machinability, crust color and longer shelf life (Smith and Circle, 1972; Wolf, 1975).

Undenatured soy protein with a high PDI is highly soluble in water, even though it is classified as a globulin (which is a water insoluble but saline soluble protein). This is because soy globulin is insoluble in water only in a narrow Ph range near the isoelectric point (pH 4.2 to 4.6) (McWatters and Holmes, 1979; Wolf and Cowan, 1962). In the pH range 3.5 to 6.0, less than 50% of protein in soy is water soluble; in the range 4.0 to 5.0 soy protein has the minimum solubility (Wolf, 1978). However, water at a neutral pH range will extract approximately 85% of the soy protein present (Snyder and Kwon, 1987).

After aqueous extraction, soy proteins may be fractionated by many methods, including isoelectric precipitation, metal cation precipitation, cryogenic precipitation, and ammonium sulfate precipitation (Wolf, 1978). However, a method developed by Thanh and Shibasaki (1976) is believed to be the best method available for simultaneous separation of the major globulins in soy at the present time (Snyder and Kwon, 1987). This method separates soy proteins into three primary fractions, each of which precipitates at a different pH value.

After separation, various methods including ultracentrifugation, isoelectric focusing, electrophoresis and column chromatography may be used to study the soy protein further. Among these methods, ultracentrifugation which

separates the proteins based on the sedimentation rates is the most widely recognized (Snyder and Kwon, 1987). Using ultracentrifugation, soy proteins have been classified into 2S, 7S, 11S and 15S fractions, with relative quantities of 22, 37, 31 and 11% of the total protein, respectively (Wolf and Cowan, 1962).

Further analysis of these fractions by ultracentrifugation revealed that the 2S and the 7S fractions consist of more than one protein (Wolf, 1970). The 2S fraction contains: cytochrome c with a molecular weight (MW) of 12,000 Daltons, Bowman-Birk trypsin inhibitor (MW: 8,000 to 24,000), Kunitz trypsin inhibitor (MW: 21,500), 2.3S globulin (MW: 18,200), and 2.8S globulin (MW: 32,600). Among these, the trypsin inhibitors are the most studied proteins in the 2S fraction probably due to the interest in their anti-nutritional effect.

The physical properties of trypsin inhibitors including their structures and aggregation properties have been identified by many researchers, and are important in investigating the proteins using SEC-HPLC. The primary structure (amino acid sequence) of the Kunitz trypsin inhibitor has been established by Koide and Ikenaka (1973). The three dimensional folding pattern of this monomer has been revealed by Blow et al. (1974) using crystallography. A recent study by Honig et al. (1987) has indicated that these trypsin

inhibitors exist as aggregated forms under the conditions normally used for their isolation. One of the trypsin inhibitors, Bowman-Birk trypsin inhibitor (MW: 8,000) is known to form dimers and trimers (MW: 24,000) if the ionic strength is low (i.e. 0.1μ) (Millar et al., 1969). In addition to self-polymerization, Kunitz trypsin inhibitor may exist in different forms of aggregates, such as a complex with other proteins present in the soy bean. For example, it has been reported that Kunitz trypsin inhibitor binds to the subunits of 11S protein upon heat treatment (Wang et al., 1972). The binding property of the trypsin inhibitors to other proteins is also supported by the fact that these enzymes are easily heat deactivated if they are in the presence of other soybean proteins even though purified trypsin inhibitors are quite heat stable. According to Tanahashi et al. (1988), binding of trypsin inhibitors to the 11S fraction is the cause of the loss of enzyme activity; specifically, heat deactivation of trypsin inhibitors is found to be a function of the sulfhydryl groups which are present both on the surface and in the interior of the molecule.

Enzymes are also found in the 7S fraction including: allatoinase (MW: 61,700), beta-amylase (MW: 61,700 to 69,000), hemagglutinin (MW: 89,000 to 110,000) which is also known as lectin, and lipoxygenase (MW: 102,000 to 108,000) (Wolf, 1970). However, the most abundant proteins in the 7S fraction

are beta-conglycinin (MW: 180,000 to 190,000) which forms dimers at low ionic concentrations, and gamma-conglycinin (MW: 104,000) (Nielsen, 1985). The 11S fraction, on the other hand, contains only 11S globulin (MW 333,000 to 356,000) which is also known as glycinin, and the 15S fraction is considered to be a polymer of glycinin. Among these proteins, beta-conglycinin and glycinin are the major constituents of soy protein, comprising 19% and 31% of the total soy protein (Nielsen, 1985; Wolf and Cowan, 1962).

Beta-conglycinin and glycinin each consist of more than one type of subunit. Beta-conglycinin, which is approximately 50% of the 7S fraction, consists of three subunits in combination, from among the following three types: alpha-prime (MW: 57,000), alpha (MW: 57,000) and beta (MW: 42,000), depending on the soybean variety (Nielsen, 1985; Snyder and Kwon, 1987). Glycinin, on the other hand, consists of two hexamers (Snyder and Kwon, 1987). Each hexamer is composed of three acidic polypeptides and three basic polypeptides. Each acidic polypeptide or subunit has a molecular weight of approximately 37,000 Daltons, and each basic subunit has a molecular weight of about 17,000 Daltons.

2.3. Protein Interactions

2.3.1. Interaction Forces

2.3.1.1. Covalent bonds

The forces involved in soy protein interactions with other ingredients such as carbohydrate, protein and water are divided into two main groups: covalent bonding and noncovalent bonding. A covalent bond, which is formed as a result of sharing pair(s) of electrons between two atoms, requires a large energy to break (Laidler, 1978). Although covalent bonds such as carbon-hydrogen and carbon-carbon are present in a protein molecule, the covalent peptide bonds and disulfide bonds are particularly important for protein structure and interactions. The peptide bond, which is responsible for the primary structure of protein is a bond between an amino nitrogen of one amino acid and the carboxyl carbon of another amino acid (Barrow, 1961). Compared with a peptide bond, which can be formed between any amino acids, a disulfide bonding involves only one type of amino acid, cysteine which contains a sulfhydryl side chain (-SH). The disulfide bond (-S-S-), which is often responsible for cross linking of a protein, is a covalent bond between two sulfhydryl groups of cysteine (Zubay, 1983).

2.3.1.2. Noncovalent bonds

Noncovalent bonds may be further divided into: ionic bonds, dipole interactions, Van del Waals dispersion force and hydrophobic interactions (Laidler, 1978). Ionic bonding, which is also called electrostatic interaction, is a result of the transfer of one (or more) electrons from one atom to another, forming ions. These two oppositely charged ions stick together due to the electrostatic attraction, the strength of which depends upon the distance between the two ions (Atkins, 1978; Laidler, 1978; Zubay, 1983). An example of an electrostatic interaction is in sodium chloride ($Na^{+}Cl^{-}$).

Another distance dependent interaction is the dipole interaction (Zubay, 1983). If electrons are not distributed evenly throughout a molecule (i.e., the electron clouds are more concentrated at one location in the molecule), the molecule is considered to have a dipole moment (Laidler, 1978). A dipole moment can be permanent or induced, and interactions due to the dipole moment of molecules (e.g. dipole-dipole, dipole-ion) are termed as dipole interactions. One special case of a dipole interaction is called a hydrogen bond which requires a relatively large energy to break. A hydrogen bond which is highly directional and less favored at higher temperatures (Oh et al., 1980) is a special case of dipole-dipole attraction of highly polar groups such as oxygen with nitrogen (O --- N) or oxygen with hydrogen (O --- H)

(Zubay, 1983). One example of hydrogen bonding is the attraction between water molecules.

The Van del Waals force which is electromagnetic in origin is related to dipole interactions (Atkins, 1978). Van del Waals force can be either attractive or repulsive (Zubay, 1983). Attractive interactions take place due to induced dipole moments of molecules, and repulsive interactions take place when molecules come too close to each other. Van del Waals force is very weak and is commonly used to explain the nonideal behavior of gases.

Hydrophobic interactions, which are favored at higher temperatures, are related to the lowering of the free energy of a protein-water system where nonpolar groups of protein molecules avoid contact with water (or other polar solvents), and the polar groups contact with water (Barrow, 1961; Hayakawa and Nakai, 1985b; Hjerten et al., 1974). At a pH level near the isoelectric point of the protein, hydrophobic interactions are favorable due to minimum charge repulsions (Reddy and Rao, 1988). Without the presence of water, little interaction exists between nonpolar groups. However, in an aqueous environment, hydrophobic interactions, which are indirect and nondirectional, emerge because of the structure of the surrounding water which is more crystalline and dense than ordinary liquid water due to formation of hydrogen bonds. Formation of rigid crystalline structures in the water

surrounding a protein lowers the free energy of the system, thus generating the hydrophobic interactions (Laidler, 1978). According to Tanford (1962, 1978), the hydrophobic interaction alone, which is based on repulsion by the solvent instead of attractive forces, can explain the stability of a protein conformation in water, even though it is the weakest force among known intermolecular forces. One example of hydrophobic interaction is detergents forming micellar complexes with proteins (Oh et al., 1980).

Of these various interaction forces, disulfide bonds, electrostatic interactions, hydrogen bonds and hydrophobic interactions are considered to be the most important in protein interactions (Areas, 1986; Cherry, 1982; Schultz and Anglemier, 1964; Whitney, 1977). Investigation of these particular forces can provide an insight into the effects of important protein interactions in a complex food system.

2.3.2. Chemical Modifying Agents

The interaction forces listed previously are altered by various chemical compounds which can, therefore, be useful in studying specific interactions. Some examples of the action of chemical modifying agents on specific interactions are described in this section.

2.3.2.1. Disulfide bonding

Many mild reducing agents are known to cleave disulfide bonds between sulfhydryl groups without affecting the rest of the molecule. Even though many such agents including dithiothreitol, sodium borohydride, sodium phosphorothioate and potassium metabisulfite are available, 2-mercaptoethanol is most frequently used to obtain this cleavage (Damodaran and Kinsella, 1982; Kelley and Pressey, 1966; Means and Feeney, 1971; Saio et al., 1971). At low concentration, 2-mercaptoethanol cleaves only the intermolecular disulfide bonds, while other disulfide cleaving agents such as potassium metabisulfite cleave intramolecular disulfide bonds (Damodaran and Kinsella, 1982).

Another method used to alter the disulfide bonding is to prevent disulfide-sulfhydryl exchange reactions. This reaction can be prevented by blocking the available sulfhydryl groups with compounds such as N-ethylmaleimide and 5,5-dithiobis(2-nitrobenzoic acid) (Means and Feeney, 1971; Saio et al., 1971).

2.3.2.2. Electrostatic interactions

Electrostatic interactions are stabilized or destabilized by shifts in the pH level of the media, depending upon the isoelectric point of the protein (Cherry, 1982). The electrostatic interactions of protein molecules are also suppressed at a high ionic strength of the media (i.e. above

0.5μ) or addition of low concentrations of neutral salts (i.e. less than 0.5M) such as sodium chloride (NaCl) and sodium thiocyanate (NaSCN) through charge neutralization (Babajimpoulos et al., 1983; Catsimpoolas and Meyer, 1970; Damodaran and Kinsella, 1982; McWatters and Holmes, 1979; Nakamura et al., 1986). However, neutral salts can work as either a "salting in" or "salting out" agent. For example, the presence of NaCl at low concentration (0.35 M) increases canola protein solubility (salting-in) at low pH (less than 5) (Paulson and Tung, 1987). On the other hand, at a high pH (above 9.5) a decrease in solubility (salting-out) has been observed. For the "salting in" effect, the formation of a soluble complex is considered to be responsible (Lapanje, 1978). A soluble complex may be formed by a possible shift in the isoelectric point of the protein to a lower pH range due to selective and direct binding of anions (e.g., chloride anions) to the amide portion of the protein (Lapanje, 1978; Paulson and Tung, 1987). The "salting out" effect, on the other hand, is due to the nonpolar hydrocarbons in the molecule and lack of interaction with the solvent (Lapanje, 1978). With larger nonpolar hydrocarbon groups, the protein-protein interactions are more favorable than protein-solvent interactions, thus precipitating the proteins (Zubay, 1983). Other agents are also known to affect the electrostatic interactions. For example, ethylene glycol (or propylene

glycol) enhances inter and intramolecular electrostatic interactions by decreasing the dielectric constant of the medium (Catsimpoolas and Meyer, 1971; Utsumi and Kinsella, 1985b).

Instead of adding salts and other agents to the solvent, direct chemical modification of the protein side chains (e.g. acetylation, succinylation and methylation) are often used in order to affect the electrostatic interactions (Damodaran and Kinsella, 1982; Franzen and Kinsella, 1976a; Hoagland, 1966; Klotz, 1967). Acetylation or succinylation mainly alters the epsilon-amino group of lysine (Franzen and Kinsella, 1976a,b). In acetylation, cationic amino groups ($-NH_3^+$) are converted to a neutral acetyl group ($-OOCCH_3$), thus reducing the opportunity for the electrostatic interactions (Franzen and Kinsella, 1976b). In succinylation, the negative charge is increased since cationic amino groups are converted to succinate anions which contain a carboxyl group ($-COO^-$) (Grant, 1973; Klotz, 1967). In methylation, the negative charge is reduced since the carboxyl group is esterified by methanol ($-COOCH_3$) (Damodaran and Kinsella, 1982; Mandell and Hershey, 1960).

2.3.2.3. Hydrogen bonding

Even though many compounds including urea, guanidine hydrochloride and dimethylformamide destabilize hydrogen bonds, urea is the most frequently utilized (Cherry, 1982).

Urea cleaves inter and intramolecular hydrogen bonds since urea functions as a hydrogen-bond competitor by favorably interacting with polar groups of a protein (Burgess and Stanley, 1976; Kelley and Pressey, 1966; Tanford, 1962; Utsumi and Kinsella, 1985a). Enhancement of hydrogen bonding, on the other hand, is accomplished by a high concentration of propylene glycol (Tanford, 1962)

2.3.2.4. Hydrophobic interactions

Urea, which destabilizes hydrogen bonds also destabilizes hydrophobic regions of a protein (Damodaran and Kinsella, 1981; Ishino and Okamoto, 1975; Utsumi and Kinsella, 1985a). The presence of urea in the solvent exposes nonpolar residues of the protein to the solvent due to changes in the water structure. Other agents are also known to alter the hydrophobic interactions. For example, the aliphatic part of propylene glycol is capable of hydrophobic interaction, which should destabilize the hydrophobic interactions among the proteins (Catsimpoolas and Meyer, 1971; Utsumi and Kinsella, 1985b). Hydrophobic interactions are also altered by salts. For example sodium nitrite ($NaNO_3$) and sodium thiocyanate (NaSCN) destabilize hydrophobic interactions, whereas sodium sulfate ($NaSO_4$) and sodium chloride (NaCl) stabilize hydrophobic interaction (Furukawa and Ohta, 1983; Damodaran, 1988; von Hippel and Scheich, 1969).

Instead of adding chemical compounds to the media, the hydrophobicity of a molecule can be measured by a spectroscopic method using hydrophobic fluorescence probes. These include 1-anilino-8-naphthalene sulfonate (ANS) which determines the aromatic hydrophobicity due to aromatic amino acid residues, cisparinaric acid (CPA) which determines the aliphatic hydrophobicity due to aliphatic amino acid residues, and 1,6-diphenyl hexatriene (DPH) (Hayakawa and Nakai, 1985a, b). In addition, the hydrophobicity of a molecule may also be determined by measuring the binding of n-heptane to the hydrophobic region of a molecule (Ishino and Okamoto, 1975).

Investigation of the protein interactions in soy fortified foods can be accomplished by adding chemical modifying agents to the protein extraction solvent in order to alter the interaction forces (Utsumi and Kinsella, 1985a,b). Converting the functional groups on the protein directly, such as blocking the sulfhydryl groups or succinylating the amino group, is more labor intensive with a longer time required to achieve similar results. The chemical modifying agents influence the interaction forces which are important in the protein interactions. The agents include (1) NaCl, to suppress electrostatic interactions and increase hydrophobic interactions, (2) Propyleneglycol, to increase electrostatic interactions and increase hydrogen bonding at high concentration, (3) Urea, to suppress both hydrogen bonding and

hydrophobic interactions, and (4) 2-mercaptoethanol, to cleave intermolecular disulfide bonding.

2.3.3. Protein-Carbohydrate Interactions

A starch gel is defined as a "filled gel" which is composed of a gel matrix containing "fillers" such as gas bubbles, liquid droplets, crystals (ice or fat) and starch granules (Oakenfull, 1987). These fillers can be either inactive, having few interaction with the gel matrix, or active meaning that they strongly interact with the gel matrix (Brownsey et at., 1986; Stainsby, 1982).

If the protein functions as an active filler, a network could form in which either the protein gel fills the amylose gel network or amylose fills the protein gel network. In addition to functioning as an active filler, protein can also become part of a matrix component in a starch gel. In this case, both starch and protein function as the gel matrix, and networks are formed which could be either phase-separated networks where interacting starch gel and protein gel form a composite, or coupled networks where starch and protein form mixed gel networks. Examples of such networks include: gelatin-agarose gel (phase-separated) and casein-carrageenan gel (coupled) (Morris, 1986).

In these various gel networks, many interaction forces are possible; however, the forces involved in protein-carbohydrate interactions are reported to be mainly

noncovalent in nature, primarily electrostatic (Cherry, 1982; Takeuchi, 1969; Yoshino and Matsumoto, 1966). This is due in part to the large number of hydroxyl groups present in sugar and starch.

Amino residues of a protein (especially amino acids such as lysine, arginine, histidine, tyrosine, methionine, tryptophan, glutamic acid and phenylalanine) also react with reducing sugars such as glucose to form glycosamines by nonenzymatic browning reactions (Racicot et al., 1981; Reynolds, 1969). For example, the solubility of extruded corn protein increased in the presence of lactose, probably because lactose was bound to the amino acid residues of the proteins, thus forming a more hydrophilic complex (Racicot et al., 1981). The same authors also suggest that with the absence of reducing sugars, the reducing end of the starch which is insoluble in water binds to the amino acid residues of the protein, forming an insoluble complex.

In addition to nonenzymatic browning, protein-carbohydrate interactions, especially protein starch interactions, are influenced by disulfide bonding. For example, Dahle et al. (1975) found that wheat protein did not bind starch significantly (0 to 8%) in the presence of a disulfide bond cleaving agent (dithiothreitol), while the control bound 28.5 to 32% of the wheat starch (and other types of starches). It is also reported that starch works against

heat aggregation of beta-lactoglobulin (Moore and Carter, 1974).

2.3.4. Protein-Protein Interactions

2.3.4.1. Structural aspects

Protein-protein interactions may include intra and intermolecular interactions. Intramolecular protein-protein interactions include forces involved in the folding of peptide chains (secondary structure), conformation of subunits (tertiary structure), and arrangement of the subunits (quaternary structure). The intermolecular interactions may involve one type of protein or multiple types, including the interaction of subunits from the different proteins.

The secondary structure of protein (e.g. alpha-helix and beta-pleated structure) in an aqueous solution is mainly driven by hydrophobic interactions, and once established, it is maintained by hydrogen bonding (Kinsella et al., 1985). Tertiary and quaternary structures of proteins involve disulfide bonding, electrostatic interactions, dipole interactions including hydrogen bonding, Van del Waals forces and hydrophobic interactions (Kinsella et al., 1985). For example, Badley et al. (1975) suggest that glycinin subunits (hexamers) are held together by electrostatic and/or hydrogen bondings, and that the peptide within each subunit is held by hydrophobic interactions. However, disulfide bonding also has a significant role in soy protein structure, especially in

glycinin (Kinsella et al., 1985). It has been reported that glycinin which has 48 mols of half-cystine per mole of protein contains mainly disulfide bonding, whereas con-glycinin which has only two moles of half-cystine per mol of protein consists of noncovalent bondings such as hydrogen bonding (Kamata et al., 1990; Mori et al., 1982; Saio, et al., 1971; Wolf, 1978). The denaturation temperature (at pH 7.0) of glycinin is 80°C, whereas that of con-glycinin is approximately 70°C (Saio et al., 1971; Waggle et al., 1989). The differences in the interaction forces involved in the conformation may explain the denaturation temperature differences since it is to be expected that the cleaving of a covalent bond such as the disulfide bond would require more energy than for a noncovalent bond such as the hydrogen bond.

Alteration of the disulfide bonds affects the conformation of the soy protein. For example, if the disulfide bondings are cleaved by an alkaline treatment (sodium hydroxide treatment at pH 11.0), the hydrophobic regions of the protein are exposed (Ishino and Okamoto, 1975).

2.3.4.2. Functional aspects

In addition to structure related studies, the interactions of soy protein have been investigated based on functional properties, such as emulsion, foaming, gelation and texturizing (spun and extruded). The major interaction forces involved in the emulsification of soy protein are considered

to be electrostatic in nature since the pH level is the primary factor in determining the emulsification property (McWatters and Holmes, 1979). The importance of electrostatic interactions in emulsion is also supported by the fact that succinylation (or acetylation) improves the emulsifying property of proteins including soy, alfalfa and canola (Franzen and Kinsella, 1976a, b; Paulson and Tung, 1988).

Succinylation (or acetylation) also improves the foaming properties of these proteins, indicating that electrostatic interactions are also important for foaming (Franzen and Kinsella, 1976a, b; Paulson and Tung, 1988). Addition of a protein such as lysozyme also improves the foaming ability of soy isolate through the electrostatic interactions of positively charged lysozyme and negatively charged soy protein at the air-liquid interface (Damodaran and Kinsella, 1986; Poole et al., 1984).

Compared with the emulsion and foaming properties, the interaction forces involved in gelation are not as simple. For example, neither the hydrophobicity of unfolded protein nor the sulfhydryl content alone can explain the functional properties of protein such as thickening and heat coagulation (Nakai and Harwalkar, 1983). In addition, the aggregation of sulfur-rich protein from soybean was enhanced by hydrogen bonding and sulfhydryl exchange and inhibited by hydrophobic interactions and disulfide bonding (Sathe et al., 1989).

Soy may be formed into a protein gel by heating or by adding specific chemical compounds (e.g. calcium ions for Tofu manufacturing). It seems that disulfide bonding is important in chemically induced soy protein gelation. The hardness, cohesiveness, and springiness of Tofu prepared from the 11S fraction of soy protein is significantly affected by the change in sulfhydryl groups, but those characteristics of Tofu made from the 7S fraction are not affected (Saio et al., 1971).

A soy protein gel may also be formed by heating the protein dispersion. Reported minimum protein concentrations required for gelation differ slightly among researchers probably due to the differences in the flour type, processing conditions used for the flour, protein isolate preparation and the experimental conditions (Catsimpoolas and Meyer, 1970; Mori et al., 1981; Shimada and Cheftel, 1988). However, a self-supporting gel is generally formed by heating an 8% (or higher) protein dispersion at 70°C for 10 to 30 minutes, if the protein meets the minimum molecular weight criteria of 23,000 for the formation of a self-supporting gel network (Schmidt and Morris, 1984; Wang and Damodaran, 1990; Wolf, 1978).

Even though a protein dispersion is not capable of forming a gel network below 8% protein concentration and 23,000 molecular weight, a protein dispersion can still form

aggregates in which many interaction forces are involved. Soy bean glycinin forms a soluble aggregate which is believed to be an intermediate for gel formation, through intermolecular disulfide exchange and hydrophobic interactions (Matsumura and Mori, 1988; Mori et al., 1981). After releasing an intermediate subunit, under conditions where disulfide bond exchange is blocked, native glycinin also forms a soluble aggregate, by polymerization mainly through hydrophobic interactions (Yamagishi et al., 1981). If the disulfide exchange is not blocked, the soluble aggregate contains both linear and branched strands. The thickness of the strands (10 to 20 nm) is approximately equal to the diameter of the native glycinin molecule which suggests that the linear portion of the strand is made of glycinin molecules held together by hydrophobic interactions (Nakamura et al., 1984). It has been shown using a hydrophobic probe and circular dichroic spectra that the secondary structure and hydrophobic regions of glycinin remain intact when it is aggregated (Yamagishi et al., 1983).

This soluble aggregate forms a macroaggregate, leading to gel formation by disulfide bonding if the protein concentration is high enough (Mori et al., 1981; Nakamura et al., 1984; Shimada and Cheftel, 1988). It has been also reported that the disulfide bonding is responsible for formation of buffer-soluble polymers made up of the acidic

subunits of glycinin upon heating (Yamagishi et al., 1981, 1987). In addition, a significant correlation between the gel strength and the sulfhydryl (-SH) content was found by Hayakawa and Nakai (1985a). However, intra- or intermolecular disulfide bonds, particularly the newly formed ones, insolubilized only 10% of the protein upon heat treatment of soy isolate (Shimada and Cheftel, 1988).

The involvement of other interaction forces such as electrostatic and hydrophobic interactions in protein gel have also been documented. For example, the presence of sodium chloride prevented the gelation of 11S protein, whereas the 7S protein formed gel at a lower protein concentration when sodium chloride is present (Shimada and Matsushita, 1980). Similar results by other researchers also confirm the involvement of noncovalent bonds in soy protein gelation. Under conditions where the effect of disulfide bonding was eliminated by presence of 2-mercaptoethanol, aggregation of glycinin was prevented by the presence of conglycinin due to formation of a soluble complex. This complex was between the beta-subunit of conglycinin and the basic subunits of glycinin through electrostatic interactions. In contrast, glycinin alone (in absence of conglycinin) aggregated through hydrophobic interactions (Damodaran and Kinsella, 1982; German et al., 1982; Nakamura et al., 1986; Utsumi et al., 1984; Utsumi and Kinsella, 1985b).

Among the noncovalent forces, electrostatic interaction which are pH dependent (by altering the ionization of charged groups) and hydrogen bonding which is temperature dependent (higher the temperature, less hydrogen bonding) are found to be particularly important in gelation of soy protein (Catsimpoolas and Meyer, 1970). This conclusion is based on the results that weaker gels are formed at both low and high pH (pH 1.0 to 2.0 and 10), and that the apparent viscosity of the gel is higher at higher gelation temperature (within the temperature range from 65 to 80°C).

According to Babajimpoulos et al. (1983), however, hydrogen bonding is the most important interaction among these noncovalent bonds based on the results of an experiment in which various salts affecting the electrostatic and hydrophobic interactions were used. The relative contributions of electrostatic and hydrophobic interactions to gel formation were considered to be minimal since the viscosity of the gel was higher (stronger gel) in the presence of sodium thiocyanate and lower (weaker gel) in the presence of sodium chloride. If electrostatic interactions were important, a weaker gel should have been formed in the presence of both of these salts since both destabilize electrostatic interactions. If hydrophobic interaction were important, gel strength opposite from the results should have been observed since sodium chloride stabilizes hydrophobic interactions, leading

to a stronger gel, whereas sodium thiocyanate destabilizes hydrophobic interactions, leading to weaker gel. This conclusion was also supported by the observation that neither sodium chloride nor sodium thiocyanate affected the free energy of the gel which is related to hydrophobic interactions. The importance of hydrogen bonding was demonstrated by the extensive unfolding of soy protein in presence of sodium thiocyanate (NaSCN), allowing the exposed functional groups to form new hydrogen bonds.

The importance of hydrophobic interactions in the gelation of other food proteins has also been documented. A study by Hayakawa and Nakai (1985a) suggested that hydrophobic interactions were partially responsible for the coagulation of ovalbumin, even though the gel strength was decreased by excessive hydrophobicity. In addition, a significant correlation was found between the hydrophobicity of unfolded protein isolates from soy, canola and sunflower and the gelation properties of these proteins (Nakai and Harwalkar, 1983). Hayakawa and Nakai (1985b) in another study showed that ANS measured hydrophobicity, due to aromatic amino acid residues, had a positive linear correlation with the protein insolubility, which related to the gelation property. The authors suggested that the exposed aromatic amino acids and the net charge were important in the solubility of proteins, including milk and soy protein.

In the gelation of soy protein, it seems that both covalent disulfide bonds and noncovalent bonds including electrostatic, hydrogen and hydrophobic interactions are important even though there are some differences among the results by different researchers. The complexity of the interaction forces may be explained by the involvement of multiple interaction forces at the same location. At the initial stage of gelation, only one type of interaction force is present at each bridging point in the gel network. However, as the gel matures, other interaction forces such as electrostatic interactions, hydrogen bonding and hydrophobic interactions are established near the bridging points, reinforcing the strength of the gel (Matsumura and Mori, 1988).

The interaction forces in extruded soy protein, on the other hand, are related to the amino groups of the protein (Simonsky and Stanley, 1982). These forces could be either noncovalent or covalent in nature since positively charged amino groups can form both electrostatic interactions and peptide bonds with carbonyl groups. It has been reported that the major forces involved in the extrusion of soy protein are intermolecular peptide bondings (Burgess and Stanley, 1976). However, according to Simonsky and Stanley (1982), the texture and structure of extruded soy is a function of the pH and is affected by the blocking of amino groups, indicating the

importance of electrostatic interactions. It has also been suggested that hydrophobic and electrostatic interactions are also involved during protein texturization by extrusion (Areas, 1986).

For a food product containing a low protein level, the addition of protein may mean major changes in the physical and chemical characteristics of the product. Chemical alteration of protein interactions (suppression or enhancement) in the food product can give an insight into the causes for these changes.

2.4. Analytical Method

2.4.1. Commonly Used Analytical Methods for Protein Analysis

Various methods are used to study proteins and their interactions. Examples include ultracentrifugation, viscosity and gel filtration which are based on the transport properties of the molecules; circular dichroism, light scattering and X-ray diffraction which are based on the optical properties; and electrophoresis, isoelectric focusing and various chromatographic methods which are based on the charges on the molecules (Barrow, 1961; Laidler, 1978; Lapanje, 1978). Aside from ultracentrifugation, the most commonly used methods for analysis of soy proteins are isoelectric focusing, electrophoresis and column chromatography (Snyder and Kwon, 1987). If the determination of molecular weight is important,

it is suitable to use ultracentrifugation, electrophoresis, especially polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), or size-exclusion chromatography (SEC) which is also called gel filtration chromatography (Comstock, 1984; Snyder and Kwon, 1987; Willard et al., 1988).

For example, Afify and Shousha (1988) used SDS-PAGE in a study of irradiation effects on soy protein. The water extractable protein was separated into 7 to 9 bands which were identified as alpha, beta, 11S-2 and 11S-1 subunits. Another SDS-PAGE study on soy protein by Sato et al. (1986) fractionated both the water soluble and salt soluble fractions into 10 bands. Addition of 6M urea to SDS-PAGE increases the resolution and sharpens the bands; Iwabuchi and Yamauchi (1987) used urea SDS-PAGE to separate various soy protein fractions into 14 distinct bands. SDS-PAGE can also be used in combination with gel permeation chromatography to study the protein subunits. For example, in an investigation of the changes in soy bean 11S globulin during high humidity storage, protein samples were first separated into 2 to 3 fractions using a gel permeation column made of either Sepharose 4B with a fractionation range of 0.1 to 3.0 million or Sepacryl S-300. These fractions were then further fractionated by SDS-PAGE into the acidic and basic subunits of glycinin (Hoshi and Yamauchi 1983). The Sepharose 4B and SDS-PAGE combination was

also used by Brooks and Morr (1985) to study the effect of phytate removal on soy protein fractions. The samples were first separated into 7 peaks even though they were broad and not completely resolved. Each fraction was then analyzed on SDS-PAGE in order to establish the subunit compositions.

In addition to the Sepharose column which is a natural material made of agarose, Sephadex which is based on dextran is commonly used for column chromatographic analysis of protein. Particularly, the Sephadex G-200 which has a MW fractionation range of 5000 to 800,000 (Kremmer and Boross, 1979) is frequently used. For example, a column of Sephadex G-200 (3 x 96 cm) was used for the fractionation of water-extractable soy protein by Obara and Kimura (1967). Using this Sephadex column, elution with a pH 7.6 phosphate buffer containing sodium chloride and dilute mercaptoethanol fractionated the soy protein into 5 peaks. Based on their conditions (5 ml per tube, 160 tubes and 10 ml per hour), the actual chromatography required 80 hours. Using the same elution buffer and the column material, Hasegawa et al. (1963) separated the water extractable soy protein into 11 peaks instead of 5 by utilizing a longer column, 210 cm instead of 96 cm.

2.4.2. The Advantages of SEC-HPLC

The major drawback of high resolution with a long column such as the column used by Hasegawa et al. (1963) is the time required for the analysis. Since over 300 tubes containing 5 ml each were collected at a flow rate of 10 ml/hr, this separation took 150 hours to complete. However, gel permeation chromatography techniques have improved over the years, and semi-rigid gel columns for HPLC applications are now readily available. It should be possible to use these available HPLC gel filtration columns to accomplish the same analyses conducted by Hasegawa et al. (1963).

As seen in Table 2.1, the time required for the SEC-HPLC procedure is less than one hour, when only one column is used. This is considerably more rapid than conventional SEC. When applied to the albumin fractions of potato protein using two size exclusion HPLC columns, the TSK SW 2000 and 3000, in series required only 80 minutes. The TSK SW-3000 HPLC gel permeation column was also used by Kruger (1984) for analysis of the molecular weight distribution of wheat proteins during germination, by Anderson et al. (1988) in determination of water-extractable proteins from hot-processed pork carcasses, and by Okumura et al. (1990) in an investigation of the foam stability of thiolated casein. A TSK SW-4000 column which separates proteins at a higher molecular weight range than TSK SW-3000 was utilized by Ma and Harwalkar (1987) for study of

Table 2.1. Time requirement and resolution of protein analysis utilizing SEC-HPLC.

Column	Time (min)	No.of peaks	Commodity	References
TSK 2000 SW TSK 3000 SW	80	14	Potato	Liedl et al., 1987
TSK 3000	32	5	Wheat	Kruger, 1984
TSK 3000 SW	10	5	Milk	Okumura et al., 1990
TSK 3000 SW	30	9	Pork	Anderson et al., 1988
TSK 4000 SW	16	5	Oat	Ma and Harwalkar 1987
I-125 I-250		7	Cod	Leblance et al., 1987

thermally coagulated oat globulin. The TSK SW columns are manufactured by Toyo Soda of Japan, but a similar type of column which is also commonly used is available from Waters Associates of the U.S. Two of these Waters' columns, the I-125 and I-250 were chosen by Leblance et al. (1987) in order to establish the HPLC profile of sarcoplasmic proteins from frozen cod under four different processing conditions.

2.4.3. Justification for SEC-HPLC

Size exclusion chromatography studies of soy protein mainly conducted during 1960's required a long time and extensive labor. Even though proteins from various food commodities have been analyzed using size exclusion HPLC in recent years, this technique has not be applied to the soy proteins. There is a need to update the size exclusion chromatography technique from the conventional method to HPLC in order to gain speed and resolution advantages, in addition to the simplifying the operation for soy protein analysis.

CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental Plan

Two experiments were planned for this study. The first experiment included separation of an enzyme active defatted soy flour with a protein dispersibility index (PDI) of 85 and a toasted defatted soy flour with a PDI of 20 into three different protein fractions, followed by analysis of the total extractable proteins in each flour and its fractions using SEC-HPLC. A complete listing of abbreviations may be found on page xiii for the convenience of the reader.

For the second experiment, soy flours (PDI 85 and 20) were used in the production of tapioca based snack chips. At four different processing stages of the chips (soy flour, batter, gel and cooked), the proteins were extracted in a buffer with and without dilute 2-mercaptoethanol, and then analyzed using SEC-HPLC. At each processing stage, the level of protein which was extractable in buffer solutions containing various chemical modifying agents (sodium chloride, urea, propylene glycol or 2-mercaptoethanol) was also determined. The chemical composition (moisture and protein content) of the chips at each processing stage, and the physical characteristics (expansion and hardness) of the finished chips were also determined.

Enough defatted soy flour (PDI 20 and 85) and tapioca flour were obtained to complete two replications of each experiment. For the second experiment, chips were produced from both soy flours. At four different processing stages (flour, batter, gel and cooked), samples were taken to complete one replication. The order in which the samples were analyzed was randomized across replications.

3.2. Experiment 1

3.2.1. Materials

Defatted soy flours (PDI 20 and 85) were obtained from Archer Daniels Midland Co. (Decatur, IL); the PDI 20 flour was a toasted type and PDI 85 flour was an enzyme active type. Eight protein standards with known molecular weights ranging from 6,500 to 2,000,000 Daltons used for preparation of the HPLC standard curves were purchased from Sigma Chemical Co. (St. Louis, MO). Other soy proteins, two Kunitz trypsin inhibitors (a molecular weight standard for gel electrophoresis and an active enzyme) and lipoxygenase (an active enzyme) were also obtained from Sigma Chemical Co. HPLC grade solvents and other chemicals were obtained from both Sigma Chemical Co. and Baxter Scientific Products (Stone Mountain, GA).

3.2.2. Protein Extraction and Fractionation

Proteins were extracted from each flour (PDI 20 and 85) in an aqueous buffer with and without 10 mM 2-mercaptoethanol (ME) as follows. First, 10 g of soy flour was extracted with 200 ml of 0.03 M Tris-buffer (pH 8.0) at room temperature for two hours on a wrist action shaker (Burrel, Model 75) at a speed setting of 10. A two hour shaking time was selected because the amount of soy protein solubilized in aqueous solutions reaches an equilibrium after 100 minutes of extraction with gentle shaking (Shen, 1976). The flour suspension was then centrifuged for 20 minutes at 10,000 rpm (Sorvall Model RC2-B centrifuge), and the supernatant containing the extracted proteins (total buffer extract) was saved. The supernatant was lyophilized after dialysis against purified water at 5°C for 24 hours with frequent change of water (i.e. at least six times), and the protein sample (total extractable protein) was stored at -18°C in a desiccator charged with calcium chloride until analyzed.

For preparation of the protein fractions from each soy flour, proteins were extracted from 10 g of flour with 200 ml of 0.03 M Tris buffer (pH 8.0) both with and without ME for two hours as described for preparation of total extractable proteins. The supernatant collected after centrifugation served as the starting point for protein fraction preparation.

The protein isolation and fractionation scheme is shown in Figure 3.1 and was reported by Thanh and Shibasaki (1976).

The 11S fraction was first precipitated by adjusting the pH of the supernatant to 6.4 with 2 N hydrochloric acid. The precipitate was washed twice with Tris buffer (pH 6.4) in order to remove as much contamination as much as possible. The precipitate was re-dispersed in a phosphate buffer (pH 7.8) prior to dialysis. The 7S fraction was precipitated next by lowering the pH of the remaining supernatant to 4.8 with addition of 2 N hydrochloric acid. After suspending the crude 7S protein precipitate in a Tris buffer (pH 8.0), the pH was adjusted to 7.6 with 2N sodium hydroxide to solubilize all the proteins. The pH was then lowered to pH 6.2 with 2N hydrochloric acid which precipitated the contaminating 11S proteins and the polymerized form of the 7S proteins. After removing the precipitate by centrifugation, the supernatant containing the 7S fraction was adjusted to pH 7.8 prior to dialysis. The supernatant, from which the crude 7S precipitate had been removed, contained the 2S fraction. From the 2S fraction, phytate was precipitated by cooling the protein dispersion overnight at 5°C and removed by centrifugation at 5°C. All three fractions were dialyzed against deionized water for 24 hours at 5°C with frequent changes of water. The dialyzed protein dispersions were lyophilized, then stored at -18°C in a desiccator until the time of analysis.

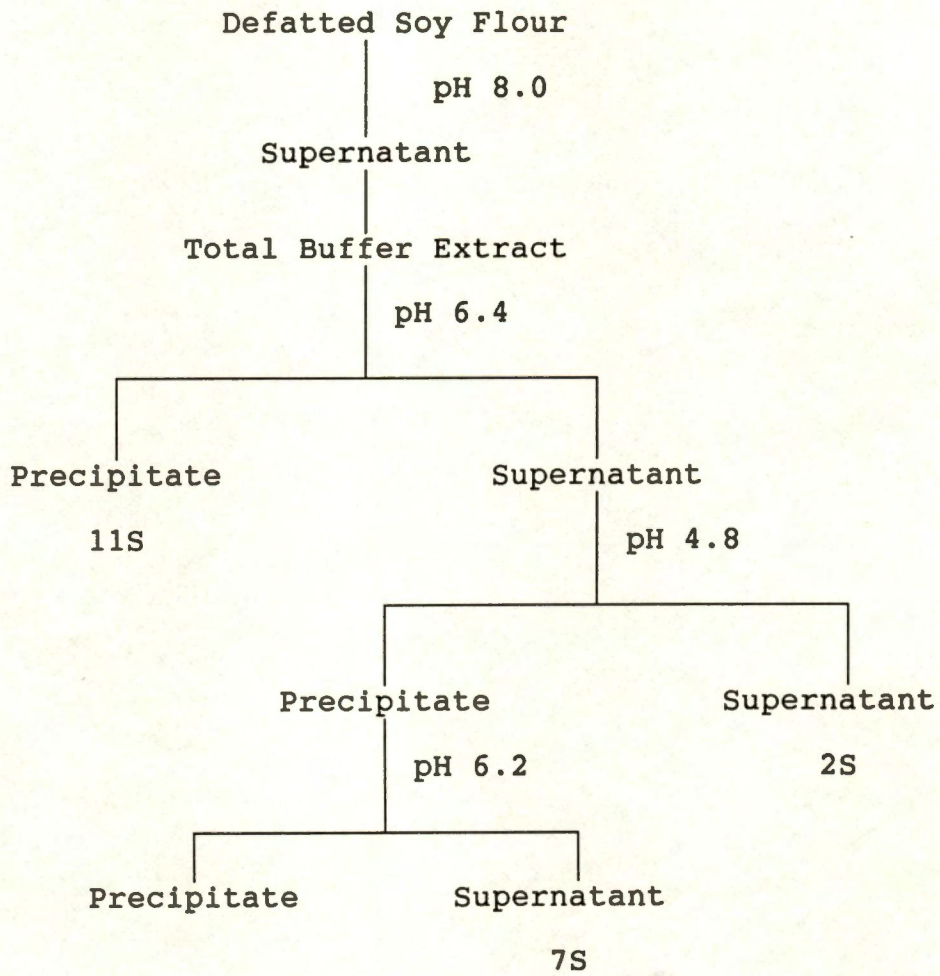


Figure 3.1. Isolation of defatted soy flour (Thanh and Shibasaki, 1976).

3.2.3. HPLC Analysis

The total soy proteins, extracted with and without ME, the 2S, 7S and 11S protein fractions from each flour (PDI 20 and 85), the Kunitz trypsin inhibitors and lipoxygenase, and eight standard proteins with MW from 6,500 to 2,000,000 Daltons were each analyzed using SEC-HPLC. One mg of each lipholized protein sample was dispersed in 1 ml of the 0.17μ ionic strength aqueous elution buffer (0.01 M phosphate, pH 6.8, 0.15 M NaCl) and filtered through a 0.45 micron filter prior to analysis. Between 25 and 100 μl of each protein sample was analyzed using a Toyo Soda TSK 3000-SW column (9.5 mm i.d. x 600 mm long) purchased from Beckman Instruments, Inc., Altex Division (San Raman, CA). The conditions for the HPLC analysis were established from the manufacture's technical bulletin (Anon, 1987) and the methods of Leblance (1987) and Anderson et al. (1988). The analysis was done on an HPLC instrument (Waters Associates, Inc, Milford, MA) equipped with a M6000A pump, U-6K injector and a Model 480 ultraviolet spectrometric absorbance detector. Proteins were eluted from the column with an elution buffer at a flow rate of 1.00 ml/min and detected at a wave length of 215 nm.

The retention times of the eight standard proteins, the enzymes, each protein peak in the total extractable protein and protein fractions were determined from the HPLC chromatograms. Three standard curves relating the molecular

weights of the protein standards ranging from 2,000,000 to 6,500 Daltons to their respective retention times from HPLC analysis were obtained during the course of Experiment 1, and are shown in Appendix A. The molecular weight of the protein in the total extractable proteins and in the 2S, 7S and 11S fractions of each soy flour were calculated using the standard curve which was normally constructed within two months of sample analysis. The accuracy of the molecular weight of the proteins calculated from the standard curve was checked by matching the elution times of blue dextrin and aprotinin between the standard curve and the protein samples.

The retention times of Kunitz trypsin inhibitor and lipoxygenase were used for positive identification of like proteins in the chromatograms of the total protein extracts and in the 2S and 7S protein fractions. The 2S fraction from PDI 85 flour was also spiked with Kunitz trypsin inhibitor in order to confirm the identification.

3.2.4. Statistical Analysis

The elution times for the molecular weight standards were analyzed statistically as a function of molecular weight (n=8). The percentage peak area of the total extractable protein at high molecular weight range including peak 1 to 3 was statistically analyzed as a function of soy flour PDI (n=2), presence of dilute ME (n=2) and their interactions. The statistical analysis was done using the GLM procedure of SAS

and the significantly different means were identified by the Student Newman Keuls' multiple range test (SNK) (SAS, 1985).

3.3. Experiment 2

3.3.1. Materials Used

The defatted soy flours (PDI 20 and 85) used for Experiment 2 were the same as for Experiment 1. The tapioca flour was obtained from National Starch and Chemical Corp. (Bridgewater, NJ). The chemical modifying agents (sodium chloride, urea, propylene glycol and 2-mercaptoethanol) and all other reagents (e.g. for Biuret and Kjeldahl analysis) were obtained from Baxter Scientific Product and/or Sigma Chemical Co. Soybean oil for frying the dried gel was obtained from a local retail store.

3.3.2. Soy Chip Production

The soy chips were produced by following the method of Siegel and Lineback (1976) and Anderson (1988) with some modifications (Figure 3.2). In the method of Siegel and Lineback (1976), the steam blanching used for gelation was replaced with baking in an impingement oven, and the two step drying at 45°C and 70°C for one hour was changed to one step drying at 44°C for 2.5 hours. The original recipe of Anderson was simplified by removing the sweeteners and flavorings, including sugar and salt, in order to eliminate the effects of these ingredients on the protein interactions. In addition,

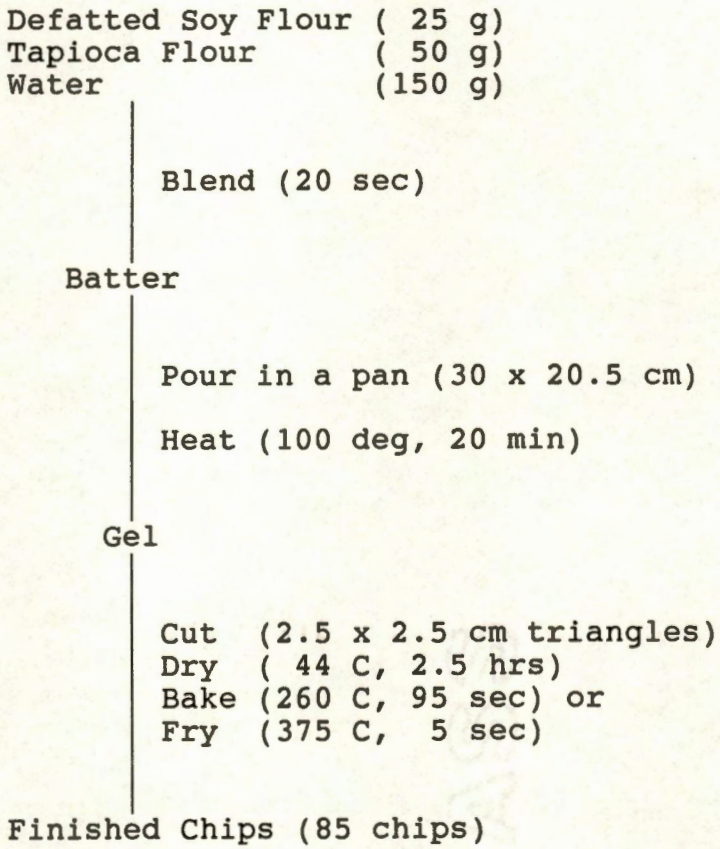


Figure 3.2. Soy chip production flow chart.

the level of soy flour was raised to 50 g per 100 g of tapioca in order to give a higher protein content than that used by Anderson (1988), so that the finished chips would have at least 9% of their calories from the protein. According to Anderson's recipe, 42 g shrimp per 100 g of tapioca is approximately equivalent to 15 g of defatted soy flour based on the protein content of shrimp (approximately 18%) and defatted soy flour (approximately 50%) (Leung et al, 1972). The 9% level of fortification was chosen based on information about the protein requirements for human nutrition (Steinke and Hopkins, 1983). The dried gel was cooked using two different methods: baking and frying. Only the baked chips were used for extraction of protein in order to avoid contamination with oil.

The raw ingredients, 25 grams of soy flour (PDI 20 or 85), 50 grams of tapioca flour and 100 ml of water, were blended in a 0.9 liter size Waring blender at high speed for 20 seconds. The resulting batter was poured on a lightly greased cookie sheet (30 x 20.5 cm) which was then covered with aluminum foil to prevent the surface from drying, and gelatinized in an impingement oven (Wear-Ever Impinger II) at 100°C for 20 minutes. The gel (approximately 1 mm thick) was allowed to cool down to room temperature, then cut into triangular shapes. Prior to drying in a forced air dryer (Stewart Warner dehydrator) at 44°C for 2.5 hours, the gel was

docked on both sides with sharp metal spikes for even drying and expansion upon cooking. The finished chip was obtained either by baking the dried gel in a toaster oven (Black & Decker) at 260°C for 95 seconds, or by frying in soybean oil at 190°C for 5 seconds. After cooking (baking or frying), the finished chips were allowed to cool down to room temperature on paper towels before storage in tightly closed containers at room temperature.

3.3.3. Sampling

Samples for protein extraction, HPLC analysis, protein interaction study, and chemical analysis were taken at each production stage: flour, batter, gel (before drying) and baked chips, for each flour (PDI 20 and 85) with the replications combined. In addition, samples of chips were obtained for both soy flour replication combinations after the chips were baked or fried for determination of physical characteristics. Samples of fried chips from one replication were also taken for chemical analysis.

3.3.4. Protein Extraction

The flour and batter were extracted without further sample preparation. However, the gel and the baked chip required further preparation. A sheet of thin gel (approximately 1 mm or less in thickness) was cut into small squares (approximately 0.5 x 0.5 cm). The baked chip was

ground with a mortar and a pestle then passed through a number 40 mesh prior to extraction. The weight of the extraction samples were: flour, 0.5 g; batter, 5 g; gel (before drying), 5 g; and baked chips, 2 g. Samples were extracted with 50 ml of 0.03M Tris buffer (pH 8.0) for two hours at 24 +/- 1°C on a twist motion shaker (Burrel, Model 75) at a speed setting of 10. After extraction, the mixture was centrifuged at 10,000 rpm for 20 minutes at room temperature and the supernatant was recovered for analysis. From each supernatant, one ml of the supernatant was taken for the protein interaction study, and the rest of the remaining extract was dialyzed against deionized water for 24 hours at 5°C with frequent changes of water. The extract from each sample was then lyopholized and stored in a desiccator at -18°C until analyzed.

The total protein content of each sample was determined by the Kjeldahl method (AOAC, 1984), and the protein level in each sample extracted was determined by the Biuret method (Layne, 1957). For construction of a Biuret standard curve, lyopholized soy protein isolate from PDI 85 flour was used as the protein standard, and was prepared by the same procedure as the total extractable protein described previously. The protein content of the same soy protein standard was determined by Kjeldahl method. A standard curve expressing the relationship between the protein level and the absorbance (A) at 540 nm from a dispersion of the protein

standard at different concentrations was constructed and is shown in Appendix B. Only the linear portion was used for protein analyses. The percentage of protein extracted from each sample was calculated using the following equation:

$$\text{Percent protein extracted} = \frac{\text{wt. extracted protein}}{\text{wt. total protein}} \times 100$$

3.3.5. HPLC Analysis

For HPLC analysis, two protein extractions were made for each sample at four production stages as described previously, one with the buffer containing 10mM 2-mercaptoethanol (with ME) and another with the buffer alone (without ME). Each protein extract (with and without ME) was analyzed under the HPLC conditions given in Experiment 1. Figures showing the chromatograms for all four production stages were constructed for visual comparison and the molecular weight of each peak in the chromatograms estimated as described in Experiment 1. In addition, from each sample extract chromatogram, peaks 1 through 3 (700 to 159 daltons) were grouped together, and the percentage peak area determined.

3.3.6. Protein Interaction Study

Each sample from the four production stages of the chips was extracted using the five different extraction solvents shown in Table 3.1. The weight of the samples, the volume of the extracting solvent and the extracting conditions were the same as given previously for protein extraction. The first solvent was the control which was 0.03 M Tris-buffer at pH 8.0. Solvents numbered 2 through 5 contained additional reagents which affected the interaction forces in various ways. The levels of the protein extracted in solvents 1, 2, 3 and 5 were determined by the Biuret method as described previously. The level of protein extracted by solvent 4 (buffer + 0.2M 2-mercaptoethanol) was measured by the Kjeldahl method. The percentage protein extracted by each solvent was determined also as before.

3.3.7. Chemical Composition

Protein and moisture contents of baked chips were analyzed in samples from each production stage. Proximate composition of tapioca flour and finished fried chips were determined. AOAC (1984) methods were used.

3.3.8. Physical Properties

The expansion and hardness of the finished chips made from PDI 20 and 85 flour were determined. The expansion of chips was calculated from the ratio of the chip volume before

Table 3.1. Solvents used for the protein interaction study.

Solvent No.	Content
1	0.03M Tris buffer (pH 8.0)
2	buffer + 0.5 M Sodium Chloride
3	buffer + 6M urea
4	buffer + 0.2 M 2-mercaptoethanol
5	buffer + 20% propyleneglycol

and after cooking (expansion = vol. of finished chip / vol. of dried gel). The base, height and thickness of the dried gel and finished chip (fried and baked) were measured with a micrometer and a caliper to calculate the volume. The hardness of the fried and baked chips were determined as the force required (kg) to break a chip using the Instron Universal Testing Machine (Model 1132) connected to a Shimazu Chromatopac C-R3A (Anderson, 1988). The conditions used for the hardness measurement were: a 50 kg cell, a 2.5 mm thick blade at a moving speed of 10 cm/min. A minimum of ten chips were evaluated for each flour type (PDI 20 and 85) and cooking method (baked and fried) in each replication. The highest and lowest values were omitted, and the remaining values averaged for each measurement.

3.3.9. Statistical Analysis

The percentage peak area of the high molecular weight range (sum of percentages for areas of peaks 1 through 3) was analyzed statistically as a function of soy flour PDI (n=2), chip production stage (n=4) and presence or absence of dilute ME (n=2) and their interactions. Significantly different means were identified by the Student-Newman-Keuls' (SNK) multiple range test (SAS, 1985).

The percentage of the protein extracted by the various solvents in the protein interaction study was analyzed statistically as a function of soy flour PDI, chip production

stage, extraction solvent (n=5) and their interactions. The PDI * stage interaction variance was partitioned by orthogonal contrasts, and the other two and three way interactions were partitioned into solvent effects for each PDI * stage combination. Significantly different means among solvents were identified by the SNK multiple range test as described previously.

The percentages protein and moisture of the samples were analyzed statistically as a function of soy flour PDI, chip production stage and their interactions. Again, significantly different means among stages were identified by the SNK multiple range test.

The physical characteristics of the cooked chips were analyzed as a function of soy flour PDI, cooking method (n=2) and their interactions.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. EXPERIMENT 1

4.1.1. Standard Curve

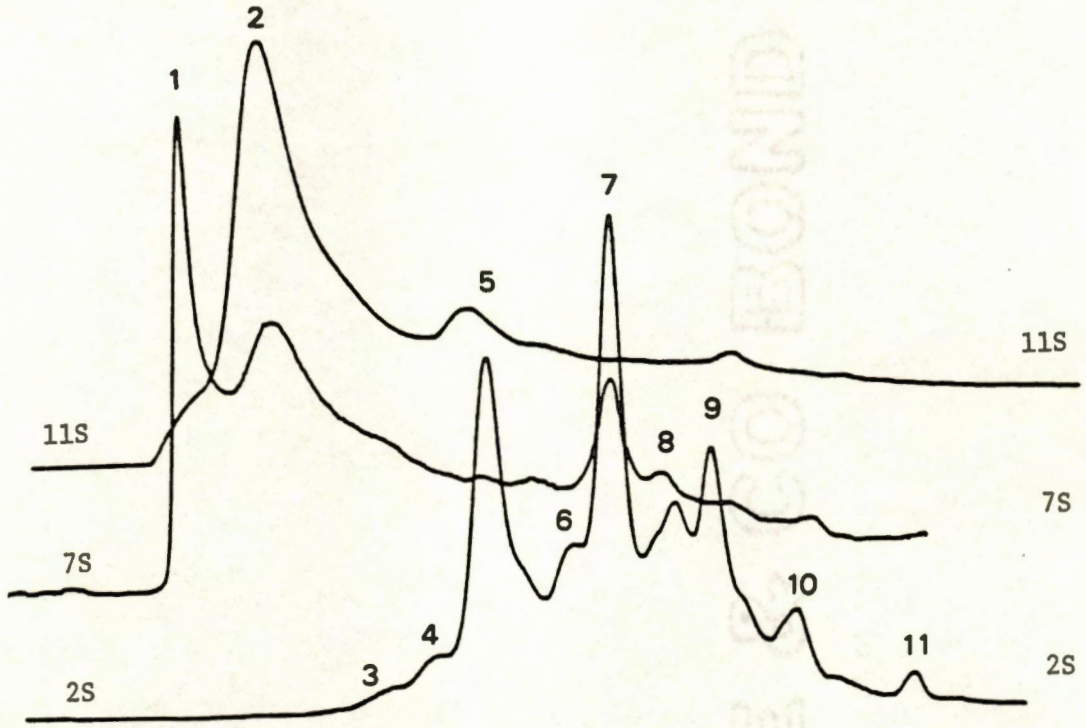
Preparation of the standard curves relating the elution time to the molecular weight for the TSK 3000-SW column using eight different molecular weight protein standards showed that the elution limits were from 12.7 minutes (669,000 Daltons) to 37.0 minutes (6,500 Daltons). The standard curves are presented in Appendix C, Table C.1. Significant differences were not found between the elution times of blue dextrin (MW 2,000,000 Daltons) and thyroglobulin (MW 669,000); however, the elution time of thyroglobulin was different ($P < 0.05$) from that of apoferritin (MW, 443,000 Daltons). These results suggest that the exclusion limit of the column was 669,000 Daltons. Total permeation of the column, on the other hand, was equal or less than 6,500 daltons since the elution times for cytochrome C (MW 12,400 Daltons) and aprotinin (MW 6,500 Daltons) were significantly different. The elution limits are comparable to those for the same type column as reported by Kruger (1984) and Anderson et al. (1988) (i.e. 450,000 to 500,000 Daltons for the exclusion limit and less than 12,500 Daltons for total permeation). According to the manufacturer of the column, the

separation range of a TSK 3000-SW column for globular protein is 500,000 to 10,000 Daltons (Kato et al., 1980). The standard curves listed in Appendix A show how the standard curve changed as the column aged; this factor must be taken into account when applying this technique.

4.1.2. Protein Fractions from PDI 85 Defatted Soy Flour

The HPLC chromatograms of the soy protein 2S, 7S and 11S fractions isolated from PDI 85 soy flour are shown in Figure 4.1. The 2S fraction had nine peaks (number 3 through 11) of which peak numbers 3, 4 and 6 were only shoulders and could be considered minor peaks. The 7S fraction had three major peaks (numbers 1, 2 and 7) and at least two minor peaks (numbers 8 and 10). The 11S fraction had one major peak (number 2) and three minor peaks (numbers 1, 5 and 9). The elution times, estimated molecular weights from the standard curves (Appendix A), and tentative identification of the peaks are given in Table 4.1.

The 2S fraction contained peaks numbered 3 and 4, identified as beta-conglycinin and lipoxygenase, respectively. These proteins are found primarily in the 7S fraction (Wolf,1970) and are considered contaminants in the 2S fraction. Peaks 5 and 6 (an estimated MW of 74,000 and 57,000 Daltons, respectively), have yet to be identified. Other tentatively identified peaks in the 2S fraction include: peak number 7, an aggregate of the Kunitz trypsin inhibitor (MW



1: 500-700	4: 98	7: 35	10: 16
2: 325	5: 74	8: 26	11: 11
3: 159	6: 57	9: 20	

Peaks 1-11 and their Molecular Weights in KDaltons

Figure 4.1. SEC-HPLC chromatograms of the 2S, 7S and 11S protein fractions from PDI 85 defatted soy flour. (Estimated molecular weight of each peak is given in kDaltons and each chromatogram represents 100 μg of protein.)

Table 4.1. Elution time, molecular weight and tentative identifications of soy protein fractions.

Peak No.	Elution time (min)	Approximate MW (KDalton)	Tentative identification
1	13.1	> 500	Aggegate of beta-conglycinin
2	15.2	325	Glycinine (317-350)
3	16.1	159	beta-conglycinin (180)
4	19.4	98	Lipoxygenase (100) Hemagglutinin (89-110)
5	20.5	74	
6	21.7	57	
7	23.9	35	Aggregate of Kunitz TI
8	25.3	26	Aggregate of BBTI (24)
9	27.0	20	Kunitz TI (20.5-21.5)
10	28.6	16	Aggregate of BBTI (16)
11	31.5	11	Cytochrome C (12) Bowman-Birk TI (8-10)

35,000 Daltons); peak number 8, an aggregate of the Bowman-Birk trypsin inhibitor (BBTI) (MW 24,000 Daltons); peak number 9, Kunitz trypsin inhibitor (MW 20,000 Daltons); peak number 10, a dimer of BBTI (MW 16,000 Daltons) and peak number 11, either cytochrome C or BBTI. All of the identified proteins listed for peaks numbered 7 through 11 have been previously identified in the 2S fraction (Honig et al., 1987; Millar et al., 1969; Wolf, 1970).

Tentatively identified proteins in the 7S fraction include: peak number 1, an aggregate of beta-conglycinin (MW > 500,000 Daltons); peak number 2, glycinin (MW, 325,000 Daltons); and peak number 7, an aggregate of the Kunitz trypsin inhibitor. Beta-conglycinin, the major 7S protein, has been reported to aggregate at low ionic strengths by Thanh and Shibasaki (1976), and the ionic strength of the elution buffer in the present experiment (0.17μ) was low enough for the beta-conglycinin to aggregate. Glycinin which is normally found in the 11S fraction (Wolf, 1970), and Kunitz trypsin inhibitor aggregate which is also found in the 2S fraction, appear to be contaminants of the 7S fraction, even though no detectable contamination of the 7S fraction was reported by Thanh and Shibasaki (1976). However, the purity of the 7S fraction by the same method of Thanh and Shibasaki (1976) was reported to be only 90%, suggesting coprecipitation of the 7S protein with other proteins such as the 11S protein through nonstructure

specific interactions, including electrostatic and hydrophobic interactions (Damodaran and Kinsella, 1982). In addition, complete isolation of the trypsin inhibitor aggregate seems to be difficult to accomplish. The trypsin inhibitor activity in the 7S fraction and the same electrophoretic bands for the 2S and the 7S fractions have been documented by Honig et al. (1987) and Thanh and Shibasaki (1976), respectively.

The major peak in the 11S fraction was peak number 2, identified as glycinin (MW 324,000 Daltons). Small amounts of the beta-conglycinin aggregate (peak number 1) and unidentified peak number 5 appear to be contaminants in the 11S fraction (Figure 4.1).

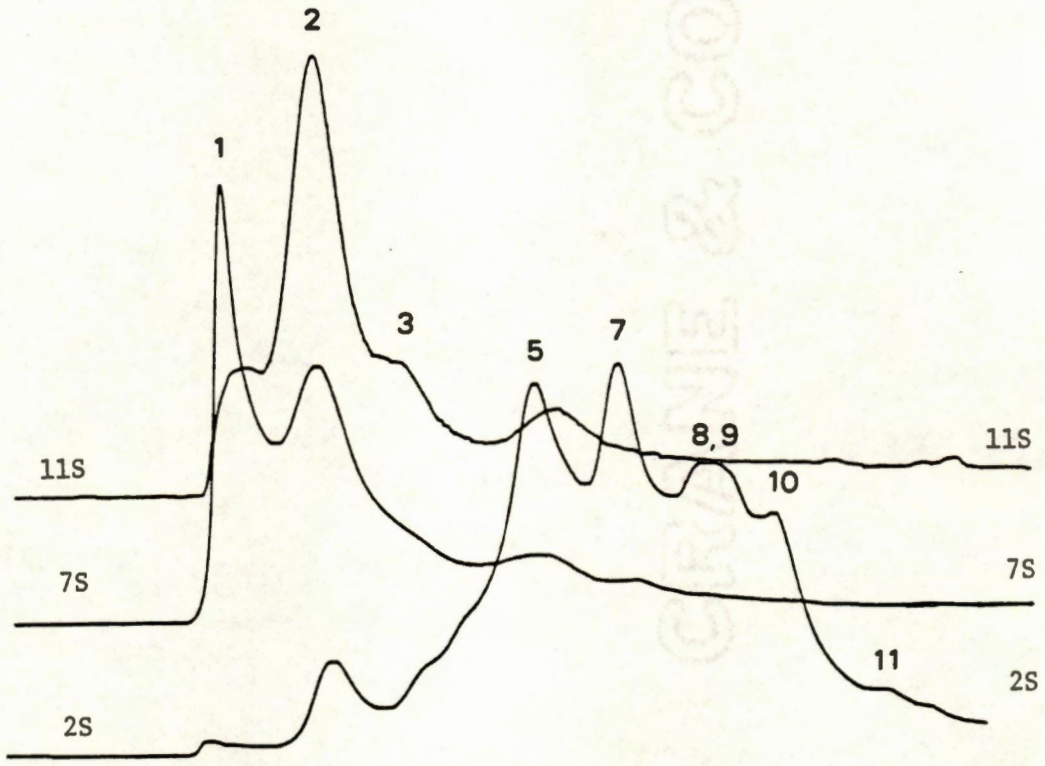
Efforts were made to identify the Kunitz trypsin inhibitor and lipoxygenase in the HPLC chromatograms of the 2S and 7S fractions. The two Kunitz trypsin inhibitors had the same elution times as that of peak number 7 when each was analyzed alone or in combination with the 2S fraction. This indicates that peak number 7 is definitely an aggregate of the Kunitz trypsin inhibitor since the molecular weight was too great to be a monomer. Unfortunately, it was not possible to positively identify lipoxygenase from the enzyme purchased. It did not disperse well in the elution buffer and when analyzed by HPLC gave a very small, broad peak, the elution time of which could not be determined. The lipoxygenase enzyme purchased was only 50% pure, and the quality of the protein

was most likely responsible for the poor results obtained. Pure forms of lipoxygenase such as a molecular weight standard could not be obtained.

4.1.3. Protein Fractions from PDI 20 Defatted Soy Flour

Compared with the chromatograms of the PDI 85 fractions (Figure 4.1), the corresponding peaks of the PDI 20 flour (Figure 4.2) are generally broader and less resolved, indicating that the PDI 20 flour has a wider molecular weight distribution, probably due to heat denaturization by toasting. As seen in Figure 4.2, in the 2S fractions of PDI 20 flour, peaks numbered 5, 6 and 7 and peaks 8 and 9 were not completely resolved. These peaks were resolved in the PDI 85 2S fraction (Figure 4.1). In addition, there appears to be a larger peak number 7 in the 2S and 7S fractions of PDI 85 soy flour than in the corresponding fractions of PDI 20 flour.

Wider molecular weight distribution of the PDI 20 proteins may be explained by unfolding of the native protein, which is the initial step of protein denaturization, and by dissociation and aggregation of the unfolded protein. Toasting of the soy flour most likely caused these protein size changes. Heat has been reported to cause localized structural changes in the 11S fraction initially which does not affect the quaternary structure of the protein molecule (Matsumura and Mori, 1988). Both the 7S and 11S proteins dissociate into



1: 500-700	4: 98	7: 35	10: 16
2: 325	5: 74	8: 26	11: 11
3: 159	6: 57	9: 20	

Peaks 1-11 and their Molecular Weights in KDaltons

Figure 4.2. SEC-HPLC chromatograms of the 2S, 7S and 11S protein fractions from PDI 20 defatted soy flour. (Estimated molecular weight of each peak is given in kDaltons and each chromatogram represents 100 μg of protein.)

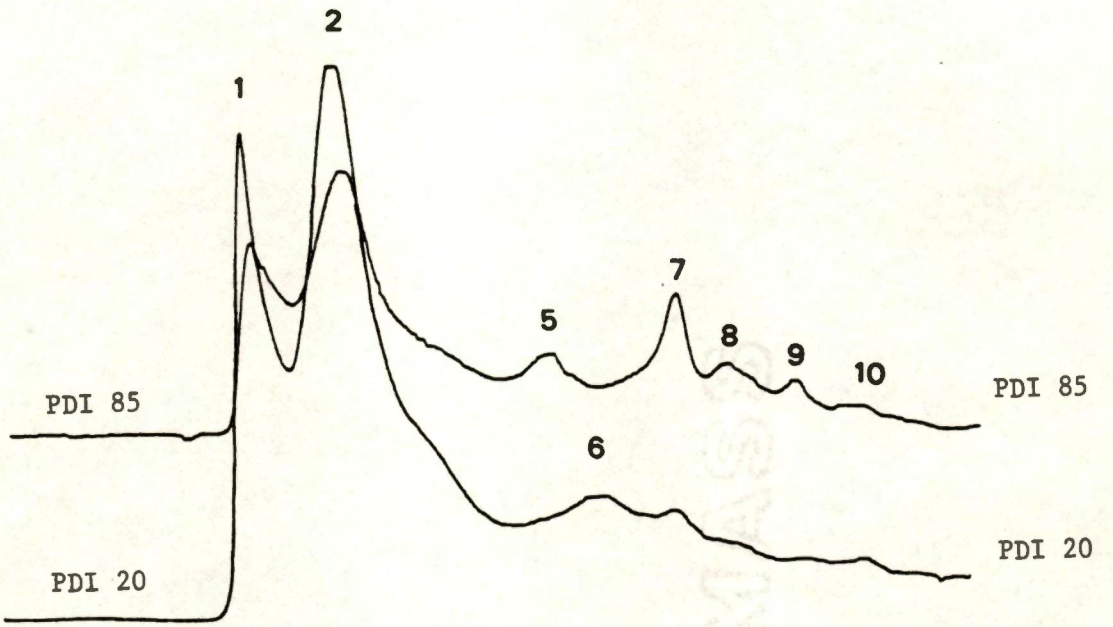
subunits upon heating which, in turn, form aggregates (Utsumi et al., 1984).

4.1.4. Total Extracted Proteins in PDI 20 and 85 Defatted Soy Flours

The proteins present in the HPLC chromatograms of proteins from PDI 20 and PDI 85 flours are shown in Figure 4.3. Proteins with molecular weights equal to or less than 35,000 Daltons (peaks number 7 and up) appear to be in higher concentration in the PDI 85 soy flour than in the PDI 20 flour. Proteins with molecular weights greater than 300,000 Daltons (peak numbers 1 and 2) appear to be present at lower levels in the PDI 85 flour than in the PDI 20 flour. These results suggest that the lower molecular weight proteins either aggregated or interacted with conglycinin and/or glycinin to form higher molecular weight proteins during the toasting of the soy flour (Wang et al., 1972).

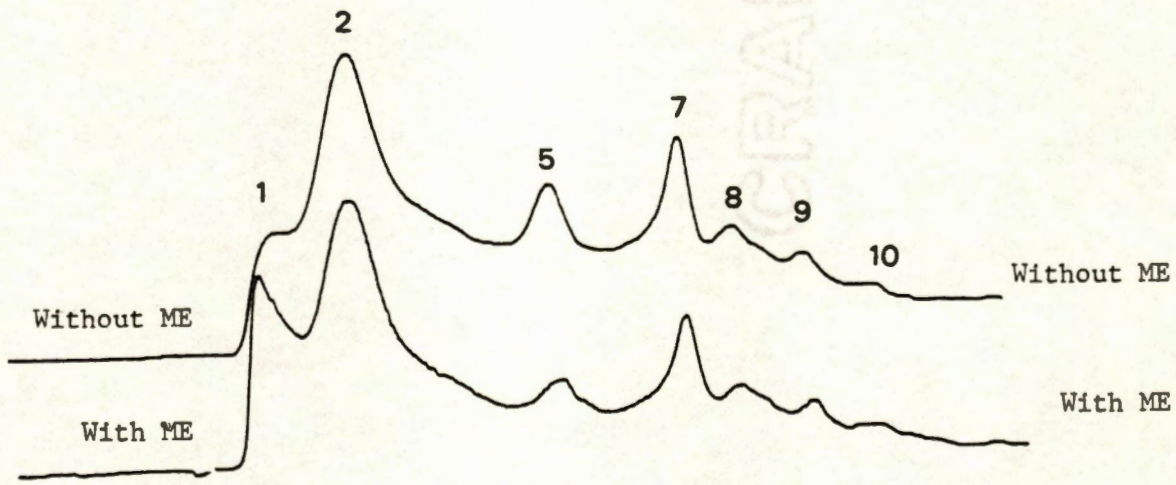
4.1.5. Effects of Dilute 2-Mercaptoethanol

The SEC-HPLC chromatograms of total extractable protein from either PDI 20 or 85 soy flour do not seem to be affected much by the presence of dilute ME in the extracting buffer. The chromatograms of the total protein extracted with and without ME from PDI 85 soy flour are shown in Figure 4.4. Peak number 1 had a larger area in the chromatogram of the proteins extracted with ME than in the chromatogram of the



SEC-HPLC Chromatogram of Unfractionated Soy Flour

Figure 4.3. SEC-HPLC chromatograms of the total protein extracted from PDI 20 and PDI 85 defatted soy flours. (Peak numbers are the same as those in Figure 4.1, and each chromatogram represents 100 μg of protein.)



Effect of Mercaptoethanol (10 mM) on PDI 85 Flour

Figure 4.4. SEC-HPLC chromatograms of the total protein extracted with and without 10 mM ME in a 0.03M Tris buffer from PDI defatted soy flour. (Peak numbers are the same as those in Figure 4.1, and each chromatogram represents 100 μg of protein.)

protein extracted without ME. The other peaks (numbers 2,6,7,8,9 and 10) appear to be similar in size in both chromatograms.

The difference in the two chromatograms is due to cleavage of the intermolecular disulfide bonds in the proteins by the ME during extraction. The smaller size of peak number 1 in the absence of ME may be due to absence of insoluble protein aggregates in the extract, especially aggregates of glycinin and conglycinin since only the buffer extractable proteins were analyzed. By extraction with ME, such insoluble aggregates might have been broken down to soluble forms including smaller aggregates, monomers and subunits which were either still large enough to elute near the exclusion limit, or were re-aggregated upon dialysis and lyopholization. The low ionic strength (0.17μ) of the elution buffer used by this study allows such re-aggregation of solubilized proteins. It has been reported that the major soy proteins, beta-conglycinin and glycinin exist as aggregates at low ionic strength (less than 0.2μ) (Kinsella et al., 1985; Nielsen, 1985) and that subunits of conglycinin and glycinin will form soluble aggregates (German et al., 1982; Nakamura et al., 1984).

4.1.6. Summary of Experiment 1

A simple and rapid analysis of soy protein was demonstrated utilizing SEC-HPLC. The exclusion limit of the TSK-3000 column was 669,000 Daltons, and the total permeation of the column was equal to or less than 6,500 Daltons. The fractionated soy proteins were separated into a total of 11 different peaks in a relatively short time (less than 45 minutes). The 2S fraction had nine peaks (numbers 3 through 11); the 7S fraction has three major peaks (numbers 1, 2 and 7) and at least two minor peaks (numbers 8 and 10). The 11S fraction had one major peak (number 2) and three minor peaks (numbers 1, 5 and 9). These peaks were tentatively identified as various proteins reported to exist in each fraction based on their molecular weights, and one of the peaks (peak 7) was positively identified as the peak containing Kunitz trypsin inhibitor. The effect of heat denaturization indicated by PDI difference in the soy flour was reflected in the chromatograms by both the resolution and relative sharpness of the peaks. The effect of dilute mercaptoethanol (10mM) which was used during extraction and fractionation of the soy protein in order to prevent intermolecular disulfide bonding was observed as a higher quantity of large proteins (MW >669,000) probably due to breakdown of insoluble aggregate.

4.2. Experiment 2

4.2.1. HPLC Analysis

Figure 4.5 illustrates the effects of chip production stages (flour, batter, gel and cooked) on the HPLC chromatograms of the buffer extractable protein from PDI flour. The numbered peaks in the chromatograms (Figure 4.5) have the same tentative identification and molecular weights as those shown in Table 4.1 and Figure 4.1. Statistically significant differences were found in the sum of the areas for peaks 1, 2 and 3 (high molecular weight range) among the production stages (Table C.2 and Table 4.2). The sum of the peak areas for the flour and batter stages were significantly larger than those of the gel and cooked stages (Table 4.2). The sum was not significantly different among other processing stages.

The percentages of extractable proteins determined by the Kjeldahl method from samples at each processing stage also confirmed that heating to form the gel significantly lowered the protein solubility (Table C.3 and Table 4.3). In the chips made from PDI 20 soy flour, the flour and batter stages had significantly higher percentages of extractable protein than did the gel and cooked stages (Table 4.3). In addition, averaged across stages, a higher ($P < .05$) percentage protein was extracted from PDI 85 flour than from PDI 20 flour (33.2% versus 17.3%, respectively). Also, a significant PDI*stage

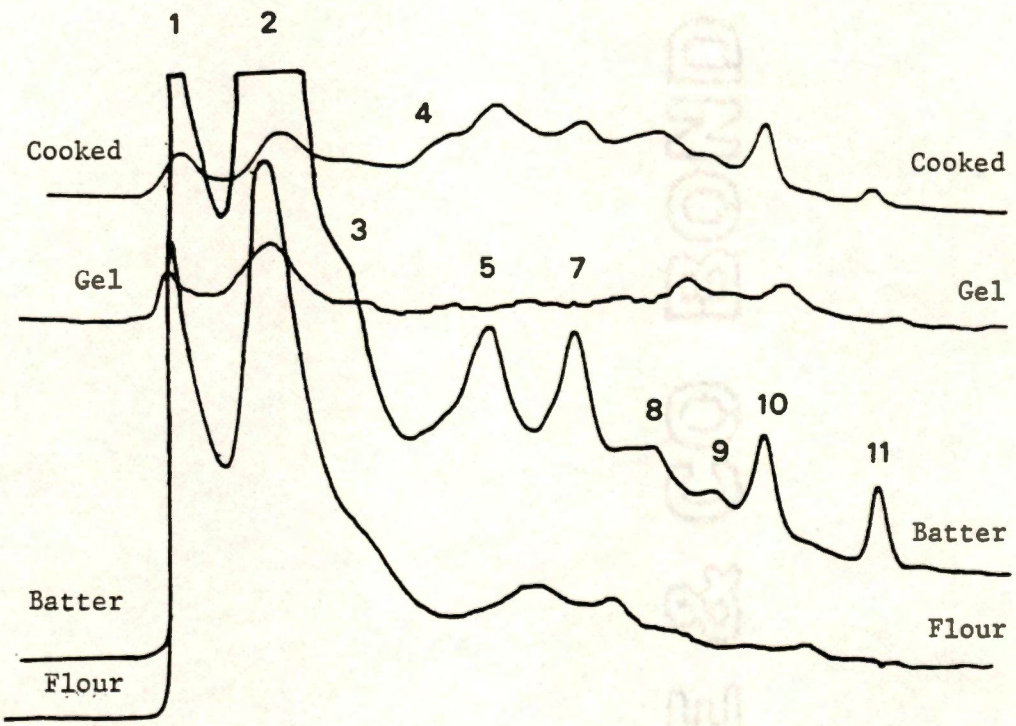


Figure 4.5. SEC-HPLC chromatograms of protein extracted at different processing stages of tapioca chips containing PDI 20 defatted soy flour.

Table 4.2. Mean percentages and standard errors of the sum of peak 1-3 (high MW range) areas in chromatograms of extractable protein from chips containing defatted soy flours at different processing stages.

Stage	Peak area sum (n = 8)
Flour	62.72 ± 3.82[a]
Batter	69.38 ± 5.68[a]
Gel	30.13 ± 8.23[b]
Chip	31.70 ± 7.62[b]

[ab] Means with unlike superscripts are significantly different ($P < 0.05$)

Table 4.3. Mean percentages[a] and standard errors of extractable protein from different processing stages of chips containing PDI 20 or 85 defatted soy flours.

Stage	Protein Extractability (%)	
	PDI20 (n=4)	PDI 85 (n=4)
Flour	29.32 ± 1.51[b]	63.90 ± 7.70[b]
Batter	30.95 ± 5.70[b]	44.37 ± 7.16[c]
Gel	2.60 ± 1.04[c]	11.00 ± 1.52[d]
Chip	6.47 ± 1.24[c]	13.72 ± 2.77[d]

[a](Weight of protein extracted/weight of total protein) x 100

[bcd]Means in the same column with unlike superscripts are significantly different ($P < 0.05$)

interaction (Table C-3) was found. This interaction shown in Table 4.3 was between flour and batter stages. For PDI 20 flour, there was a trend for more protein to be extracted from batter than from the flour stage, but for PDI 85 flour, significantly less protein was extracted from the batter than the flour stage.

Results similar to those found for PDI 20 flour were observed with the PDI 85 flour. The chromatograms of the chips made from PDI 85 flour at each processing stage were very similar to those of the PDI 20 flour. Statistical analysis of the sum of the areas for peaks 1, 2 and 3 showed no significant differences between the two PDIs (Table C.2). However, the protein extractability of the PDI 85 sample was different from that of the PDI 20 sample between the flour and batter stages (Table 4.3). In addition to the major decrease going from the batter to the gel stage, the PDI 85 flour had a higher percentage of soluble protein than the PDI 85 batter. This decrease before the heat treatment may be explained by the surface denaturization of the native protein caused by high speed blending during batter preparation (Kinsella et al., 1985).

According to Kinsella (1976), the loss of protein solubility is an indication of protein denaturization. In the chip production, the loss of the protein solubility between the batter and gel stages was caused by heating a thin layer

of batter at 100°C for 20 minutes. During heat treatment in aqueous media, the major proteins in soy flour, beta-conglycinin and glycinin undergo several protein denaturization steps: localized unfolding of the proteins, dissociation of protein molecules by releasing the subunits, and polymerization to form aggregates, leading to a gel (Kinsella et al., 1985; Matsumura and Mori, 1988; Mori et al., 1981; Yamagishi et al., 1981).

The most severe condition for protein denaturization in the chip production steps was for the formation of the gel (high humidity and high temperature). According to Hoshi and Yamauchi (1983), high humidity (RH 96%) and elevated temperature (50°C) causes a drastic decrease in the solubility of glycinin after only 4 hours of storage. The temperature for drying the gel (44°C) prior to baking was not high enough to denature the soy proteins; the denaturation temperature for soy proteins is in the range of 70 to 80°C (Saio et al., 1971; Waggle et al., 1989).

Although the dried chips were baked at 260°C for 95 seconds, due to the very low moisture content of the dried gel compared with that of the batter, the high temperature at the baking stage was probably not enough sufficient to cause further insolubilization of protein through aggregation (Kinsella et al., 1985). The amount of heat required to denature a protein significantly increases as the water

content decreases (Kinsella, et al., 1985). This could explain for the lack of any additional loss of protein solubility between the gel and cooked stages in Table 4.3.

In addition to the processing stages, the effect of the dilute 2-mercaptoethanol was also significant on the sum of areas from peaks 1, 2 and 3 (Table C.2 and Table 4.4). Higher percentages of protein were extracted by the buffer containing 10mM ME than by the buffer without ME (Table 4.4). The preparation with dilute ME might have managed to solubilized a greater amount of proteins by cleaving the intermolecular disulfide bonds (Damodaran and Kinsella, 1982).

4.2.2. Protein Interaction Study Using Chemical Modifying Agents

4.2.2.1. Results of protein interaction study

Statistical analysis of the protein extractability revealed that the individual effects of PDI, processing stage of the chips, and extraction solvent were all significant. Two way interactions (PDI * extraction solvent, and processing stage * extraction solvent) and three way interactions (PDI * processing stage * extraction solvent) were all significant. The analysis of variance table is given in Table C.4.

Table 4.4. Mean percentages[a] and standard errors of the sum of peak 1-3 (high MW range) areas in chromatograms of extractable proteins from chips containing defatted soy flours

Mercaptoethanol	Peak area sum (n = 4)
With	56.62 ± 5.98[b]
W/O	40.35 ± 6.14[c]

[a] See Table 4.3.

[bc] Means with unlike superscripts are significantly different ($P < 0.05$)

The effect of PDI and processing on protein extractability

Averaged across all stages and solvents, a higher percentage of protein was extracted from PDI 85 soy flour than from PDI 20 soy flour as shown in Table 4.5. Orthogonal comparisons of the flour and batter stages versus the gel and cooked stages for each soy flour were also significant (Table C.4). In addition, flour versus batter and gel versus cooked for PDI 20 flour were also significant (Table C.4). Higher percentages of protein were extracted from the flour and batter stages than from the gel and cooked stages for each flour (Table 4.5). In addition, for PDI 20 flour a higher level of protein was extracted from the flour than from the batter, and a lower percentage of protein was extracted from the gel than the cooked chips. For PDI 85 flour, no significant differences were found between the flour and batter stages or between the gel and cooked stages.

The effect of extraction solvents for each PDI and processing stage

(1) Extraction solvent at Flour and batter stage

Significant differences among the extraction solvents for PDI 20 and 85 flours at both the flour and batter stages of chip production are listed in Table 4.6. For the PDI 20 and 85 flour and batter, no significant differences were found in the protein solubility in either the buffer, the 0.5M NaCl-buffer or the 20% PG-buffer solvents. These three solvents

Table 4.5. Mean percentages[a] and standard errors of protein extracted from each chips production stage for each defatted soy flour by all solvents.

Stage	Soy flour	
	PDI 20 (n=10)	PDI 85 (n=10)
Flour	37.50 ± 7.56[b]	52.86 ± 9.79[b]
Batter	31.49 ± 6.30[c]	52.12 ± 8.37[c]
Gel	8.07 ± 1.95[e]	19.67 ± 2.90[c]
Cooked	15.29 ± 2.67[d]	22.80 ± 3.87[c]
Mean (n=40)	23.09 ± 3.14[x]	36.86 ± 4.15[y]

[a] See Table 4.3.

[bcd] Means with unlike superscripts in a column are significantly different ($P < 0.05$).

[xy] Means with unlike superscripts in a row are significantly different ($P < 0.05$).

Table 4.6. Mean percentages[a] and standard errors of proteins extracted by different solvents from flour and batter stages of chips containing PDI 20 or 85 defatted soy flour.

Extraction solvent[b]	Soy flour	
	PDI 20 (n=2)	PDI 85 (n=2)
	Flour	
Buffer	24.00 ± 1.67[e]	58.97 ± 2.68[d]
NaCl	22.13 ± 1.39[e]	47.55 ± 1.25[d]
Urea	55.15 ± 4.13[d]	11.73 ± 4.21[e]
ME	72.62 ± 4.19[c]	100.72 ± 11.84[c]
PG	13.61 ± 0.70[e]	45.32 ± 6.13[d]
	Batter	
Buffer	18.76 ± 0.02[e]	56.22 ± 0.50[d]
NaCl	19.23 ± 1.09[e]	44.47 ± 0.71[d]
Urea	42.58 ± 3.30[d]	17.99 ± 2.64[e]
ME	63.36 ± 1.05[c]	93.58 ± 11.20[c]
PG	13.50 ± 0.83[e]	48.33 ± 6.97[d]

[a] See Table 4.3.

[b] Buffer was 0.03M Tris, pH 8.0; NaCl, buffer + 0.5M NaCl; Urea, buffer + 6M urea; ME, buffer + 0.2M 2-mercaptoethanol and PG, buffer + 20% propylene glycol.

[cde] Means with unlike superscripts in a column for flour or batter are significantly different ($P < 0.05$).

extracted the lowest levels of protein among all the extracting solvents used for PDI 20 flour and batter. The lowest ($P < 0.05$) protein extraction for PDI 85 flour and batter occurred in the solvent containing urea. The solvent containing 0.2M ME extracted the highest ($P < 0.05$) percentage of all extraction solvents from the both PDI 20 and 85 flour and batter.

(2) Extraction solvent at gel and cooked stages

The percentage of protein extracted by different solvents from the gel and cooked chips containing PDI 20 and 85 flours are given in Table 4.7. Although not shown in this table, it is apparent that the protein solubility from the gel or baked chips in each solvent was lower than from the flour or batter with one exception (the urea solvent extraction of PDI 85). Urea apparently extracted higher levels of protein from gel and cooked than flour and batter stages for PDI 85 flour.

The only significant difference found among extracting solvents for the gel and cooked chips was in the cooked chips containing PDI 85 flour. The ME solvent extracted significantly more protein than did the buffer or NaCl solvents.

Table 4.7. Mean percentages[a] and standard errors of proteins extracted by different solvents from gel and cooked stages of chips containing PDI 20 or 85 defatted soy flour.

Extraction solvent[b]	Soy flour	
	PDI 20 (n=2)	PDI 85 (n=2)
	Gel	
Buffer	4.08 ± 0.63[c]	14.96 ± 3.86[c]
NaCl	4.11 ± 0.63[c]	12.81 ± 2.31[c]
Urea	12.86 ± 0.47[c]	26.72 ± 8.99[c]
ME	15.59 ± 5.39[c]	28.00 ± 2.68[c]
PG	3.72 ± 3.41[c]	15.84 ± 8.25[c]
	Cooked	
Buffer	9.90 ± 0.23[c]	11.04 ± 1.35[d]
NaCl	13.88 ± 0.82[c]	13.55 ± 2.97[d]
Urea	26.19 ± 7.09[c]	29.33 ± 1.40[cd]
ME	20.22 ± 3.50[c]	39.56 ± 10.26[c]
PG	6.26 ± 1.28[c]	20.53 ± 1.87[cd]

[a] See Table 4.3.

[b] Buffer was 0.03M Tris, pH 8.0; NaCl, buffer + 0.5M NaCl; Urea, buffer + 6M urea; ME, buffer + 0.2M 2-mercaptoethanol and PG, buffer + 20% propylene glycol.

[cd] Means with unlike superscripts in a column for gel or cooked are significantly different ($P < 0.05$).

4.2.2.2. Discussion of protein interaction study

According to Utsumi and Kinsella (1985a, b), the effects of chemical modifying agents on protein in a food matrix, such as a gel, should reflect the type and magnitude of the protein interaction forces present in the system. The chemical effect of these reagents is summarized later. For this study, in addition to extraction with dilute buffer (0.03 M), four chemical modifying reagents (NaCl, urea, ME and PG) were added to the extraction buffer to investigate the soy protein interactions during production of the soy fortified tapioca based chips.

The presence of a low concentration of sodium chloride (NaCl) (less than 1 M) in the protein extraction buffer decreases electrostatic interactions and stabilizes hydrophobic interactions among the protein (Babajimpoulos et al., 1983; Utsumi and Kinsella, 1985a, b). Urea (6M or above) destabilizes both hydrogen bonding and hydrophobic interactions in soy protein (Damodaran and Kinsella, 1981; Utsumi and Kinsella, 1985a). Intermolecular disulfide bonds in protein are cleaved during extraction with 2-mercaptoethanol (ME) (0.2M) (Utsumi and Kinsella, 1985a, b). Propylene glycol (20%) (PG) enhances both electrostatic interactions and hydrogen bonding (Utsumi and Kinsella, 1985a).

The effects of PDI and processing on protein extractability

The significantly lower percentage of protein extracted by the buffer solvent from PDI 20 flour than from the PDI 85 flour (Table 4.5), shows that more denatured proteins were present in the PDI 20 flour than the PDI 85 flour. According to Kinsella (1976), loss of solubility is one indicator for protein denaturization.

The lower level of protein extracted from the gel and cooked stages for both PDI 20 and 85 flours were expected since these stages involved heat treatment (above 100°C). Heating causes denaturation and insolubilization of protein, and the denaturing temperature for soy protein ranges from 70 to 90°C (Kinsella et al., 1985; Saio et al., 1971; Waggle et al., 1989).

However, the degree of protein denaturization depends upon the intensity of heat and other factors including mechanical shear (Wolf, 1978; Kinsella et al., 1985). Proteins from the PDI 20 flour which had been toasted were already denatured prior to chip production and were less soluble than the PDI 85 proteins. The preparation of the batter caused a further loss of solubility in the denatured proteins (PDI 20), probably due to the high speed blending (Table 4.5). The lack of a significant decrease in the protein extractability from the flour to the batter stage in the PDI 85 flour seems to be in conflict with the decrease found in Table 4.3. However, the

protein extractability in Table 4.3 represents the result of extraction by buffer solvent only. More protein was extracted from the native protein in PDI 85 flour which was slightly denatured by high speed blending, but not from the denatured protein in PDI 20 flour, due to the effects of the chemical reagents in the extracting solvents which destabilize and/or enhance the interaction forces in protein. The interaction of the solvent for each PDI and processing stage will be discussed later.

The reduced extractability of protein (PDI 20) at the gel stage (Table 4.5) may be explained by the interactions of protein with other ingredients in the chip. Upon heating, starch from the tapioca (and/or soy) flour, which is insoluble in water, could have bound the protein, causing a decrease in the protein extractability. This means that the soy proteins were active part of the gel matrix (Brownsey et al., 1986; Stainsby, 1982), and represents the first time soy protein has been investigated in combination with starch in a gel. Many proteins including gelatin and casein are known to interact with a carbohydrate gel matrix (Morris, 1986). Other carbohydrate components of the tapioca and soy flour, such as sugar might have reacted with the protein. It has been reported that reducing sugars increase the solubility of textured protein by formation of glucosamine through nonenzymatic browning reactions (Racicot et al., 1981;

Reynolds, 1969). Since a decrease rather than an increase in the level of protein extracted after gelation was observed, the involvement of reducing sugar-protein interactions do not seem to be important.

The higher percentages of protein extracted from the cooked chip (PDI 20) than the gel (Table 4.5) may indicate that the denatured soy protein dissociated to soluble subunits and/or polypeptides. Wolf (1978) reported an increase in protein solubility when soy proteins were heated above 100°C in an autoclave.

The effect of extraction solvents for each PDI and processing stage

(1) Extraction solvent at flour and batter stage

No significant differences were found in the percentages of extractable protein among three solvents (buffer, NaCl and PG) for either PDI 20 or PDI 85 soy flour (Table 4.6). The results indicates that electrostatic interactions are not very important to the proteins from PDI 85 flour and are not involved in the denaturation for the PDI 20 flour. As reported previously, the NaCl solvent decreases electrostatic interactions and stabilizes hydrophobic interactions while PG enhances both hydrogen bonds and electrostatic interactions (Catsimpoolas and Meyer, 1971; Utsumi and Kinsella, 1985a, b).

However, the interaction forces involved in soy protein (foaming and emulsifying properties (Franzen and Kinsella, 1976a; McWatters and Holmes, 1979; Paulson and Tung, 1988; Poole et al., 1984) and starches (Cherry, 1982; Takeuchi, 1969; Yoshino and Matsumoto, 1966) are mainly electrostatic intervention. This discrepancy may be explained due to interference from other interaction forces since negatively charged soy protein (Damodaran and Kinsella, 1986; Poole et al., 1984) and the large number of hydroxyl groups in tapioca starch (Balogopalan et al, 1988) are also capable of generating induced dipole interactions including hydrogen bonds (Laidler, 1978).

For PDI 20 flour, the large increase ($P < 0.05$) in percentage protein extracted by the urea solvent compared with that by the buffer solvent indicated that hydrogen bonding and hydrophobic interactions were important forces in preventing the denatured protein from being soluble in the buffer. For the PDI 85 flour, directly opposite results from the PDI 20 was observed; a significantly lower percentage of protein was extracted by the urea solvent than by the buffer solvent (Table 4.6).

Destabilization of the hydrogen bonding and hydrophobic interactions solubilizes denatured protein from the PDI 20 flour which is normally insoluble in the buffer alone. Hydrogen bonds are reported to be the most important

among the noncovalent bonds in heat denatured protein, whereas the effect of the hydrophobic interactions are considered to be minimal (Babajimpoulos et al., 1983). Destabilization of hydrogen bonding and hydrophobic interactions among the insoluble proteins in PDI 20 flour possibly caused formation of hydrogen bonds between the protein molecule and the solvent, solubilizing the protein as a result.

In the case of PDI 85 flour, destabilization of hydrogen bonding (and hydrophobic interactions) on the already soluble protein caused the exposure of the hydrophobic groups to the polar solvent, causing insolubility. It has been reported that the secondary structure of protein involves mainly hydrophobic interactions and hydrogen bonding (Kinsella et al., 1985) and that urea favors exposure of nonpolar groups in protein to the solvent (Damodaran and Kinsella, 1981).

The importance of disulfide bonds in the interaction forces of the denatured proteins in PDI 20 soy flour and the proteins in PDI 85 soy flour was clearly demonstrated. The percentage protein extracted from the PDI 20 flour by the buffer more than doubled in the presence of ME which reduces disulfide bonds (Utsumi and Kinsella, 1985a, b) (Table 4.6). The percentage protein extracted from the PDI 85 flour increased from 59.0% in the buffer solvent to 100% in the ME solvent. Disulfide bonds have been reported to be involved in thermal denaturation of protein by Utsumi and Kinsella (1985a)

and Sathe et al. (1989). Disulfide bonds are also important to the native structure of soy proteins (Kinsella et al., 1985).

(2) Extraction solvent at gel and cooked stages

No significant differences were found for gels made from PDI 85 and 20 flours among all the extracting solvents (Table 4.7). There are two possible explanations for this phenomena. First, the protein insolubility caused by gelation may involve multiple interaction forces, so that extraction with selected chemical reagents which affect only one or two types of interaction was not sufficient to cause solubilization of the protein. The presence of such multiple interactions forces in glycinin gel are documented by Matsumura and Mori (1988). Second, gelation could have involved interaction forces other than disulfide bonding, electrostatic interaction, hydrogen bonding and hydrophobic interactions. One such interaction force is peptide bonding which is a strong bond and is not affected by any of the reagents used in this study. According to Simonsky and Stanley (1982) and Burgess and Stanley (1976), intermolecular peptide bonds are formed by severe heat treatment of protein.

In summary, the results indicate that:

1. heat denaturization of native soy protein by toasting involves hydrogen bonding, hydrophobic interactions and disulfide bonds,

2. disulfide bonds are important in the structure of both native and denatured protein, and

3. heat treatment during chip production involved multiple interaction forces in protein including electrostatic interactions, hydrogen bonds, hydrophobic interactions and disulfide bonds, and possibly protein-starch interactions.

4.2.3. Chemical Composition of the Baked Chip

4.2.3.1. Results of chemical composition

The PDI of the soy flour did not affect ($P < 0.01$) the percentages of protein and moisture in samples from different processing stages of the chip; however, significant differences were found among the processing stages for protein and moisture contents as shown in the analysis of variance in Table C.5.

The mean percentages of protein and moisture for each processing stage are given in Table 4.8. In general, higher levels of protein were found at the flour and cooked stages where the moisture contents were low, and significantly lower protein levels were found in the batter and gel stages which contained higher levels of moisture (Table 4.8).

4.2.3.2. Discussion of chemical composition

(1) Protein content

Since the baked chips contained hardly any fat as opposed to the fried chips, the high protein content of 17.5%

Table 4.8. Mean percentages and standard errors of moisture and protein contents in different processing stages of chips containing defatted soy flour.

Stage	Protein (%, wet basis) (n=4)	Moisture (%) (n=4)
Flour	51.14 ± 0.13[a]	6.35 ± 0.30[c]
Batter	5.85 ± 0.09[d]	68.95 ± 0.16[a]
Gel	6.88 ± 0.40[c]	54.90 ± 2.67[b]
Cooked	17.52 ± 0.31[b]	5.04 ± 0.27[c]

[abcd] Means with unlike superscripts in a column are significantly different ($P < 0.05$)

obviously meets the recommended protein fortification level of foods that 9% of the total calorie content should be supplied from protein (Steinke and Hopkins, 1983).

Although not statistically analyzed, the chemical composition of the fried chips was also determined. The fried chips contained 3.8% moisture, 12.2% protein, 30.2% fat and 1.5% ash. Based on these figures, the percentage of calories derived from the protein in the fried chips was calculated to be 9.2%, which also satisfies the recommended protein fortification level of 9%.

(2) Moisture Content

The moisture level in the batter (Table 4.8) reflects the level of water added to the soy and tapioca flours (Figure 3.2). The amount of water (150 g) in the chip formula represents 66.7% added water; the higher percentage moisture of 68.95% in the batter is due to the additional moisture present in the soy and tapioca flours. The moisture level of the tapioca flour was determined to be 12.0%, even though it was not statistically analyzed. Based on the moisture contents of the chip ingredients, the moisture content of the batter should have been 70.0%.

The lower level of moisture ($P < 0.05$) in the gel than in the batter reflects the heating of the batter to form the gel. The significantly lower level of moisture in the baked

chips than in the batter or gel is also indicative of the amount of heat treatment received.

4.2.4. Physical Properties

4.2.4.1. Visual Observation

Functional property differences due to the difference in PDI were observed throughout the processing. The batter made from the PDI 85 soy flour looked thicker and foamier than that from the PDI 20. The gel made from the PDI 85 flour was also more adhesive and fragile and the punch holes made by metal spikes had tendency to reclose. As seen in Figures 4.6, 4.7, and 4.8, the PDI 85 flour produced unevenly colored and unevenly puffed chips upon baking and darker chips upon frying compared with PDI 20 flour.

The excessive browning of the PDI 85 baked chip on the edges and darker color on the bottom side of the PDI 85 fried chips may be explained by nonenzymatic browning reactions between reducing sugars (or the reducing end of the starch) and amino acids (or free amino groups of the protein). Reducing sugar such as glucose and fructose could have been produced by the actions of alpha- and beta-amylase present in soybean (Snyder and Kwon, 1987) since the PDI 85 flour was the enzyme active type. These amylases break down the sugars and starches in soy flour and tapioca. The major sugars in soybean are sucrose (5%), raffinose (1%) and stachyose (4%) (Snyder and Kwon, 1987). The starch content of the soybean is very

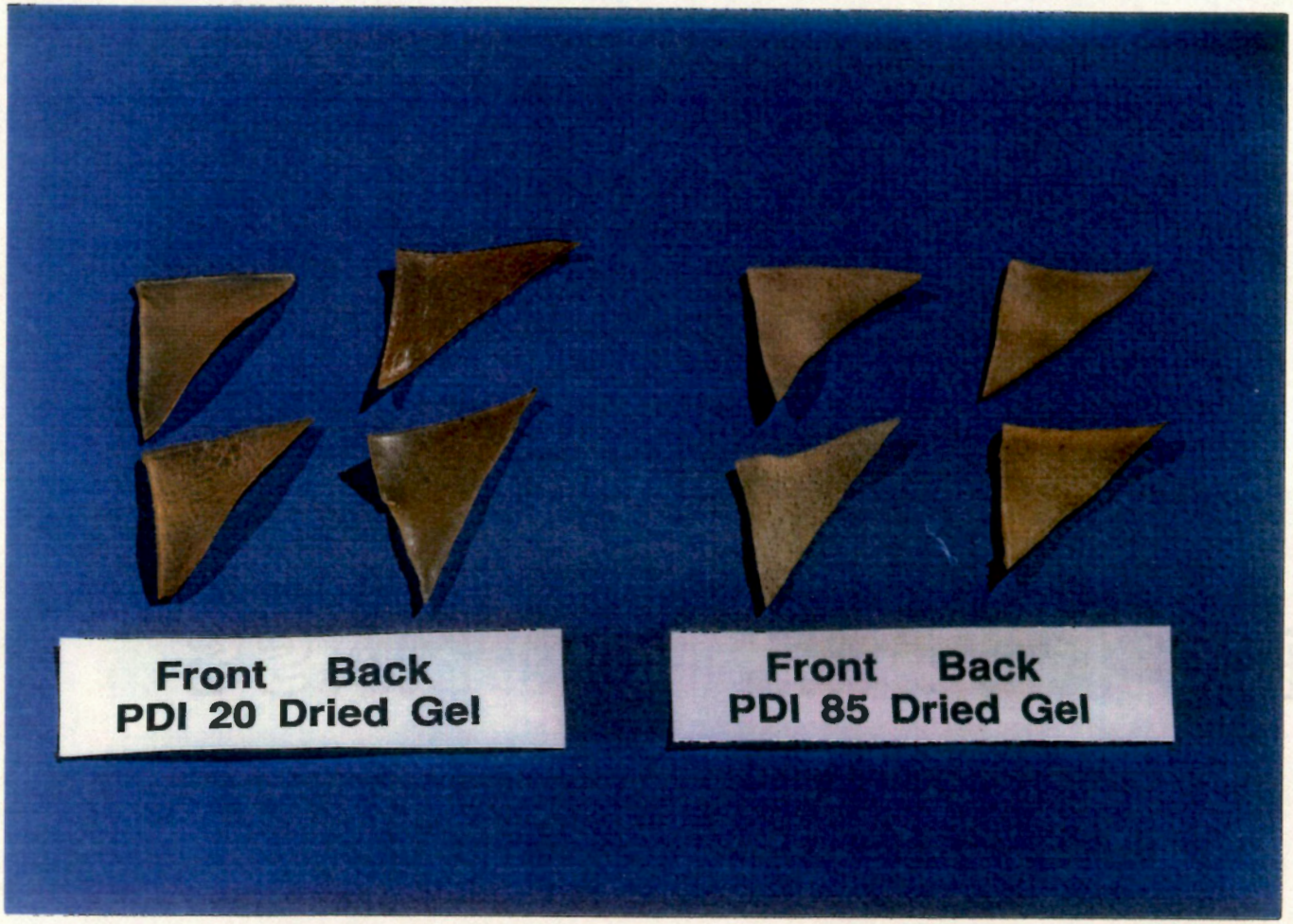


Figure 4.6. Photograph of the tapioca chips at the gel stage.

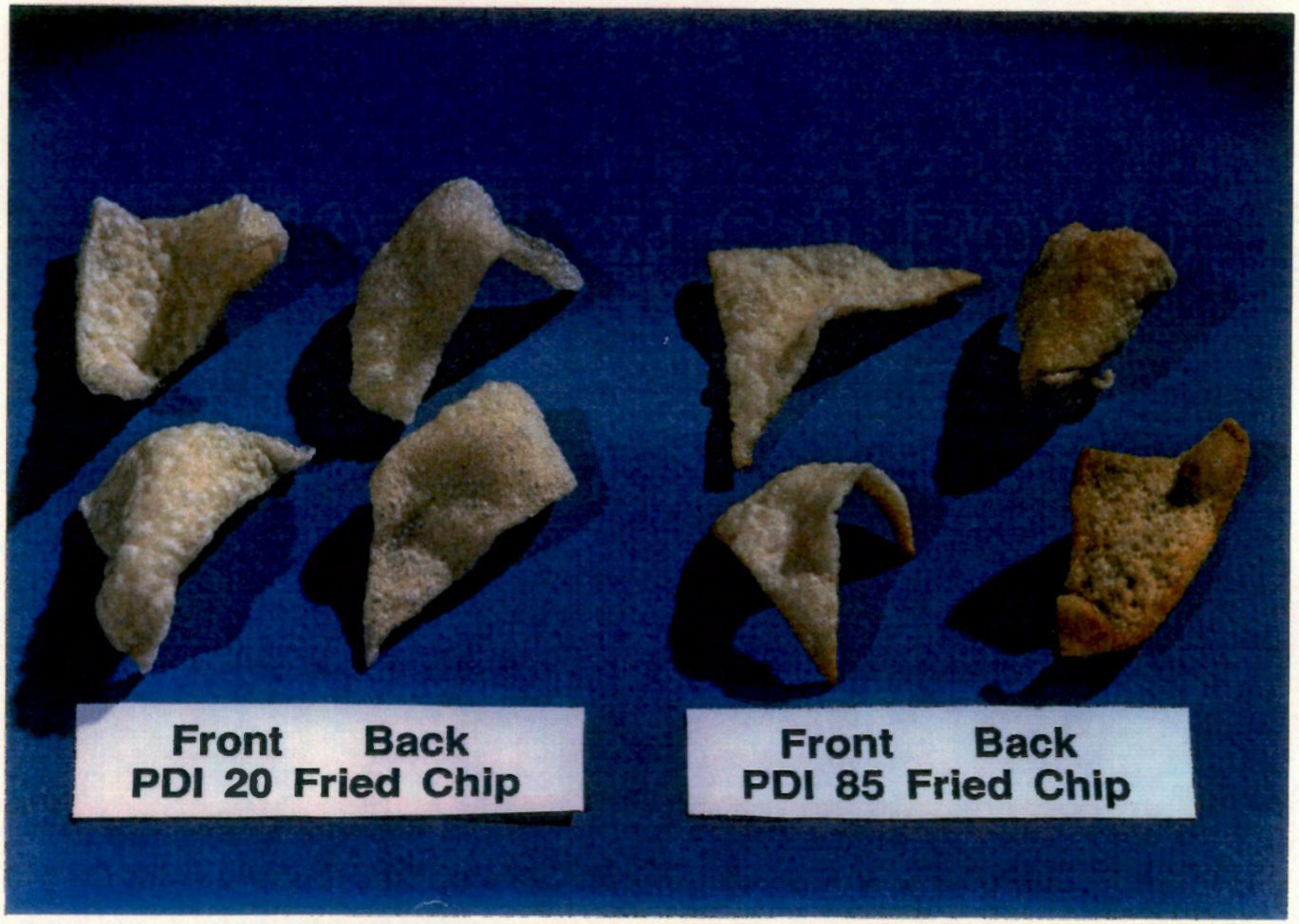


Figure 4.7. Photograph of the tapioca chips after frying.

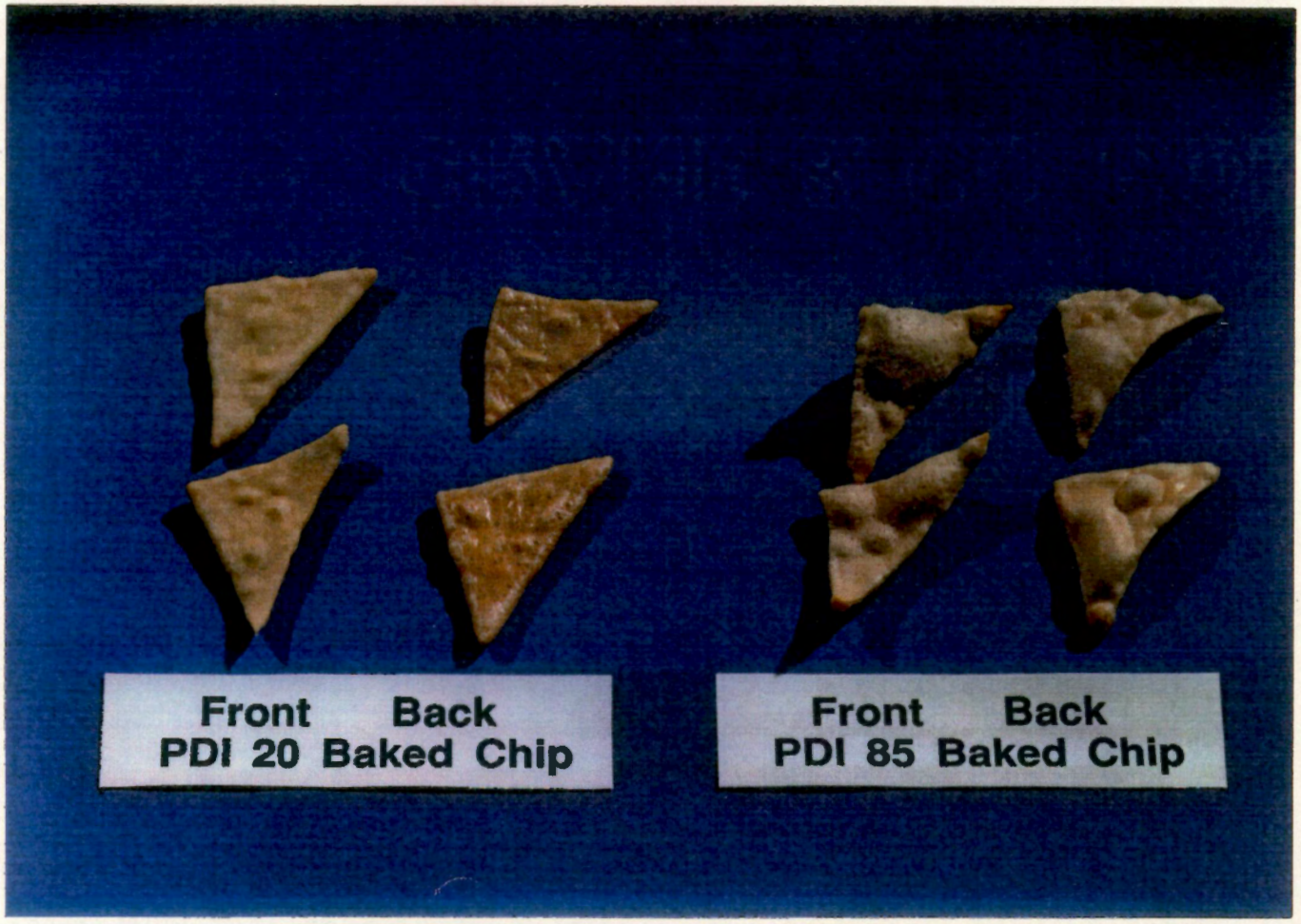


Figure 4.8. Photograph of the tapioca chips after baking.

low, being less than 1% (Snyder and Kwon, 1987), but a large quantity of starch is found in tapioca. According to Balogopalan et al. (1988), the starch content of tapioca is above 70%. Formation of the other component of the nonenzymatic reaction, free amino groups, also could be explained by the presence of active proteolytic enzymes in the PDI 85 soy flour (Smith and Circle, 1972).

4.2.4.2. Hardness of Chips

The hardness of the cooked chip was affected by the PDI of the soy flour ($P < 0.027$) and the cooking method ($P < 0.052$). The interactions between PDI and cooking method also affected the hardness ($P < 0.103$) (Table C.6). Since only eight observations were made in this part of the experiment, this level of significance should be considered. The means of the chip hardness for each soy flour and cooking method are shown in Table 4.9. For chips containing PDI 20 soy flour, the baked chips were much harder than the fried chips, but there was not much difference in the hardness of the baked and fried chips containing PDI 85 flour. This explains the PDI * cooking method interaction.

It was expected that the more porous, softer-looking fried chips would require less force to break than the small and stiff-looking baked chips (see Figure 4.8), and this was found to be true. Other factors such as the differences in protein interaction forces, may also contribute to the

Table 4.9. Mean and standard errors of the hardness[a] of cooked chips containing PDI 20 and 85 defatted soy flours.

	Soy flour		
	PDI 20 (n=2)	PDI 85 (n=2)	Mean (n=4)
Baked (n=2)	16.45 ± 4.4	4.16 ± 0.96	10.32 ± 2.56[x]
Fried (n=2)	5.62 ± 0.07	2.77 ± 0.43	4.20 ± 0.35[y]
Mean (n=4)	11.05 ± 1.54[b]	3.47 ± 0.67[c]	

[a] Kg required to break chips by Instron (50 kg cell).

[bc] Means in a row followed by unlike superscripts are significantly different ($P < 0.05$).

[xy] Means in a column followed by unlike superscripts are significantly different ($P < 0.05$).

hardness difference since heat denaturation of native soy protein involves hydrogen bonding and hydrophobic interactions, as indicated by the protein interaction study.

The difference in the chip hardness might also have been affected by the differences in the physical shape of the chips. It is reasonable to assume that the thinner chip wall at the large air bubbles of the unevenly puffed PDI 85 chips (Figure 4.8) would require less force to break.

4.2.4.3. Expansion of Chips

The frying method expanded the gel more evenly, creating big foamy looking chips even though the PDI 85 chips tended to curl up. The baking method resulted in flatter and smoother chips, similar to Japanese style rice crackers. These differences are reflected in the expansion of the chips during cooking. Statistical analysis of the expansion revealed that the cooking method and the interaction of the cooking method and PDI were significant (Table 4.10). For PDI 20, the frying method expanded the dried gel better than baking probably due to more rapid vaporization of water. With PDI 85, the cooking method was not significantly different because of uneven puffing by baking and curling by frying. As seen in Figure 4.8, PDI 85 chips created large hollow bubbles upon baking which were counted as part of the volume, which resulted in a large expansion calculation.

Table 4.10. Means and standard errors of the expansion of cooked chips containing PID 20 or 85 defatted soy flour.

	Soy flour		
	PDI 20 (n=2)	PDI 85 (n=2)	Mean (n=4)
Baking (n=2)	2.48 ± 0.21	5.87 ± 0.22	4.18 ± 0.26[y]
Frying (n=2)	7.45 ± 0.15	4.67 ± 0.10	6.06 ± 0.13[x]
Mean (n=4)	4.96 ± 0.26	5.27 ± 0.16	

[xy] Means in a column followed by unlike superscripts are significantly different ($P < 0.05$).

4.2.5. Summary of Experiment 2

Tapioca starch based snack chips fortified with defatted soy flour (PDI 20 or 85) were produced. During the chip production, heat treatment (gelation followed by cooking) caused insolubility of the soy protein. According to the results of HPLC analysis, the differences in buffer extractable protein at four processing stages (flour, batter, gel and cooked) were mainly quantitative.

Based on the protein interaction study utilizing various chemical modifying agents, the interaction forces involved in the denaturization of native soy protein by toasting were found to be mainly hydrogen bonding, hydrophobic interactions and disulfide bonds. Disulfide bonds were also important to the structure of undenatured soy protein. The results also indicated that heat treatment during chip production involved multiple interaction forces in protein including hydrogen bonding, disulfide bonds and hydrophobic interactions. Decreased levels of protein extractability from the gel stage of the chips containing PDI 20 flour compared to cooked chips also indicated the possibility of protein starch interactions.

These differences in the protein interaction forces may be related to the chemical composition and physical properties of the chips. PDI 20 flour produced harder chips than PDI 85 flour probably due to differences in the level of

protein denaturization and/or protein interactions, due mainly to hydrogen bonding and hydrophobic interactions, since the protein content of the chips made from PDI 20 and PDI 85 was not different. Cooking method also affected the physical properties of the finished chips. Frying produced larger chips than baking probably due to more rapid vaporization of moisture.

CHAPTER 5

CONCLUSION

Two experiments were conducted. The first experiment evaluated a rapid method for analysis of proteins from two different soy flours (PDI 20 and 85), and the second experiment, which had three parts, investigated the interactions of soy proteins during production of tapioca based snack chips and the physical and chemical characteristics of those chips. For the first experiment, total proteins were extracted from each soy four by a 0.03M Tris buffer (pH 8.0) with and without 2-mercaptoethanol (ME). These proteins were also separated into 2S, 7S and 11S fractions. Total proteins and their fractions were analyzed by size exclusion high performance liquid chromatography (SEC-HPLC) on a TSK 3000-SW column. For the second experiment, soy proteins at different production stages of tapioca based snack chips: flour, batter, gel and cooked (baked), were extracted and analyzed by SEC-HPLC. The percentage protein extracted from each stage by a buffer alone or with an extractant containing one of the following: 0.5M NaCl, 66M urea, 0.2M ME or 20% propylene glycol was also measured to determine specific protein interaction forces. In addition, the percentage moisture and protein of each stage of production the chip (baked and fried) and the hardness and expansion during cooking of the chips (baked and fried chips) were

determined. For the first experiment and each part of the second experiment (protein analysis by SEC-HPLC, protein interaction study, chemical analyses of baked chip production stages and physical analyses of baked and fried chips), two replications were run.

In the first experiment, the TSK 3000-SW column used in SEC-HPLC had an exclusion limit of 669,000 Daltons and a total permeation equal to or less than 6,900 Daltons. The soy proteins were separated into 11 different peaks in less than 45 min by SEC-HPLC. The 2S fraction had nine peaks (numbers 3 through 11) and the 7S fraction had three major peaks (numbers 1, 2 and 7) and at least two minor peaks (numbers 8 and 10). The 11S fraction had one major peak (number 2) and three minor peaks (numbers 1, 5 and 9). Peak 1 was tentatively identified as an aggregate of beta-conglycinin, peak 2 as glycinin, peak 3 a beta-conglycinin, peak 4 as an aggregate of lipoxygenase and/or hemagglutinin, peak 8 as an aggregate of the Bowman Birk trypsin inhibitor (BBTI), peak 9 as the Kunitz trypsin inhibitor, peak 10 as an aggregate of BBTI and peak 11 as the BBTI and/or cytochrome C. Peaks 5 and 6 with MW of 74,000 and 57,000 Daltons, respectively, were not identified. Peak 7 was positively identified as an aggregate of the Kunitz trypsin inhibitor from the analysis of two standard Kunitz trypsin inhibitors with MW of 20,500 to 21,500 Daltons each. The heat denaturation of protein indicated by the PDI

differences of the soy flours was reflected in the chromatograms mainly by the better resolution between peaks and sharper peaks from PDI 85 flour than PDI 20 flour. Also, smaller peaks were observed for peak numbers 5 through 10 in PDI 20 flour than in PDI 85 flour, and larger peaks for number 1 and 2 were found in the PDI 21 flour. Dilute ME which was used during extraction and fractionation of the soy protein in order to prevent intermolecular disulfide bonding extracted a higher quantity of high molecular weight proteins than buffer alone probably due to breakdown of insoluble protein aggregates during extraction. Results of this experiment represent the first report of soy proteins having been analyzed by SEC-HPLC.

SEC-HPLC analysis of the soy proteins extracted from different stages of chip production: flour, batter, gel and cooked, showed mainly quantitative differences among the stages. Heating the batter to form the gel which was then dried and baked resulted in significant decreases in the sum of the areas of peak numbers 1 through 3 in SEC-HPLC chromatograms. Significantly lower percentages of protein were also extracted by the buffer from the gel and cooked stages than from the flour and batter stages for both soy flours. Peaks 1, 2 and 3 were an aggregate of beta-conglycinin, glycinin and beta-conglycinin, respectively.

In the flour and batter stages, higher ($P < 0.05$) levels of protein extracted from both PDI 20 and 85 soy flours by the ME reagent compared to the buffer alone showed that disulfide bonds were important in causing protein insolubility. In comparison with the percentage of protein extracted by the buffer, however, higher ($P < 0.05$) percentages of protein extracted by the urea solvent from the PDI 20 flour and batter indicated that both hydrogen bonding and hydrophobic interactions were important in preventing the heat denatured protein in the buffer from solubilizing. Contrary to this result, the lower ($P < 0.05$) levels of protein extracted from PDI 85 soy flour and batter by the urea reagent compared with the buffer showed that hydrogen bonding and hydrophobic interactions were important in maintaining solubility of the undenatured soy proteins in the buffer.

When averaged across all extracting solvents, lower percentages of protein were extracted from the gel and cooked stages than from the flour and batter stages for both soy flours. In addition, no significant differences were found among the extracting solvents in the percentage of soluble protein for the gel and cooked stages containing PDI 20 soy flour or for the gel stage containing PDI 85 flour. In the cooked chips containing PDI 85 flour, higher ($P < 0.05$) percentages of protein extracted by ME than by buffer, showed that intermolecular disulfide bonding was an important force

in protein interactions during cooking. The possibility of protein interaction with the tapioca starch was also a possibility for PDI 20 flour, since lower ($P < 0.05$) levels of protein were extracted by all solvents from the gel stage containing this flour than from the baked chips. This represents the first time interactions of soy proteins with tapioca starch have been found.

Baked chips made from either soy flour contained 17.5% protein and 5.0% moisture. Fried chips contained 12.2% protein and 3.8% moisture. Baked or fried chips containing either soy flour met the recommended protein fortification levels of foods for developing countries since more than 9% of their total calories were from protein. Chips containing PDI 20 soy flour were harder ($P < 0.05$) to break than chips containing PDI 85 soy flour, mainly due to the extreme hardness of baked PDI 20 chips. Baked chips were harder and expanded less ($P < 0.05$) during cooking than fried chips. In general, chips that expanded the least during cooking were the hardest. However, the expansion depended upon the type of soy flour in the chip combined with the cooking method.

The fact that ME and urea extracted more protein than the buffer from the flour and batter stages of the chips containing PDI 20 soy flour, but not from the gel and cooked stages, indicates that disulfide bonding, hydrogen bonding and hydrophobic interactions occurred in the proteins during the

production of the baked chip. In addition, extraction of a higher percentage of protein by ME than the buffer from baked chips containing PDI 85 flour compared to no significant difference between these solvents for PDI 20 four chips, indicates that the protein in PDI 20 chips was more denatured than that in the PDI 85 chips. This may have been the reason why baked chips containing PDI 20 flour expanded less during baking and were harder than baked chips containing PDI 85 flour.

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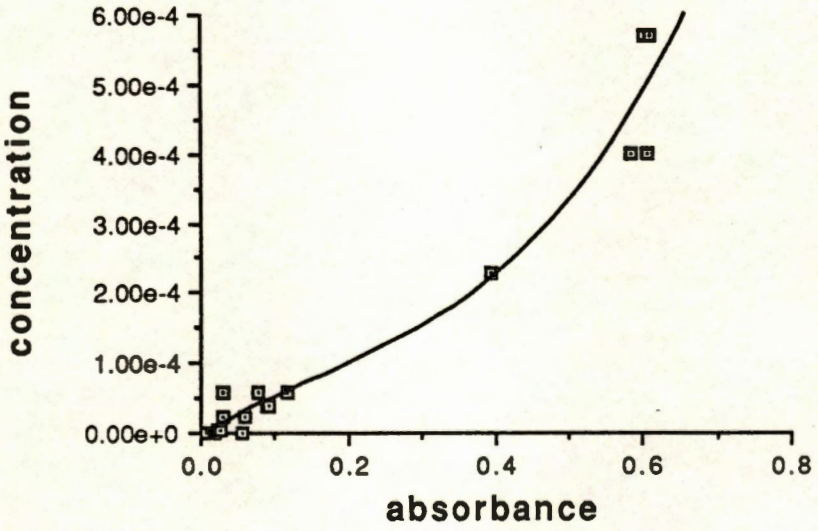
DALTON MASS

APPENDICES

APPENDIX A



y = - 5.1659e-6 + 6.4705e-4x - 1.1168e-3x^2 + 2.3219e-3x^3 R^2 = 0.963

Figure A.1. Standard curves for determination of MW of proteins on TSK SW-3000 SEC-HPLC.

APPENDIX B

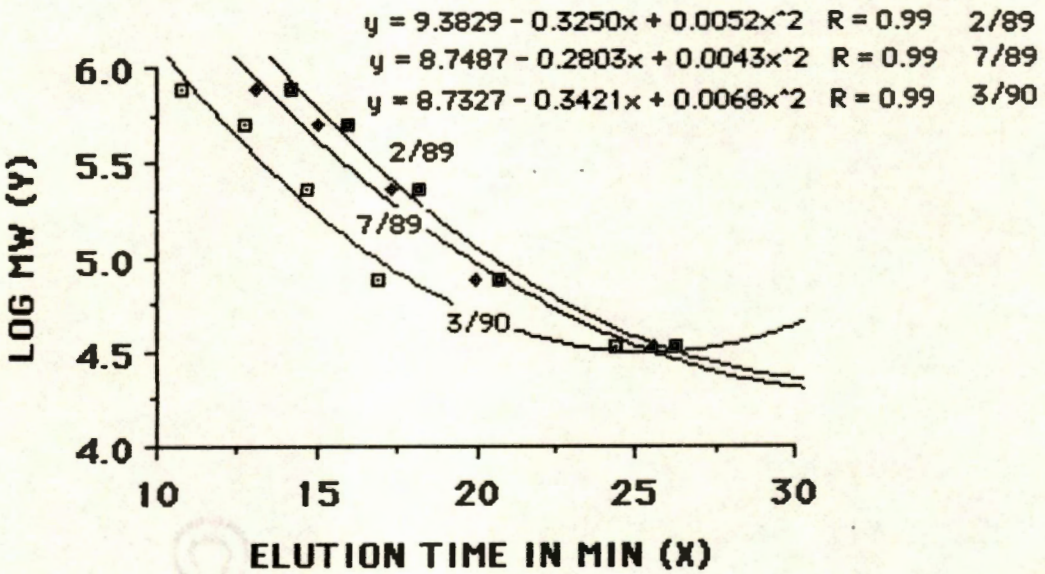


Figure B.1. Standard curve for determination of protein level by Biuret method.

APPENDIX C

Table C.1. Molecular weight standards and their elution time

MW Standard	MW (Daltons)	Elution Time (min.)
Blue dextrin	2000,000	12.6 ± 0.1
Thyroglobulin	669,000	12.7 ± 0.1
Apoferritin	443,000	14.7 ± 0.1
beta-Amylase	200,000	17.0 ± 0.2
Albumin	66,000	19.6 ± 0.2
Carbonic anhydrase	29,000	25.2 ± 0.1
Cytochrome C	12,400	32.2 ± 0.7
Aprotinin	6,500	37.0 ± 1.3

Table C.2. Analysis of variance for peak area (sum of peaks 1 to 3) as a function of flour PDI, processing stage and dilute ME

Source	df	MS	F value
PDI	1	369	1.42
Stage	3	3354	12.89**
ME	1	2118	8.14*
PDI * Stage	3	738	2.84
PDI * ME	1	0	0.00
Stage * ME	3	94	0.36
PDI * Stage * ME	3	175	0.67
Error	16	260	

** $P < 0.01$
* $P < 0.05$

Table C.3. Analysis of variance for protein extractability as a function of flour PDI, processing stage and dilute ME[a]

Source	df	MS	F value
PDI	1	2027	24.19**
Stage	3	3148	37.58**
ME	1	139	1.67
PDI * Stage	3	324	3.87*
PDI * ME	1	59	0.71
Stage * ME	3	67	0.80
PDI * Stage * ME	3	51	0.62
Error	16	84	

[a] ME = 2-mercaptoethanol

* P < 0.05
** P < 0.01

Table C.4. Analysis of varience for interaction study as a function of soy flour PDI, processing stage and extraction solvent.

Source	df	SS
PDI	1	3794*
Stage	3	14998*
PDI * Stage	3	467
PDI 20 F&B vs. G&C[a]	1	5205*
PDI 20 F vs. B	1	181*
PDI 20 G vs. C	1	260*
PDI 85 F&B vs. G&C	1	9768*
PDI 85 F vs. B	1	3
PDI 85 G vs. C	1	49
Extsol	4	12190*
PDI * Extsol	4	3698*
Stage * Extsol	12	5530*
PDI * Stage * Extsol	12	3638*
PDI 20 * F	4	5068*
PDI 20 * B	4	3550*
PDI 20 * G	4	260
PDI 20 * C	4	511
PDI 85 * F	4	8210*
PDI 85 * B	4	5947*
PDI 85 * G	4	406
PDI 85 * C	4	1106*
Error	40	1688

[a] F = Flour; B = Batter; G = Gel; C = Cooked.

* $P < 0.05$

Table C.5. Mean squares from analyses of variance for moisture and protein contents as a function of flour PDI and processing stage

Source	Moisture		Protein	
	df	MS	df	MS
PDI	1	0.05	1	0.30
Stage	3	3527.19*	3	1333.33*
PDI * Stage	3	8.91	3	0.71
Error	6	13.89	7	0.30

* $P < 0.05$

Table C.6. Mean squares from analyses of variance for expansion and hardness of the cooked chips as a function of soy flour PDI and cooking method

Source	df	Mean squares	
		Hardness	Expansion
PDI	1	115**	0.18
Cooking	1	75*	7.10**
PDI * Cooking	1	44*	19.06**
Error	4	10	0.06

** P < 0.05
* P < 0.01

VITA

Ikuyo Kida was born in Kanazawa, Japan. Shortly after graduating from Hokuriku University in her home town with a Bachelor of Science degree in Pharmacy, she moved to the U.S.A. to pursue an advanced degree.

She entered The University of Tennessee, Knoxville, in 1986 majoring in Food Technology and Science. She is a member of both the American Chemical Society and the Institute of Food Technologists.