PROTEIN-PROTEIN INTERACTION OF SOY PROTEIN ISOLATE FROM EXTRUSION PROCESSING

A Thesis presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment of the Requirements for the Degree

Master of Science

---

by
ANGELA CHIANG

Dr. Fu-Hung Hsieh, Thesis Supervisor

DECEMBER 2007

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

PROTEIN-PROTEIN INTERACTION OF SOY PROTEIN ISOLATE FROM EXTRUSION PROCESSING

presented by Angela Chiang,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

Fu-Hung Hsieh, Ph.D., Department of Food Science

Andrew D. Clarke, Ph.D., Department of Food Science

Gang Yao, Ph.D., Department of Biological Engineering

ACKNOWLEDGEMENTS

It has been a long way for the past two years. I would not have this fulfilling journey without the support and encouragement from many people. I would like to thank Dr. Fu-Hung Hsieh for giving me the opportunity to conduct this research with his professional guidance. His understanding in the hardships of students traveling aboard is most appreciated. I would also like to thank my thesis committee members, Dr. Andrew D. Clarke and Dr. Gang Yao for their professional advice in my research and thesis. Special recognition is also extended to Harold Huff for his assistance and knowledge in extrusion processes. Thank you to JoAnn Lewis for always having the answers to the questions I have through these two years. Special thanks to my friends, including Atreyee Das, Hsin-Ming Lu, and Yu-Wei Lin, Chia-En Wu for their assistance, encouragement and companionship throughout the master study.

Most of all, I would like to express my sincere appreciation to my mom and dad, my sister, and my boyfriend Jason for endless faith and understanding when it was most needed. Your support meant the world to me and it's what pushed me forward through the difficult and stressed times.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

EFFECT OF EXTRUSION ON PROTEIN-PROTEIN INTERACTIONS IN SOY PROTEIN ISOLATE

Angela Chiang

Dr. Fu-Hung Hsieh, Thesis Supervisor

ABSTRACT

The objective of this study was to investigate the effect of low moisture, high moisture, and repeated high moisture extrusion on protein-protein interactions in soy protein isolate. Six solvents combinations were used to extract soluble proteins in extrudates from low moisture, high moisture, repeated high moisture, and dead-stop extrusion studies. The protein solubility results were used to elucidate changes in chemical bonds before and after various extrusions. SDS-PAGE was applied to some soluble protein samples to examine the molecular weight distribution of protein subunits. SEM was also used to observe the microstructure and texture of extrudates from repeated high moisture extrusion.

In both low moisture and high moisture extrusion, product temperature changes from 125 to 140°C did not cause significant difference on the amount of soluble proteins. However, the samples between low moisture and high moisture extrusion did show significant differences. The results of the repeated extrusion suggested that the chemical

bonds that contributed to the texturization of the extrudates could be broken apart and reform by the extrusion process. The repeated extrusion resulted in a darker and more fibrous extrudate as shown in the microstructure. In the dead stop experiment, the results suggested that major changes in the network of polypeptide chains and protein solubility occurred in zone 3 and zone 4 of extruder barrel. The chemical bonds that contributed to the texturization of proteins were almost the same from zone 4 on to the end of the extruder. In addition, melted proteins were realigned by the directional shear force at the cooling die to form the fibrous structure. The reduction in protein solubility of extrudates from low moisture extrusion, high moisture extrusion and of samples taken from zone 3 and zone 4 from the dead stop study were probably due to the formation of three dimensional network of soy protein isolate polypeptide chains. As a result of extrusion, these polypeptide chains aggregated together and became less accessible to the solvents used to extract soluble proteins.

The solvent system consisted of PBS+2-ME+Urea extracted most soluble proteins from all extrudates. This was followed by PBS+SDS+Urea. The results from SDS-PAGE of soluble proteins showed very little changes in the protein subunit molecular weight distribution and any changes in the protein subunits were very small. It appears that the

major effect of extrusion was to disassemble soy proteins into protein subunits and then reassemble them together by disulfide bonds, hydrogen bonds, and noncovalent interactions resulting in fibrous extrudates.

CHAPTER 1
INTRODUCTION

1.1 Justification of the Research

Soy protein has been known as an abundant and cost competitive source of protein ever since it was noticed in the 1930s. The advances in soybean production and soy protein processing technology give soy protein a broader and more versatile utilization in human foods (Snyder and Kwon 1987; Hettiarachchy and Kalapathy 1997; Liu 1997). In recent years researchers have kept discovering the benefits of consuming soy protein in substitute of animal proteins such as decreasing total serum cholesterol and decreasing the risks for several cancers (Messina and Barnes 1991; Anderson and others 1995; Messina 1997). These advantages let soy protein perform many functions in foods while maintaining their excellent nutritional quality and benefits to human health. Therefore, the food industry and researchers have placed increased efforts in the development of foods containing soy proteins that are acceptable to the general public (Faller and others 1999; Drake and others 2000; Friedeck and others 2003). As a result of soy proteins' versatility and abundant advantages, food products incorporated with soy proteins have been widely used and accepted in virtually every food system. One notable development

out of the numerous efforts for soy-based foods to be acceptable to human consumption is the texturization of extruded soy protein into meat analogs (Atkinson 1970; Rhee and others 1981; Snyder and Kwon 1987).

Both low moisture (up to 35%) extrusion and high moisture (over 50%) extrusion have been studied extensively (Burgess and Stanley 1976; Jeunink and Cheftel 1979; Hager 1984; Noguchi 1989; Cheftel and others 1992; Prudencio-Ferreira and Areas 1993; Akdogan 1999). However, most research only focused on either low moisture extrusion or high moisture extrusion. The resulting products from these two distinct extrusion processes differ greatly in appearance. Their comparison has not been reported in the literature. In addition, the feasibility of reusing ingredients made of products that do not meet the specifications or that from extruder start-up or shut-down operations, which are important to food industries, has not been reported in the literature.

1.2 Hypotheses and Objectives

The overall hypotheses in this research were: 1) the use of low moisture (35%) and high moisture (60%) extrusion with different product temperature would result in significant differences in chemical and physical properties of extruded products, 2) both

extrusion moisture and temperature would affect the protein to protein interaction in extrudates; 3) repeated high moisture extrusion would have significant effects on extrudate's appearance, texture and chemical characteristics. Therefore, the objectives of this research were: 1) to understand and compare chemical characteristics of low moisture and high moisture extrusion by using protein solubility and SDS-PAGE; 2) to study changes of protein to protein interaction in high moisture extrusion within the extruder with the dead stop procedure; 3) and to observe the differences in texturization and the effect on protein to protein interaction of the first and second high moisture extrusion using SEM, protein solubility tests and SDS-PAGE.

CHAPTER 2

LITERATURE REVIEW

2.1 Soybean Characteristics

2.1.1 Soybeans

Soybeans are typical legume seeds, which differ in size, shape, color and composition based on the variety. The proximate composition of soybeans is given in Table 2.1.1. The soybeans were introduced into the United States in the early 1800s. It was not until the early 1930s when the U.S. started to recognize and exploit their value for feed and food oil. In the mid-1930s, large portions of the oil began to be used in foods such as shortening, cooking oil and margarine. During this time, soybean meal was considered a by-product. Due to its high protein content and good nutritional value, soybean meal was primarily used as animal feed (Wolf and Cowan 1975). In the early 1950s, soybean meal became available as a low-cost, high-protein feed ingredient, triggering an explosion in U.S. livestock and poultry production and assuring a vast and continuing market for soybean farmers' output (Anonymous 2006). By 1990, the United States accounted for 51% of the world's soybean production, and soybeans were America's second largest crop in cash sales (Hettiarachchy and Kalapathy 1998).

Table 2.1.1 Proximate Composition of Soybeans and Seed Parts (Wolf and Cowan 1975).

	%(Moisture-free basis)			
	Protein (N x 6.25)	Lipid	Carbohydrates (Include fiber)	Ash
Whole bean	40%	21%	34%	4.9%
Cotyledon	43%	23%	29%	5.0%
Hull	8.8%	1%	86%	4.3%
Hypocotyl	41%	11%	43%	4.4%

2.1.2 Soy Protein

Compared to other legumes which have 20 to 30% protein content, soybean can contain about 40% to 45% (w/w) of protein depending on variety and growing conditions (Berk 1992). With each ton of crude soybean oil, approximately 4.5 tons of soybean meal with a protein content of about 44% is produced (Berk 1992). Although the unit price of soybean oil is more than twice of soybean meal, the total value of soybean meal produced by a ton of soybean still exceeds the value of soybean oil (Hettiarachchy and Kalapathy 1997). Therefore, soy protein is abundant and cost competitive. Soybean protein is also particularly valuable; because it contains sufficient lysine and can serve as a valuable supplement to cereal foods where lysine is a limiting factor as shown in Table 2.1.2 (Snyder and Kwon 1987).

Plant proteins can be separated and characterized based on their solubility in various media, as shown in Table 2.1.3. The majority of the soybean protein is globulin which is soluble in salt solution (Berk 1992). The solubility of soybean proteins in water is strongly affected by pH (Fig 2.1.1). Close to 85% of the protein in raw soybean is soluble at a pH range 6.4 to 6.6; whereas the minimum solubility for soybean protein is at pH 4.2 to 4.6, which is the isoelectric region (Berk 1992).

Soy protein is classified based on proteins' relative rate of sedimentation (Snyder and Kwon 1987). Four major fractions (2S, 7S, 11S and 15S), shown in Table 2.1.4, have been studied (where S stands for Svedburg units, calculated as the rate of sedimentation per unit field of centrifugal strength: $S = (dx/dt)/w^2x$, where x is the distance from the center of the centrifuge, t is time, and w is angular velocity). 7S and 11S fractions make up 70% of the total proteins in soybeans. The ratio 11S/7S may vary from 0.5 to 3 (Berk 1992). Both 7S and 11S were found to have great influence on the texturization of soy protein. (Ning and Villota 1994) stated that there are significant differences in texturization behavior when the 11S/7S ratio is adjusted in the feed formulation for soy protein extrudates, and an 11S/7S ratio of 1.5 in the feed formulation would result in the best textural characteristics under selected extrusion conditions investigated. Utsumi and

Kinsella (1985) found that in an aqueous solution, the 7S, 11S proteins and soy protein isolate have a three-dimensional network, shown in Table 2.1.5, which may contain hydrogen bonds, ionic interactions, disulfide bonds, and hydrophobic bonds.

Table 2.1.2 Essential amino acid composition of soybeans, wheat gluten, milled rice, corn and broad bean (g/16g N) (Snyder and Kwon 1987).

	Soybeans	Wheat gluten	Rice	Milled corn	Broad bean
Isoleucine	5.1	3.9	4.1	3.7	4.5
Leucine	7.7	6.9	8.2	13.6	7.7
Lysine	6.9	1.0	3.8	2.6	7.0
Methionine	1.6	1.4	3.4	1.8	0.6
Phenylalnine	5.0	3.7	6.0	5.1	4.3
Threonine	4.3	4.7	4.3	3.6	3.7
Tryptophan	1.3	0.7	1.2	0.7	NR
Valine	5.4	5.3	7.2	5.3	5.2
Histidine	2.6	1.8	NR	2.8	2.8

NR: Not Reported.

Table 2.1.3 Plant proteins named and separated by solubility pattern (Snyder and Kwon 1987).

Names	Solvents
Albumins	Soluble in water
Globulins	Soluble in salt solution
Prolamines	Soluble in 50-70% ethanol
Glutenins	Soluble in dilute acid or base

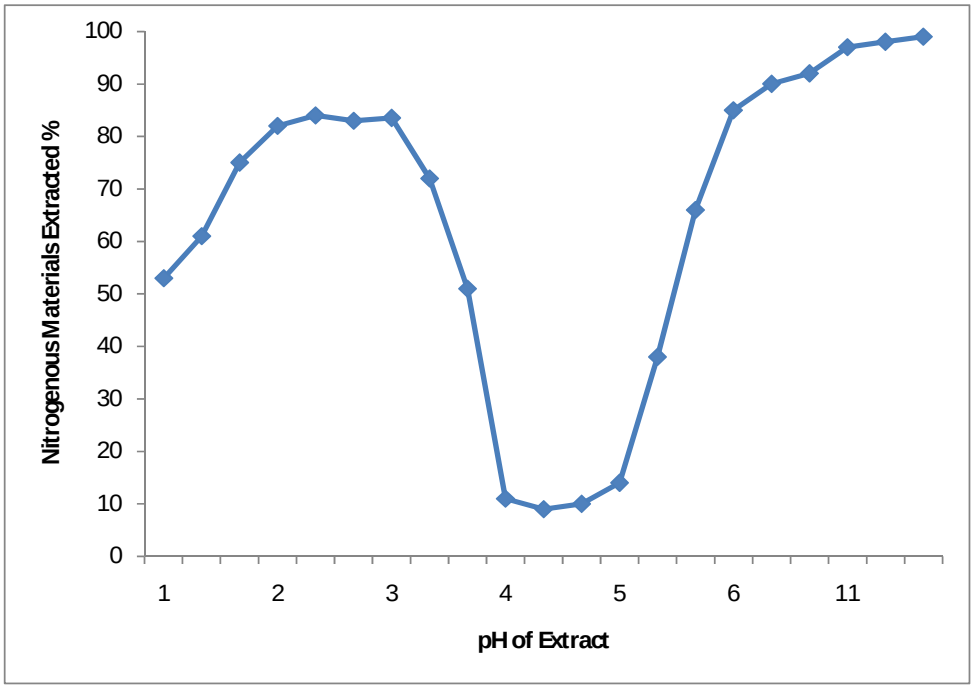


Fig 2.1.1 Effect of pH on solubility of soybean proteins (Wolf and Cowan 1975).

Table 2.1.4 Approximate amounts and components of ultracentrifuge fractions of water-extractable soybean proteins (Wolf and Cowan 1975).

Fractions	Percentage (%)	Component	Molecular weight (Da)
2S	22	Trypsin inhibitors	8,000-21,500
		Cytochrome C	12,000
7S	37	Hemagglutinins	110,000
		Lipoxygenases	102,000
		β-amylases	617,000
		7S globulin	180,000-210,000
11S	31	11S globulin	350,000
15S	11	----------	600,000

Table 2.1.5 The forces for forming and maintaining the structural matrix of 7S, 11S and soy isolate gel (Utsumi and Kinsella 1985).

Fractions	Formation force	Maintaining force
7S	Hydrophobic interactions Hydrogen bonds	Hydrogen bonds
11S	Hydrophobic interactions Electrostatic interaction	Disulfide bonds Hydrogen bonds
Soy Isolate	Hydrogen bonds Hydrophobic interactions	Disulfide bonds Hydrogen bonds

2.1.3 Soy Protein Flours, Soy Protein Concentrates (SPC) and Soy Protein Isolate (SPI)

One of the objectives for the production of soy protein concentrates (SPC) and soy protein isolates (SPI) is to enhance the protein level in soy protein products. The traditional process of producing defatted soy meals or flakes is shown in Fig 2.1.2, which produces a product with a protein content of 40 to 50%. It is necessary to further process soy meal and flakes to remove some low molecular weight components in order to have a higher protein content. SPC, which contains at least 70% protein on a dry-weight basis, is obtained by removing soluble carbohydrate, ash, and other minor constituents as shown in the three commercial processes (Fig 2.1.3). These three processes differ mainly in the method used to insolubilize the major proteins while removing the low molecular weight

components. As a result, the protein products from the three processes have different water solubilities. The proteins in alcohol leached and moist-heat water leached concentrates are denatured and insoluble, while acid leached concentrates are more soluble (Wolf and Cowan 1975). The alcohol leached concentrates, however, retains most of the functional properties such as slurry, viscosity, emulsification power, etc. (Berk 1992).

The process shown in Fig 2.1.4 is one step further than the soy protein concentrates process by removing water insoluble polysccharides, soluble sugars and other minor constituents producing products, i.e. soy protein isolates, which contain more than 90% protein. The traditional procedure for SPI production is by using aqueous or mild alkali extraction (pH 7-10) of the protein and soluble carbohydrates. The extract is then centrifuged, where suspension is used in the isoelectric precipitation procedure (pH 4.5). The precipitated protein is then washed, neutralized (pH 6.8) and spray dried. The produced SPI is therefore almost pure protein, making it to be practically free of odor, flavor, and color.

The other objective of producing SPC and SPI is that the major objectionable characteristic of soybean for usage in food products is the green-beany flavor. It is very

difficult to avoid the green-beany flavor of soybeans in soy flour that are prepared by the conventional method. Through the soy protein concentrate or soy protein isolate processes, the removal of low molecular weight components, soluble carbohydrates, ashes, and particular components that are responsible for the bitterness and beany taste could not only exclude the undesirable flavor, but also remove the raffinose and stachyose that cause adverse flatus (Snyder and Kwon 1987). These processes are a distinct improvement in defatted soy meals for human use and produce soybean protein products that have broader and more versatile utilization in human foods.

Due to these advances in soy protein production and the processing technology in soy protein products, soy protein can perform many functions in foods while maintaining their excellent nutritional quality and benefits to human health. (Snyder and Kwon 1987; Hettiarachchy and Kalapathy 1997; Liu 1997). Therefore, food industries and researchers have put more and more efforts in the development of foods containing soy proteins that are acceptable to the general public (Faller and others 1999; Drake and others 2000; Friedeck and others 2003). As a result of soy proteins' versatility and abundant advantages, food products incorporated with soy proteins have been widely used and accepted in virtually every food system.

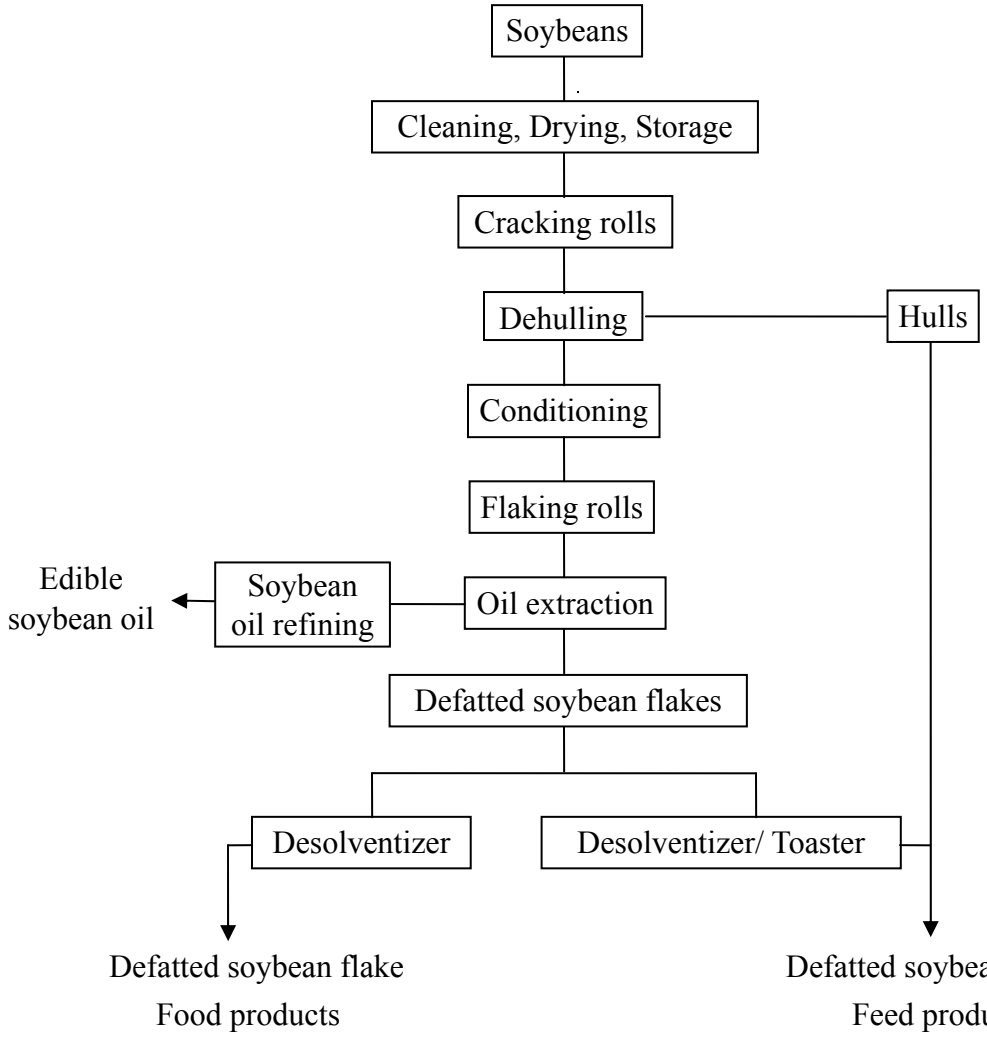


Fig 2.1.2 Outline of process for preparing defatted soy protein meal and flake (Wolf and Cowan 1975).

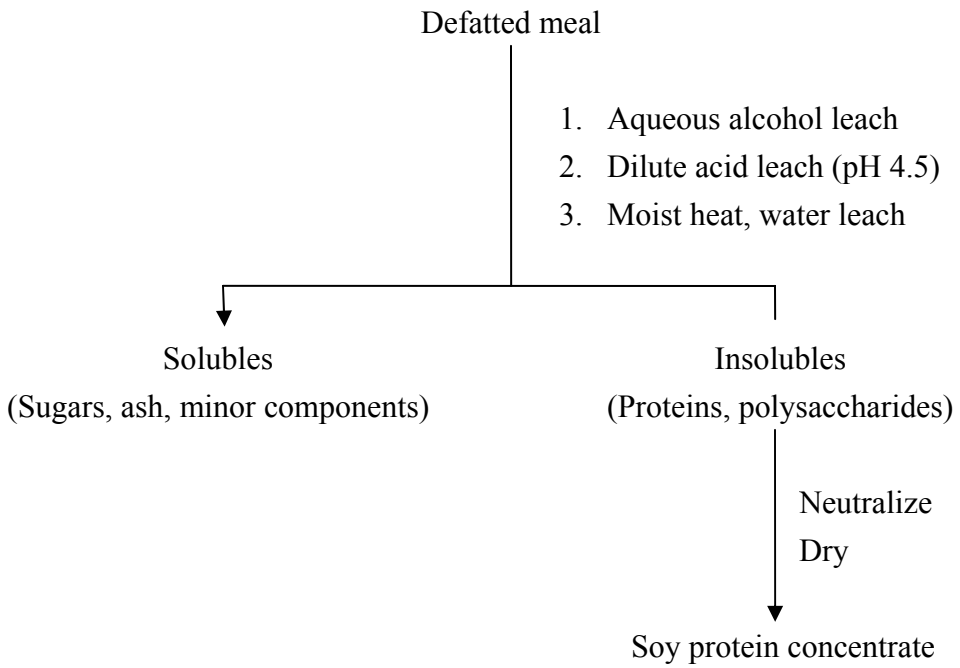


Fig 2.1.3 Outline of commercial processes for soy protein concentrates (Wolf and Cowan 1975).

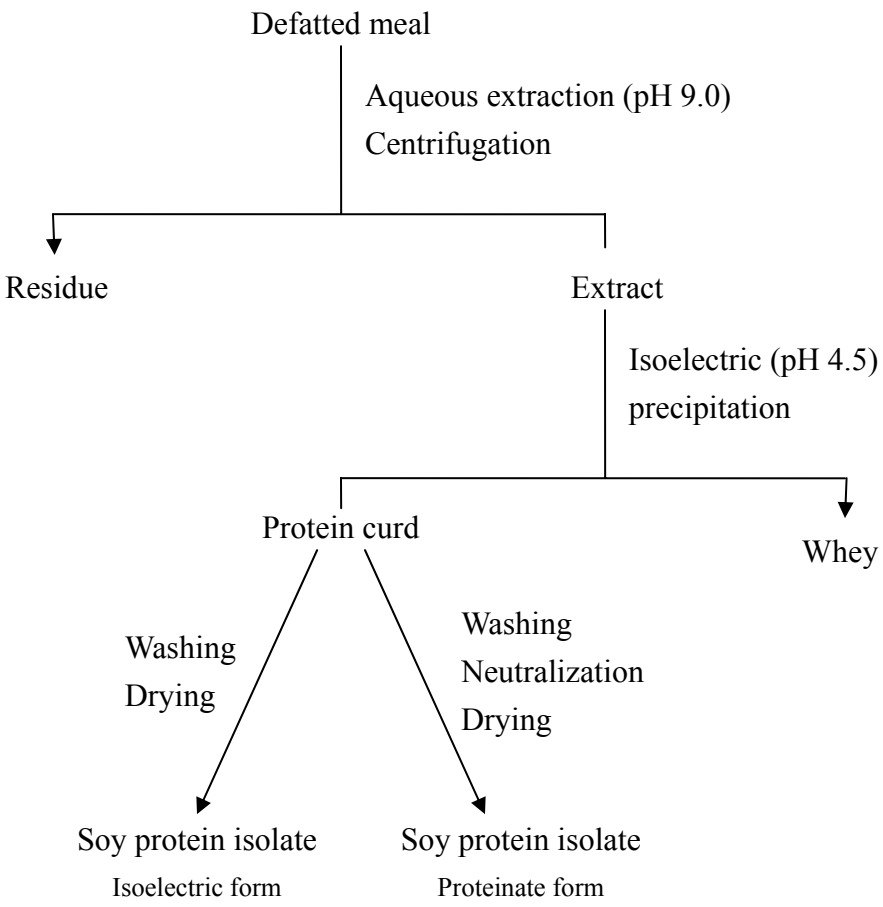


Fig 2.1.4 Outline of commercial processes for soy protein isolates (Hettiarachchy and Kalapathy 1997).

2.2 Food Extrusion

2.2.1 Extrusion Cooking

Food extrusion has been used to produce a variety of food for over 60 years. Extrusion cooking is now widely used in the food industry due to its versatility, high productivity, energy efficiency, and low cost. Extrusion cooking is a continuous

thermomechanical process with multi-step or multifunction operation. It is a high-temperature, short-time process and may involve one or more of the following unit operations: mixing, hydration, shear, homogenization, compression, de-aeration, pasteurization or sterilization, stream alignment, shaping, expansion and fiber formation (Harper 1989; Cheftel and others 1992).

The extruder basically consists of a feeder/live bin that feeds the ingredient; screws that rotates inside a cylindrical barrel; and a die that dictates the shape of the extruded products. An example of a single-screw extruder is shown in Fig 2.2.1. The barrel is divided in to six sections from the feeder to before the cool die (zone 1 to 6) according the sensors to record the temperature. The feed is mixed with water and compressed by the screws as they rotate and pushes the feed forward though the heated barrel. Due to the friction and the heat provided inside the barrel, the feed is quickly heated. As the mixture advances along the barrel, pressure and heat build up. This pressurized cooking transforms the mass into a thermoplastic “melt” (Berk 1992). While the proteins undergo extensive heat denaturation, the directional shear force causes alignment of the high molecular components (Berk 1992). At the end of the barrel the melt is forced through the die. The sudden release of pressure leads to instant evaporation of some of the water. This

causes puffing of the extrudates, thereby resulting in a porous structure. The extrudate's puffing or porous structure could be partially controlled by manipulating the melt temperature within the die.

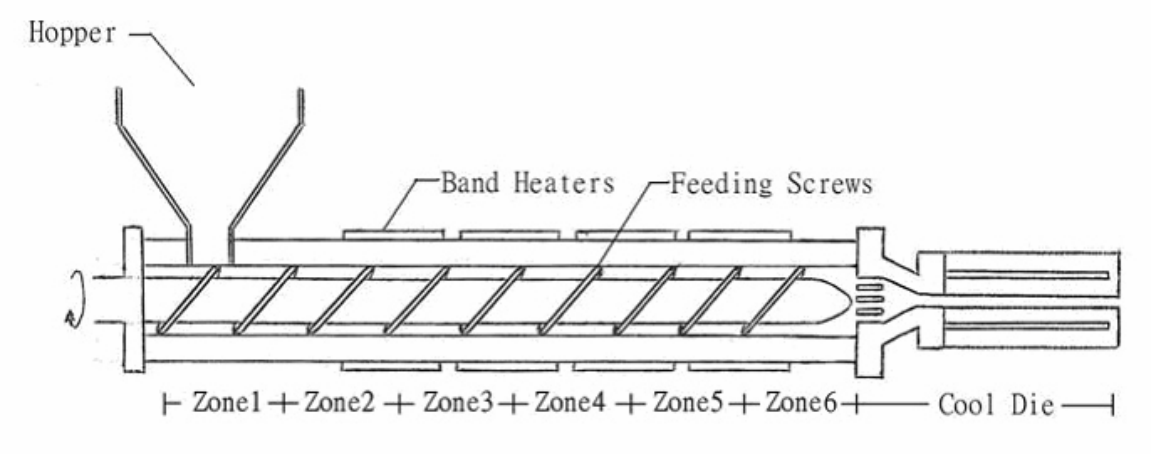


Fig 2.2.1 Example of a single-screw extruder.

2.2.2 Food Extruders

The extruders that were used in food production over 60 years ago are single-screw extruders. Their initial application was to mix and form macaroni and ready-to-eat cereals. As a result of continuous development effort, their versatility expanded and the single-screw extruders were used to produce a variety of foods such as cereals, snacks, croutons, dry pet foods, and precooked infant food in the 1960s. The twin-screw

extruders were developed in the 1970s for its expanded operational capabilities and extended range of application (Harper 1989). In addition to manufacturing foods similar to those produced by the single-screw extruders, the twin-screw extruders are used to produce confectionery products.

In comparison to the twin-screw extruders, the single-screw extruders are relatively ineffective in transferring heat from the barrel jackets to the products. This is caused by the poor mixing within the extruder channel (Harper 1989). In single-screw extruders, heat is generated by friction or conversion of mechanical energy to heat or supplied by heated barrels. Twin-screw extruders have considerably more heat exchange capability than single-screw extruders, which expand their application to heating and cooling of viscous pastes, solutions, and slurries. The twin-screw extruders, therefore, are more suitable for processing high moisture materials, due to better heat transfer. In addition, the direction of screw rotation, screw shape, screw configuration and relative position of screw sections minimize pressure and leakage flows (Harper 1989; Noguchi 1989). Twin-screw extruders have less interaction of process variables than single-screw extruders, making them easier to operate and control (Harper 1989). Both types of extruder are widely used in the food industry. Due to their low cost, single-screw

extruders remain to be an effective and economical choice to produce pet foods. The twin-screw extruders are mainly applied to products that require better control and operating flexibility. In this study, to have a better control, flexibility and the capability to extrude both dry materials (<35%) and high moisture materials (>50%), a twin-screw extruder is used.

2.2.3 Low Moisture Extrusion

Texturized vegetable protein (TVP), a commercialized meat analog, is produced by thermoplastic extrusion (Atkinson 1970). In this process, defatted soy flour with a mixture of 20-25% moisture is passed through a high pressure extrusion cooker producing a product that is porous and expanded. Although it does not have well defined fiber, it produces particulates that upon hydration have good mouth feel of chewiness and elasticity that symbolizes meat (Berk 1992; Liu 1997). Extrusion of defatted soy flour with moderate water content (up to 35%) have been studied extensively (Kelley and Pressey 1966; Cumming and others 1973; Burgess and Stanley 1976; Jeunink and Cheftel 1979; Hager 1984; Prudencio-Ferreira and Areas 1993).

2.2.4 High Moisture Extrusion

Although low moisture extrusion (up to 35% moisture) produces extrudates that have structured and textured features, it is hard to call the expanded and spongy looking extrudates “meat analogs” based on their appearance. Further disadvantages of low moisture extruded products are the time needed to rehydrate them with water or flavored liquid before use; their lack of meat flavor; and the low level of fat limiting their use as a meat alternative (Noguchi 1989). Therefore, researchers have been investigating the potential of using high moisture extrusion to improve the texturized vegetable protein for the last 20 years.

When soy protein is extruded under high pressure, high temperature and low moisture conditions, the sudden release of pressure upon exiting from the die causes instant water evaporation from the extrudates. This creates expanded and spongy structure of common texturized vegetable protein. To reduce extruder die pressure and extrudate expansion, soy protein needs to be extruded at a higher moisture content (>50%). In addition, a cooling die is essential in high moisture extrusion to increase the viscosity of the hot melt and reduce its fluidity so the necessary pressure and temperature can be maintained (Noguchi 1989). When proper cooling is applied, high moisture

protein melt forced through the cooling die is the alignment of proteins due to directional shear force (Noguchi 1989). Unlike low moisture extrusion, the products from high moisture extrusion are dense and fibrous.

2.2.5 Repeated Extrusion

The reuse of material or semi-finished material is essential for the control of the ingredient cost in the food industries. For extrusion operation, it is common to blend ingredients with up to 15% of materials from start-up or shut-down operations or from extruded products that are out of specifications. The experiment of repeated extrusion gives an idea of the physical appearance and chemical characteristic of soy protein extrudates that have been extruded more than once. Isobe and Noguchi (1987) extruded defatted soy flour at 60% moisture with the barrel temperature setting at 130, 140 and 150°C, respectively. The extrudates were cut into small pieces and extruded two more times. The shape of extrudates was similar after repeated extrusion while the soluble protein fractions decreased or disappeared following the first extrusion and gradually decreased after additional extrusions. Extrudates that were extruded at 130°C were not much different from those extruded at 140 or 150°C. These results suggested that

multiple extrusions appeared to have little effect on the extrudates.

2.3 Soy Protein Texturization and Effects of Texturization

2.3.1 Texturization of Soy Protein

Soybeans have been a major source of protein source in the eastern countries for centuries. Even though food products incorporated with soy protein are accepted in the western part of the world, the consumption of foods directly converted by soybean protein are still very limited (Berk 1992). Due to differences in culture and preference in texture and flavor, traditional Asian soy foods such as tofu, miso and yaba are not fully accepted in the American household. However, in recent years more and more Americans are becoming aware of the soy-based foods that could provide high quality protein, low fat, no cholesterol, and high fiber. Therefore, numerous efforts have been made to develop soy-based foods that might be acceptable to the western countries.

One notable effort is the texturization of soy protein into meat analogs. Several methods of texturing soy protein have been reported including spinning, thermoplastic extrusion, steam texturization, and enzymatic texturization. Thermoplastic extrusion of soy protein based on several patents, in particular, produces meat-like products that are

known as TVP (textured or texturized vegetable protein). These products were first introduced in the 1970s and remain to be an important texturized soy protein food (Atkinson 1970; Liu 1997).

2.3.2 Heat and Shear Effects on Soy Protein

Food extrusion is considered a high-temperature short-time bioreactor that transforms raw feed material into modified intermediate and finished products (Harper 1989). The thermal extrusion exposes the proteinaceous ingredients to high temperature, high pressures and mechanical shear. This converts the soy protein into a continuous plastic “melt”, resulting in protein denaturation, reduce solubility and decrease extrusion effectiveness (Harper 1989). Within the process, water soluble fractions of soy protein (7S and 11S globulins) undergo a complex pattern of association-dissociation reaction (Cheftel and others 1985). Stanley (1989) concluded that the major influence of extrusion is to disassemble the proteins and then reconnect them into a fibrous, oriented structure possessing a characteristic texture.

It is known that thermal treatment of protein results in structural changes such as hydrolysis of peptide bonds, modification of amino acid chains and the formation of new

covalent isopeptide cross-links. The effect of heat in the extrusion of soy protein has been studied systematically. Harper (1989) suggested that during extrusion, the protein is denatured and unfolded by shear and high temperature. Berk (1992) stated that while proteins undergo extensive heat denaturation, the directional shear force causes alignment of the high molecular components which lead to the texturization and the fiber formation on the extrudates.

Early works reveal that the obvious consequence of heat treatment to soy protein is the lost of solubility due to the formation of disulfide bonds, hydrogen bonds and hydrophobic bonds (Stanley 1989). Protein solubility is influenced by extrusion temperature, and with the increase of extrusion temperature a more textured product could be produced (Stanley 1989). Hayakawa and Kajiwara (1992) stated that the solubility of soy protein drastically decreased when it was heated to a temperature around 110-120°C. However, the solubility increased at temperatures above 150°C with over 10 min of heating time. Li and Lee (1996) found that when extruding wheat flour extrudates, the increase of die temperature in the extrusion process caused the intensity of higher molecular weight regions (> 25,000) to decrease with a concomitant increase of the intensity of low molecular weight regions (<25,000). They suggested that the

fragmentation of high molecular weight proteins might be the reason that the content of soluble proteins in extrudates obtained at a higher temperature was a little higher than at a lower extrusion temperature. The aggregation of proteins during extrusion caused an increase in their molecular weight, which also resulted in a decreased protein solubility.

2.3.3 Pressure Effects on Soy Protein

The pressure produced in the extruder is usually less than 100 MPa (Noguchi 1989). During high moisture extrusion, the pressure is even lower due to low viscosity. Noguchi (1989) summarized the pressure effects on proteins: 1) carboxyl, phenol and amino residues on the protein dissociate and ionize as the pressure increases; 2) formation of hydrogen bonds reduces the volume of the system; 3) hydrophobic bonds increase with the increase of pressure; and 4) covalent bonds are not influenced by pressure.

In the study of the effect of pressure on protein solubility, soy protein isolate was either pressurized at 0, 50, 100 and 250 kg/cm^2 or heated at 110, 140, 170 and 200°C (Hayakawa and Kajiwara 1992). The result shows that protein solubility was strongly dependent on the heating temperature but not dependent on the pressure. Noguchi (1989) also stated that pressure less than 50 MPa do not influence the protein reactions during

high temperature treatment.

2.3.4 Ingredient Effects on Extrusion

Major components in the extrusion of texturize soy protein may include soy proteins, water and carbohydrates. Soy proteins are the foundation of texture formation. The increase of protein levels would greatly influence the rheological properties of extrudates (Maurice and Stanley 1978; Rhee and others 1981; Sheard and others 1985). Stanley (1989) concluded that both protein quality and quantity have an important effect on soy protein texturization. Water is also an important factor in extrusion due to the effect on heat transfer during extrusion. Higher moisture content would result in a better heat transfer from the extruder barrel to the feed material and it would also lower viscosity, shear and friction during extrusion.

Another important role of water is in the separation of proteins, which helps the formation of protein fibrous structure (Noguchi 1989). During extrusion, protein undergoes a plastic melt in which protein denatures and aggregates (Harper 1989). While protein is melted into this elastic mass, large amount of water combined with carbohydrate such as starch would lead to a phase separation which enhances protein to

protein interaction (Noguchi 1989). Such phase separation with the help of shear force at the die would induce the formation of proteinaceous fibrous structures (Harper 1989; Noguchi 1989). Starch is found to distribute throughout the protein fibrous matrix and never incorporated into the protein fibers (Noguchi 1989). Low moisture extrusion has less fibrous structure due to the limited amount of water that could be absorbed by starch resulting in a weaker phase separation.

2.3.5 Protein Texturization Mechanisms

Many efforts have been made to investigate protein-protein reactions and texturization during extrusion cooking. Intermolecular disulfide bonding was considered the texturization mechanism during extrusion due to its importance in food systems such as wheat dough and spun soy fibers (Kelley and Pressey 1966; Li and Lee 1996). Jeunink and Cheftel (1979) extruded soy protein concentrate at 145°C with 32% moisture. Prudencio-Ferreira and Areas (1993) studied soy protein isolates samples that were extruded at 140,160, and 180°C with 30 and 40% moisture. Lin and others (2000) extruded soy protein isolate that were extruded under 137.8, 148.9 and 160°C with 70, 65 and 60% moisture. All three studies concluded that the major forces responsible for

protein insolubilization in the extrudates were due to hydrophobic interaction, hydrogen bonding, and covalent intermolecular disulfide bridges. However, others (Burgess and Stanley 1976; Hager 1984) found that after extrusion of soy protein concentrate there was a major increase in sulfhydryl groups, and the disulfide bond decreased rather than increased (Table 2.3.1). Some researchers also reported that during extrusion of soy protein, there was no significant loss of sulfur amino acids (Jeunink and Cheftel 1979). With these findings Stanley (1989) concluded that because the disulfide bond formation during extrusion was solely based on the indirect evidence from solubility experiments, it cannot be used to infer that intermolecular disulfide bonds contribute significantly to the texturization of soy protein during extrusion. Harper (1989) suggested that during extrusion, cross-linking reactions occur. However, Noguchi (1989) reported that there is little change in the distribution of protein fractions before and after extrusion based on the electrophoresis study. He concluded that the proteins are hardly modified by extrusion.

Table 2.3.1 Disulfide and sulfhydryl concentrations in native and extruded soy concentrate (Hager (1984); Burgess and Stanley (1976)).

Researcher	Extrusion Temp.	(mol/mg)	Native soy concentrate	Extruded soy concentrate
Hager (1984)	140ºC	-S-S- content	22.7×10^{-8}	19.6×10^{-8}
		-SH content	0.5×10^{-8}	4.1×10^{-8}
Burgess and Stanley (1976)	178ºC	-S-S- content	4.5×10^{-8}	0.9×10^{-8}
		-SH content	3.3×10^{-8}	48.9×10^{-8}

2.4 Analysis of Soy Protein

2.4.1 Protein Solubility

The effects of extrusion on proteins are difficult to isolate and determine since the proteins are exposed to several processes simultaneously. There are several ways to analyze extrudate for their chemical properties. One of which is the use of different solvents to analyze protein solubility as a tool to investigate protein-protein interaction and protein texturization of both raw materials and extrudate. As early as 1966, protein solubility was used to investigate the fiber formation of spun soy fibers (Kelley and Pressey 1966). They concluded that hydrogen bond, ionic bond and disulfide bond contribute to the formation of spun soybean fibers. Burgess and Stanley (1976)

investigated the mechanism of thermal texturization of low moisture extruded soybean protein. Their result was that disulfide bonds do not play the most important role in texturization as in the fibers formed during thermal texturization. The intermolecular peptide bond instead contributes mainly to the thermal texturization. This finding from Burgess and Stanley (1976) was widely disputed. Other researchers suggested that the disulfide bond and the noncovalent bond stabilize and texturize soybean protein (Jeunink and Cheftel 1979; Hager 1984; Prudencio-Ferreira and Areas 1993).

It should be noted that different solvents with various concentrations were used in these studies. Different solvents, each with a specific chemical reaction on protein, may reveal which and to what extent a specific chemical bond contributes to the texturization of extrudates. For example, phosphate buffer extracts water soluble proteins in their native states; 2-mercaptoethanol (2-Me), dithiothreitol (DTT), sodium sulfite are known to disrupt the disulfide bonds; acrylonitrile minimizes the effect of reoxidation of free sulfhydryls after the disulfide bonds are broken by the reagents; sodium dodecyl sulfate (SDS) are used for their ability to interrupt hydrophobic and ionic interactions and urea was applied to dissolve the proteins with hydrogen bonds and hydrophobic interactions (Burgess and Stanley 1976; Jeunink and Cheftel 1979; Hager 1984; Harvath and Czukor

1993; Prudencio-Ferreira and Areas 1993). Thus, protein to protein interaction of the extrudates could be investigated by finding the protein solubility difference before and after extrusion in different solvents. Table 2.4.1 summarizes solvents used by six groups of researchers. As soy protein is receiving increased attention throughout the late twentieth century, it is expected that more and more researchers will use protein solubility to study protein to protein interaction of textured soy proteins.

Table 2.4.1 Protein solubility[a] of extruded soy meal in various solvents

	Reference[b]					
Reagent[c]	1	2	3	4	5	6
Neutral buffer (B)	V	V	V		V	V
Basic buffer	V		V			
B + urea	V		V		V	V
B + urea + SDS	V					V
B + urea + 2ME	V					V
B + DTT		V				
B + 2ME				V	V	V
B + SDS		V		V	V	V
B + SDS + DTT		V				
B + SDS + 2ME				V		
Extrusion moisture (%)	27	32	27	35	30, 40	60, 65, 70
Product temp (°C)	178	145	135	X		X
Extrusion temp (°C)	X	X	X	120, 180	140, 160, 180	138, 149, 160

[a]Percent of total protein extracted. [b]1: Burgess and Stanley (1976); 2: Jeunink and Cheftel (1979); 3: Hager (1984); 4: Sheard and others (1985); 5: Prudencio-Ferreira and Areas (1993); 6: Lin and others (2000). [c]SDS = sodium dodecyl sulfate, 2ME = 2-mercaptoethanol, DTT = dithiothreitol.

2.4.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis has become an indispensable tool in biochemistry because of its

versatility. The principle of electrophoresis is based on the migration of a charged particle such as a peptide molecule in an electric field (Dunn 1993b). The driving force for migration, which is equal to the frictional resistance, is complicatedly influenced by many factors, such as voltage and the buffer applied to the electrical field; particle size and particle charge of the peptide sample; pore size and the presence of additives of the supporting media. The size and charge of the particle are determined by the composition of amino acids in the peptide. Different amino acids containing different charges would form unique peptides that fold and aggregate accordingly.

The supporting media most commonly used in electrophoresis is the polyacrylamide gel. Polyacrylamide gels are polymerization products of acrylamide monomer and cross-linking agent N, N'-methylene-bis-acrylamide, in which polymerization is initiated by free-oxygen radicals. The advantages of polyacrylamide are: 1) it is charge free, therefore free of electroendosmotic flow effects on the components separated electrophoretically; 2) it is a clear gel making it suitable for quantitative densitometric techniques; 3) by changing the concentration of the monomer or comonomer, the pore size may be varied over a wide range giving a wide choice of conditions for size separation (Allen and Budowle 1994).

Gel electrophoresis often is used for the molecular weight determination of proteins based on the comparison of migration distance to known proteins in the same gel. Native proteins would migrate through the gel influenced by its size, shape and net surface charge density. However, this makes it hard to estimate the molecular weight. The pioneer work of Shapiro and others (1967) solved this problem by the addition of sodium dodecyl sulfate (SDS). Shapiro and others (1967) found that when heated with SDS, thiol-reduced proteins disaggregate into subunits. In which these denatured proteins, mobility through the ploy acrylamide gel are inversely related to their molecular weight. SDS-PAGE is currently the most commonly used electrophoretic technique for the analysis of proteins. SDS has the ability to solubilize, dissociate, and denature the majority of oligomeric proteins into their constituent subunits (Dunn 1993a). SDS can mask the intrinsic charge of the polypeptide chains so that the net charge per unit mass becomes approximately constant. The resulting SDS-protein complexes can then be separated depending only on the effective molecular radius, which is relative to the molecular mass (M_r).

The most popular general protein staining procedures for gels after electrophoresis is the use of Coomassie Brilliant Blue (CBR) R-250. CBR requires an acidic medium for

electrostatic interaction between the dye and the amino groups of proteins. Staining methods using CBR are simple and convenient, and have a detection sensitivity of approximately 0.2-0.5μg of protein per band (Dunn 1993b).

2.4.3 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy is a popular technology for studying the microscopic textural characteristics of extruded product. Protein matrix, structural integrity, carbohydrate distribution and fibrous structure could be observed by SEM. Lin (2002) used SEM to investigate the effects of extrusion moisture (60, 70%) and temperature (138, 149,160°C) to the texturization of the extrudates and found that under the same extrusion temperature with the decrease in moisture content the more fibrous and texture could be observed. With 60% extrusion moisture the fibrous all showed a tight network structure, while with 70% moisture the increase of extrusion temperature produced a more organized structure. Other researchers used SEM to observe the textures of rice flour extrudates and rice flour fortified with soy protein isolate that were extruded under 20, 30, or 40% moisture and 200, 220, or 240°C extrusion temperature (Noguchi and others 1982). They found that the rice extrudates

had a film-like structure composed mainly of gelatinized starch with insoluble starch skeletons whereas the fortified extrudates had a fine string structure that was contributed by SPI.

CHAPTER 3

EFFECT OF LOW MOISTURE AND HIGH MOISTURE EXTRUSION ON PROTEIN-PROTEIN INTERACTIONS IN SOY PROTEIN ISOLATE

3.1 Introduction

Extrusion cooking is widely used in the food industry due to its versatility, high productivity, energy efficiency, and low cost. It is a continuous thermomechanical process with multi-step or multifunction operation. Texturized vegetable protein (TVP) is one of the patented products produced by thermoplastic extrusion (Atkinson 1970). In this process, defatted soy flour with a mixture of 20-25% moisture is passed through a high pressure extrusion cooker producing a product that is porous and expanded. These low moisture extrusion (up to 35%) soy protein products have been studied extensively (Burgess and Stanley 1976; Jeunink and Cheftel 1979; Hager 1984; Prudencio-Ferreira and Areas 1993). However, the products of low moisture extrusion are expanded and spongy which are hard to call them “meat analogs” based on their appearance. Therefore, researchers have been investigating the potential of using high moisture extrusion to improve the texturized vegetable protein for the last 20 years (Noguchi 1989; Cheftel and others 1992; Akdogan 1999).

When soy protein is extruded under high pressure, high temperature and low moisture conditions, the sudden release of pressure upon exiting from the die causes instant water evaporation from the extrudates. This creates expanded and spongy structure of common texturized vegetable protein. To reduce extruder die pressure and extrudate expansion, soy protein needs to be extruded at a higher moisture content (>50%). In addition, a cooling die is essential in high moisture extrusion to increase the viscosity of the hot melt and reduce its fluidity so the necessary pressure and temperature can be maintained (Noguchi 1989). When proper cooling is applied the result of high moisture protein melt forced through the cooling die is the alignment of proteins due to directional shear force (Noguchi 1989). Unlike low moisture extrusion, the products from high moisture extrusion are dense and fibrous.

Although the development of both low moisture and high moisture soy protein extrusion products have been successful, the mechanism of protein-protein reactions and texturization during extrusion cooking are been disputed. Intermolecular disulfide bonding was considered the texturization mechanism during extrusion due to its importance in food systems such as wheat dough and spun soy fibers (Kelley and Pressey 1966; Li and Lee 1996). Burgess and Stanley (1976) however, investigated the

mechanism of thermal texturization of low moisture extruded soybean protein. Their result was that disulfide bonds do not play the most important role in texturization as in the fibers formed during thermal texturization. The intermolecular peptide bond instead contributes mainly to the thermal texturization. Jeunink and Cheftel (1979) extruded soy protein concentrate at 145°C and 32% moisture. Prudencio-Ferreira and Areas (1993) studied soy protein isolates samples that were extruded at 140, 160, and 180°C with 30 and 40% moisture. Lin and others (2000) extruded soy protein isolate at 137.8, 148.9 and 160°C with 60-70% moisture. All three studies concluded that the major forces responsible for texturization of the soy protein extrudates were due to disulfide bonds and noncovalent interaction.

All these studies have been done on either low moisture extrusion products or high moisture extrusion products. Thus, the objective of this study is to investigate: 1) the effect of extrusion temperature on low moisture and high moisture extrusion and 2) comparison between low moisture with high moisture extrusion soy protein isolate products. In addition, 3) changes of protein to protein interaction in high moisture extrusion within the extruder was investigated by the dead stop procedure.

3.2 Materials and Methods

3.2.1 Materials

Soy protein isolate (SPI) (Profam 974) was obtained from Archer Daniels Midland (Decatur, IL) containing a minimum 90% w/w protein. Starch (Midsol 50) was provided *in gratis* by MGP Ingredients, Inc. (Atchison, KS). Their proximate compositions are shown in Table 1. The ingredients were mixed in 9:1 ratio using a Double Action™ food mixer (Model 100DA70, Leland Southwest, Fort Worth, TX) for 10 min to ensure the uniformity of the feeding material.

3.2.2 High Moisture Extrusion and Dead Stop Procedure

An MPF 50/25 co-rotation intermeshing twin-screw extruder (APV Baker, Inc., Grand Rapids, MI) was used. The extruder has a screw length to diameter ratio of 15 to 1 and the diameter is 50 mm. A cooling die with dimensions W × H × L of 30 × 10 × 300 mm was attached at the end of the extruder with 4.4ºC cold water as the cooling media. The screw profile from feed to exit were 100 mm twin lead feed screws, 50 mm 30º forward paddles, 100 mm single lead feed screws, 87.5 mm 30º forward paddles, 175 mm single lead feed screws, 87.5 mm 30º forward paddles, 50 mm 30º reverse paddles,

100 mm single lead feed screws and finally the cooling die. The barrel was divided into six sections and the barrel temperature settings from zone 1 to zone 5 were 22.9, 24, 42.1, 96.3, and 136.1ºC, respectively. Zone 6 barrel temperature and other independent extrusion variables are listed in Table 3.2.1. Dead stop extrusion was also conducted to investigate changes in feed material within the extruder barrel. The temperature at zone 6 was set at 137.8ºC. After steady state was reached, the extruder was abruptly stopped and the barrel was cooled immediately and split opened within 5 min. Samples were collected from zone 2 to 6, cooling die and product. All samples were sealed and stored at -20ºC for further analysis.

3.2.3 Low Moisture Extrusion

The low moisture extrusion was operated with the same extruder as the high moisture extrusion. The screw configuration from feed to exit were 25 mm single lead feed screws, 200 mm twin lead feed screws, 125 mm 30º forward paddles, 50 mm single lead feed screws, 37.5 mm 60º forward paddles, 37.5 mm 60º reverse paddle, 50 mm single lead feed screws, 25 mm 90º paddles, 87.5 mm 30º forward paddles, 37.5 mm 30º reverse paddle, 75 mm single lead feed screws. In order for high moisture extrusion and

low moisture extrusion to have similar product temperatures, the final zone temperature of dry extrusion was set according to Table 3.2.2. The temperature settings in the extruder from zone 1 to zone 5 were 19.7, 24.2, 49.8, 89.8ºC, respectively. Zone 6 was set at 118.3, 120.1, 125.2ºC for the product temperatures of 125.3, 134, 140.6ºC, accordingly. All samples were preserved by freezing at -20ºC after extrusion.

Table 3.2.1 Experimental design for high moisture extrusion.

Conditions	Levels
Final barrel temperature setting (zone 6)	137.8, 148.9, 160ºC
Product temperature	124.2, 134, 140.6ºC
Moisture content	60%
Screw speed	200 rpm
Water feed rate	12.2 kg/h (26.8 lb/h)
Dry feed rate	9.1 kg/h (20 lb/h)
No. of Replications	4
Formula	90% SPI, 10% wheat starch

Table 3.2.2 Experimental design for low moisture extrusion.

Conditions	Levels
Final barrel temperature setting (zone 6)	119.6, 130.3, 140.7ºC
Product temperature	121.7, 130.1, 138.6ºC
Moisture content	35%
Screw speed	200 rpm
Water feed rate	4 kg/h (8.8 lb/h)
Dry feed rate	9.1 kg/h (20 lb/h)
No. of Replications	4
Formula	90% SPI, 10% wheat starch

3.2.4 Protein Solubility

Protein solubility was tested on soy protein isolate, extruded products from the high and low moistrue, and samples collected from the dead stop procedure. The following were six solvents used in this study: 1) 0.035 M, pH 7.6 phosphate buffer solution (PBS) (known to extract proteins in their native state); 2) 8 M urea in the phosphate buffer solution (known to dissolve the proteins with hydrogen bonds and hydrophobic interactions); 3) 2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution (known to disrupt the disulfide bonds); 4) 8 M urea + 2% 2-ME in the phosphate buffer solution; 5) 1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution (used for their ability to interrupt hydrophobic and ionic interactions); and 6) 8 M urea +

1.5% SDS in the phosphate buffer solution. All phosphate buffer solutions from 2 to 6 were the same as the first one. All chemicals were of reagent grade and obtained Fisher Scientific (Fair Lawn, NJ).

Extruded samples were defrosted and finely chopped with a blender to approximately 3 mm cubes. One gram of chopped sample was weighed in duplicate and dried in a vacuum oven at 103ºC overnight to determine the moisture content. Twenty ml of six different solvents mentioned above were used to extract 1 g of defrosted and chopped sample or 0.5 g of the soy protein isolate and starch mix (control) or 0.5 g of zone 5 and zone 6 samples from the dead stop procedure. Each sample in duplicate was slowly added and well-mixed into 20 ml of solvent. Solutions containing the sample were placed into a water bath set at 40ºC and shaken at 100 rpm for 2.5 h. After extraction, the solutions were centrifuged at 12,500 rpm (10000×g) in a centrifuge (Beckman J2-21M/E, Schaumburg, IL) for 30 min. Protein content of all solutions, except for samples extracted by sodium dodecyl sulphates, were determined with Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL) at 595 nm based on the Bradford method. The protein contents for samples extracted by sodium dodecyl sulphates were measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) at 560 nm. A microplate reader (Bio-Rad, Hercules, CA)

with standard curves that were made for each solvent was used to determine the protein concentration of all solutions.

3.2.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to observe the differences in protein distribution. Samples were obtained from solutions in the protein solubility study. Twelve percent acryl amide gels (Mini-Protean II Ready Gel, Bio-Rad, Hercules, CA) were used to analyze the samples. To get sharp and clear bands, protein extracts were diluted to approximately 15-17% of protein content according to the protein solubility test before mixing with sample buffer (Laemmli sample buffer, Bio-Rad, Hercules, CA) at 1:1 ratio, then heated at 95°C for 5 min. Running buffer (Tris/Glycine/SDS, Bio-Rad, Hercules, CA) was added into the inner and outer chambers of the Mini-Protean II Cell (Bio-Rad, Hercules, CA) at approximately 270 ml. Twenty μL of samples and 6 μL molecular weight marker (SDS-PAGE Standards, Broad Range, Bio-Rad, Hercules, CA) were loaded in each well. The gel ran about 1.5 h with the power supplier (1000 V microprocessor power supply, Buchler, Lenexa, KS) set at 100 V, 50 mA, and 10 W.

Gels were stained by Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) for

1 h and destained by a destaining solution (Bio-Rad, Hercules, CA) for 2 h. After destaining, gels were sealed by cellophane (Bio-Rad, Hercules, CA) and dried at room temperature 23ºC overnight. The bands from the samples were compared with the molecular weight markers (GE Healthcare, Piscataway, NJ) of 200, 116, 97.4, 66, 45, 31, 21.5 and 14.5 kD.

3.2.6 Data Analysis

Analysis of variance (ANOVA), general linear model (GLM), and multivariate analysis of variance (MANOVA) in the SPSS program (version 11.5, SPSS Inc., Chicago, IL.) were used for data analysis. When analysis of variance (ANOVA) revealed a significant effect, treatment means were compared using the least significant difference (LSD) test.

3.3 Results and Discussion

3.3.1 Protein Solubility of Low Moisture and High Moisture Extrusion

Protein solubility test was performed to investigate the forces responsible for stabilizing the texturization of extruded soy protein isolate. Table 3.3.1 shows the

percentage of proteins extracted by different solvents that were extruded at 35% moisture or 60% moisture with three different product temperatures. There was a significant drop in protein solubility after extrusion, as shown in Fig. 3.3.1. This might be due to the formation of new chemical bonds of which participated in the aggregation forming texture and fibrous structure that were not soluble to the solvents used. Thus, many researchers suggested that these polypeptide chains of soy protein isolate form three dimensional network during extrusion (Jeunink and Cheftel 1979; Hager 1984; Prudencio-Ferreira and Areas 1993). The phosphate buffer solution (PBS) that was known to extract proteins in their native states had the lowest protein solubility in both the control and the extrudates. This was because when soybeans underwent through a series of processing steps to produce soy protein isolates, water soluble proteins and low molecular weight proteins were removed. By subtracting the percentage of soluble native proteins out of all other solvents used in both control and extrudates, the results (Table 3.3.2) might provide a rough estimation with respect to the percentage of various chemical bonds that were accessible by solvents and contributed to the fibrous extrudate texture. Although these percentages differed between extrudates from low and high moisture extrusion, the main difference was the amount of protein extracted by PBS

which lowered from 43.9% to an average of 4.2%. This indicates that the main reason for the significant drop from control (no extrusion) to the extrudates was due to the denaturation of native proteins due to their participation in the aggregation and texturization of extrudates.

Protein solubility increased with the combinations of PBS and another solvent, suggesting that more than one kind of chemical bonds contributed in the proteins of control and extrudates. Of all six solvents used, the amount of proteins extracted from the combination of urea and 2-mercaptoethanol was the highest for both low moisture extrusion and high moisture extrusion. The second highest is the combination of SDS and urea. These observations were similar to many others indicating that the forces responsible for the formation of protein network were mainly disulfide bond, hydrogen bonds, and noncovalent bonds (Jeunink and Cheftel 1979; Prudencio-Ferreira and Areas 1993; Lin and others 2000; Liu and Hsieh 2007).

The increase of product temperature had no significant effect on protein solubility at both low moisture extrusion and high moisture extrusion shown in Table 3.3.3. The data also show that temperature and the moisture used for extrusion did not have any interactions on the effect of protein solubility of the extrudates. Lin (2000) examined soy

protein isolate samples extruded at 137.8, 148.9 and 160ºC and found that only moisture content had a significant effect on protein solubility; barrel temperature and product temperature did not. However, Prudencio-Ferreira and Areas (1993) found protein solubility of soy protein isolate extruded at 140ºC, 160ºC and 180ºC differed significantly. The difference in their findings may be due Prudencio-Ferreira and Areas (1993) used a larger extrusion temperature range (40ºC) than that used by Lin (2000) and in this study (22.2ºC).

Significant difference of protein solubility between low moisture extruded samples and high moisture extruded samples were found in protein extracted by PBS, 2-ME and SDS+Urea (Table 3.3.4). Protein solubility of high moisture extrusion was higher than low moisture extrusion for PBS and 2-ME; and low moisture extrusion was higher than of high moisture extrusion for SDS+Urea. This suggests that there were slightly more native protein and disulfide bonds and less hydrogen bonds, hydrophobic interactions and ionic interactions in high moisture extruded samples than low moisture extruded samples. As for urea, 2-ME+Urea and SDS there were no significant differences in the amount of protein extracted by each solvent. These data suggest that although low moisture and high moisture extrudates differed in appearances, the protein to protein interactions that

contributed to the texturization were of the same kind.

Table 3.3.1 Percentage of protein extracted with different solvents.

Solvents	Control	Moisture content (%)					
		35			60		
		Product temperature (ºC)					
		125.3	134	140.6	125.3	134	140.6
PBS[1]	43.9 ± 2.2	3.4 ± 0.3	4.1 ± 0.6	4.1 ± 0.6	4.8 ± 0.5	4.2 ± 0.3	4.4 ± 1.0
Urea[2]	71.5 ± 13.8	20.0 ± 1.8	21.3 ± 0.3	20.8 ± 1.6	20.4 ± 4.1	17.6 ± 3.3	17.9 ± 1.7
2-ME[3]	58.5 ± 5.8	12.8 ± 0.4	13.5 ± 1.2	12.7 ± 1.1	20.1 ± 1.3	18.1 ± 0.5	18.6 ± 1.8
2ME+Urea[4]	81.6 ± 12.8	38.7 ± 11.9	40.4 ± 12.9	35.4 ± 13.6	35.1 ± 4.8	34.0 ± 8.5	35.8 ± 3.9
SDS[5]	63.2 ± 13.6	12.8 ± 1.4	13.8 ± 1.2	13.2 ± 1.1	15.6 ± 2.7	13.3 ± 2.1	13.8 ± 1.2
SDS+Urea[6]	60.9 ± 12.3	25.8 ± 3.8	26.9 ± 2.6	24.0 ± 0.9	21.6 ± 4.1	21.4 ± 4.1	23.0 ± 2.0

[1]0.035 M, pH 7.6 phosphate buffer solution; [2]8 M urea in the phosphate buffer solution; [3]2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution; [4]8 M urea + 2% 2-ME in the phosphate buffer solution; [5]1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution; [6]8 M urea + 1.5% SDS in the phosphate buffer solution.

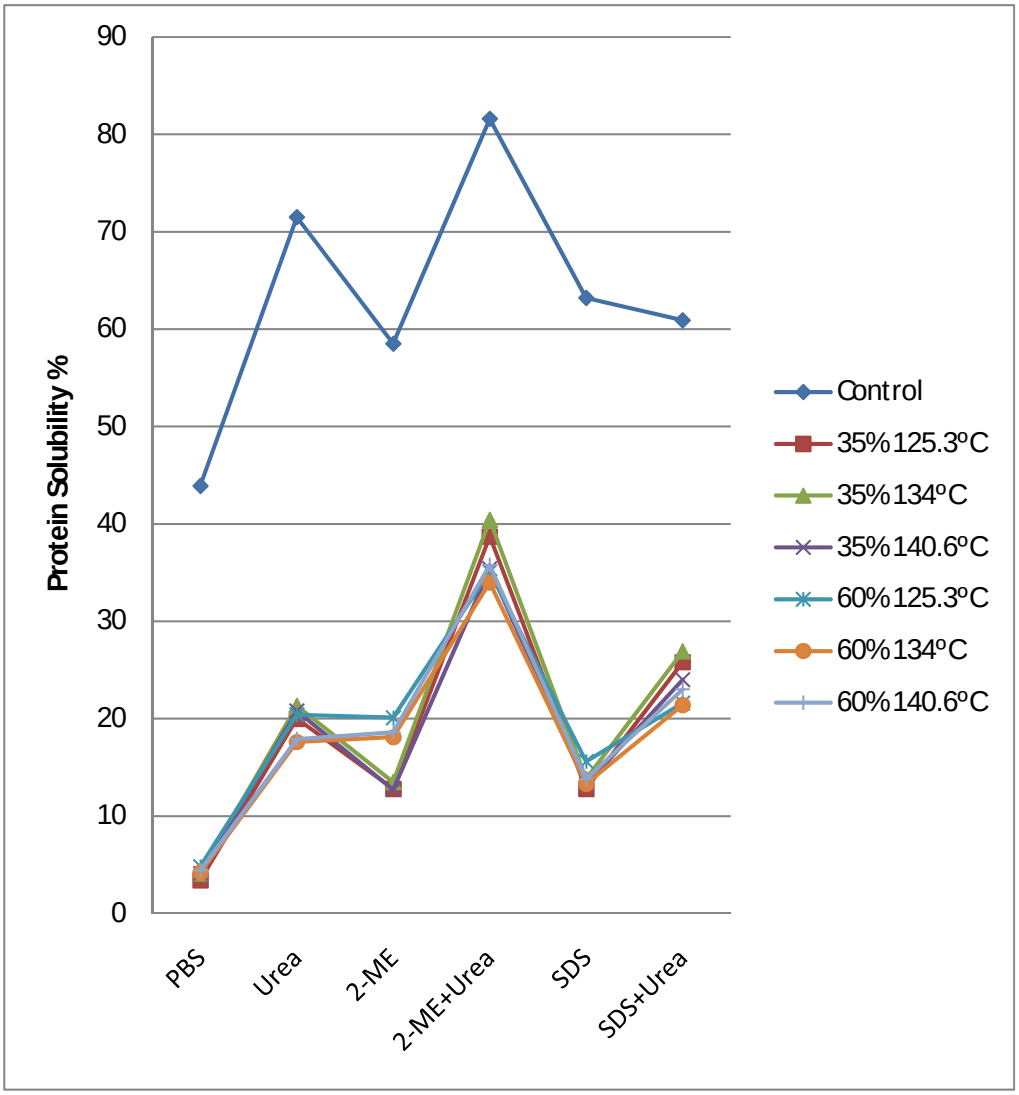


Fig 3.3.1 Comparison protein solubility of extrudates. PBS= 0.035 M, pH 7.6 phosphate buffer solution; 2-ME+Urea= 8 M urea + 2% 2-ME in the phosphate buffer solution; Urea= 8 M urea in the phosphate buffer solution; 2-ME= 2% 2-mercaptoethanol in the phosphate buffer solution; SDS+Urea = 8 M urea + 1.5% SDS in the phosphate buffer solution; SDS= 1.5% sodium dodecyl sulphate in the phosphate buffer solution.

Table 3.3.2 Protein solubility due to specific chemical bonds.

	Control	Low	High
Native protein (%)	43.9	3.9	4.5
Protein soluble due to hydrogen bonds and hydrophobic interactions (%)	27.6	16.8	14.2
Protein soluble due to disulfide bonds (%)	14.6	9.1	14.5
Protein soluble due to hydrogen bonds, hydrophobic interactions and disulfide bonds (%)	37.7	34.3	30.5
Protein soluble due to hydrophobic and ionic interactions (%)	19.3	9.4	9.8
Protein soluble due to noncovalent bonds (%)	17	21.7	17.5

Table 3.3.3 Temperature effect on the protein solubility with different solvents.

Solvent / Temp.	PBS[1]	Urea[2]	2-ME[3]	2ME+Urea[4]	SDS[5]	SDS+Urea[6]
Control	43.9 ± 2.2	71.5 ± 13.8	58.5 ± 5.8	81.6 ± 12.8	63.2 ± 13.6	60.9 ± 12.3
125.3ºC	4.2 ± 0.8	20.2 ± 3.1	16.8 ± 4.0	36.7 ± 8.2	14.4 ± 2.6	23.4 ± 4.3
134ºC	4.2 ± 0.4	19.5 ± 2.9	15.8 ± 2.6	37.2 ± 10.7	13.5 ± 1.6	24.1 ± 4.3
160ºC	4.2 ± 0.8	19.4 ± 2.2	15.6 ± 3.4	35.6 ± 9.3	13.4 ± 1.0	23.5 ± 1.5

[1]0.035 M, pH 7.6 phosphate buffer solution; [2]8 M urea in the phosphate buffer solution; [3]2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution; [4]8 M urea + 2% 2-ME in the phosphate buffer solution; [5]1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution; [6]8 M urea + 1.5% SDS in the phosphate buffer solution.

Table 3.3.4 Moisture effect on the protein solubility with different solvents.

Solvent / Moisture	PBS[1]	Urea[2]	2-ME[3]	2ME+Urea[4]	SDS[5]	SDS+Urea[6]
Control	43.9[a] ± 2.2	71.5[a] ± 13.8	58.5[a] ± 5.8	81.6[a] ± 12.8	63.2[a] ± 13.6	60.9[a] ± 12.3
35%	3.9[b] ± 0.6	20.7[b] ± 1.4	13.0[b] ± 1.0	38.2[b] ± 11.8	13.2[b] ± 1.1	25.6[b] ± 2.7
60%	4.5[c] ± 0.7	18.8[b] ± 3.3	19.0[c] ± 1.5	35.0[b] ± 5.5	14.3[b] ± 2.2	22.0[c] ± 3.3

1. [1]0.035 M, pH 7.6 phosphate buffer solution; [2]8 M urea in the phosphate buffer solution; [3]2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution; [4]8 M urea + 2% 2-ME in the phosphate buffer solution; [5]1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution; [6]8 M urea + 1.5% SDS in the phosphate buffer solution.
2. [a-c]Within each column, values with the same superscript were not significantly different at $p<0.05$.

3.3.2 Protein Solubility of Dead Stop Procedure

Dead stop procedure was performed to investigate the changes in protein solubility during extrusion. The control was fed through the hopper, mixed with water, and pushed by screws through the extruder while heat and shear were applied. The protein and starch in the feed mix were mixed with water at room temperature in zone 2 (Table 3.3.5). The protein solubility was significantly lowered after being mixed with water in the less penetrative solvents (PBS, 2-ME, SDS and SDS+Urea) (Table 3.3.6 and Fig. 3.3.2). This

might be due to the physical barrier or the hydrophobic interactions that formed as a result of the insufficient water mixed with the feed. The same effect occurred in zone 3 where the feed was heated to 37.8ºC. As the feed passed through zone 4 it was gradually heated to 96.1ºC (Table 3.3.5), as a result, proteins were denatured and protein solubility significantly decreased in all solvent combinations except the SDS+Urea.

From zone 4 to zone 6, there was no significant texture formation but a dough liked protein mixture. The cooling die and the final product had fibrous textures and no difference in protein solubility in all solvents used. Noguchi (1989) reported that the result of high moisture protein melt forced through the cooling die, where proper cooling is applied, is the alignment of proteins due to directional shear force. However, protein solubilities in the PBS, and PBS plus Urea, 2-ME or SDS were not significantly different from zone 4 on to the product. This suggests that during zone 3 and 4, the soy protein isolate formed a network of polypeptide chains that were not soluble to the solvents used, though the texture and fibrous structure was not formed yet. For the combination of 2ME+Urea there was a significant drop in protein solubility at zone 4 and increased in zone 5 and zone 6 (Table 3.3.6). This could be due to the reverse paddles in zone 5 resulting in a slightly longer residence time of the feed in zone 4.

Table 3.3.5 Temperature and screw settings of the dead stop procedure.

	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Zone 6
Set temperature (ºC)	22.9	24.4	42.1	96.3	136.1	137.8
Product temperature (ºC)	21.9	22.8	37.8	96.1	106.7	124.2
Screws configuration (mm)	100 TL[a] 25 FP[b]	25 FP 100 SL[c]	87.5 FP 37.5 SL	125 SL	12.5 SL 87.5 FP 25 RP[d]	25 RP 100 SL

[a]twin lead feed screws; [b]30º forward paddles; [c]single lead feed screws; [d]30º reverse paddles.

Table 3.3.6 Dead stop procedure extruded at 137.8ºC with 60% moisture content.

Solvents / Location	PBS[1]	Urea[2]	2-ME[3]	2ME+Urea[4]	SDS[5]	SDS+Urea[6]
Control	43.9^{d} ± 2.2	71.5^{c} ± 13.8	58.5^{c} ± 5.8	81.6^{e} ± 12.8	63.2^{c} ± 13.6	60.9^{d} ± 12.3
Zone 2	9.0^{c} ± 2.4	60.0^{bc} ± 12.4	49.3^{b} ± 6.6	70.5^{de} ± 11.2	38.3^{b} ± 10.1	43.0^{c} ± 8.5
Zone 3	7.9^{bc} ± 1.4	57.1^{b} ± 14.8	46.9^{b} ± 7.2	70.7^{de} ± 11.5	33.4^{b} ± 17.1	37.8^{bc} ± 15.8
Zone 4	4.2$_{a}$ ± 0.6	30.9^{a} ± 8.1	20.7^{a} ± 2.8	43.5^{ab} ± 10.4	15.6^{a} ± 5.3	30.8^{abc} ± 14.5
Zone 5	4.8^{a} ± 0.3	29.4^{a} ± 5.7	20.9^{a} ± 1.8	59.8^{cd} ± 10.1	15.1^{a} ± 6.6	38.4^{bc} ± 17.4
Zone 6	6.1^{ab} ± 1.0	27.1^{a} ± 3.2	21.7^{a} ± 1.5	51.9^{bc} ± 15.2	14.6^{a} ± 5.0	25.3^{ab} ± 10.7
Cool Die	5.1^{a} ± 0.4	24.6^{a} ± 2.4	21.2^{a} ± 1.9	32.7^{a} ± 7.2	12.3^{a} ± 3.6	19.0^{a} ± 8.7
Product	4.8^{a} ± 0.5	20.4^{a} ± 4.1	20.1^{a} ± 1.3	35.1^{a} ± 4.8	15.6^{a} ± 2.7	21.6^{a} ± 4.1

1. [1]0.035 M, pH 7.6 phosphate buffer solution; [2]8 M urea in the phosphate buffer solution; [3]2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution; [4]8 M urea + 2% 2-ME in the phosphate buffer solution; [5]1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution; [6]8 M urea + 1.5% SDS in the phosphate buffer solution.
2. [a-e]Within each column, values with the same superscript were not significantly different at $p<0.05$.

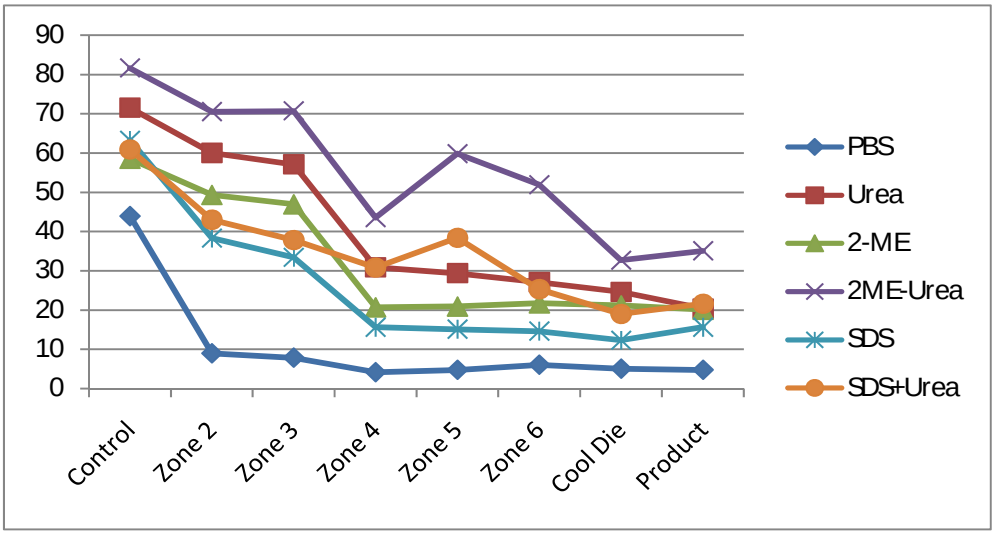


Fig. 3.3.2 Dead stop procedure extruded at 137.8ºC with 60% moisture content. PBS= 0.035 M, pH 7.6 phosphate buffer solution; Urea= 8 M urea in the phosphate buffer solution; 2-ME= 2% 2-mercaptoethanol in the phosphate buffer solution; 2-ME+Urea= 8 M urea + 2% 2-ME in the phosphate buffer solution; SDS= 1.5% sodium dodecyl sulphate in the phosphate buffer solution; SDS+Urea = 8 M urea + 1.5% SDS in the phosphate buffer solution.

3.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Low Moisture Extrusion and High Moisture Extrusion

The samples from the protein solubility tests of low moisture extrusion, high moisture extrusion and dead stop procedure were used in electrophoresis in the presence of SDS and 2-ME to observe the difference in the molecular weight distribution. The addition of SDS and 2-ME would not affect protein subunits since the proteins that were soluble to solvents without 2-ME were those with non-disulfide linkages and would not

be affected by the reducing environment introduced by adding SDS and 2-ME. The samples from the protein solubility tests were diluted into different concentrations according to the solvents used to get sharp and visible bands. These bands could provide qualitative characteristics of the soy protein subunits present in the samples, but not quantitative characteristics. Protein subunits of the extrudates extracted by PBS were very low, even without any dilution it showed very low intensity on the SDS-PAGE (Fig 3.3.3 (I)-(III) lane A and Fig. 3.3.4 (I)). This result was in agreement with the findings of the protein solubility test that most of the native proteins were denatured and participated in the aggregation and texturization of the extrudates. Only a small amount of protein was present under 21.5kD due to the processing steps used to produce soy protein isolates and remove water soluble proteins and low molecular weight proteins.

A comparison of Fig 3.3.3 (I)-(III) to (IV) shows that before extrusion, the bands were identical despite different solvents were used to extract soluble proteins. Because PBS extracted only native proteins, this suggests that the protein subunits were not aggregated or linked together by chemical bonds and existed in their native state. After extrusion there was a difference in the protein subunit distribution especially the protein subunits extracted by 2-ME (Fig 3.3.3 (I)-(III) Lane C and Fig 3.3.4 (III)). The band at

34-44kD which was more pronounced was the acidic polypeptides of the 11S fraction (Liu 1997; Hua and others 2005). When using the solvent system of PBS+2-ME+Urea, the protein subunit molecular weight distribution was almost identical to the control (Fig 3.3.3 (I)-(III) Lane D and Fig 3.3.3 (IV)). These observations are consistent with the protein solubility tests findings that during extrusion, by forming disulfide bonds, hydrogen bonds and hydrophobic interactions, protein subunits participated in the aggregation and texturization. Similar results have been reported (Jeunink and Cheftel 1979; Noguchi and others 1981).

All three extrusion temperature had the same distribution of protein subunits (Fig 3.3.3 and Fig 3.3.4), indicating that the extrusion temperature did not have a significant effect on the protein subunits extracted from the protein solubility test. Fig 3.3.4 also shows that the extrusion moisture content did not change the protein subunit distributions of the extrudates. With these observations and the statistical analysis of protein solubility, one could conclude that changes in product temperature from 125.3 to 140.6ºC did not have any effect on the chemical bonds contributing to texturization of the extrudates. Although moisture content did have a significant effect on the amount of chemical bonds, it did not affect the proteins at a molecular level.

The protein subunits of the dead stop procedure showed essentially no significant difference in distribution (Fig 3.3.5). The only difference was the distribution of subunits in the proteins extracted by 2-ME (Fig 3.3.5 (III) lane 8-5 to lane 4-1), the disappearance of the β subunits of the 7S globulin which has a molecular weight at 42kD and the increase of acidic polypeptides of the 11S fraction (Hua and others 2005). When comparing the extrudate with the control, the differences shown in protein subunits extracted by 2-ME indicates the importance of noncovalent bonds contribution to aggregation and extrudate texturization. The distribution of subunits extracted by other solvents showed no differences except the concentrations of proteins, affirming the fact that the protein subunits participated in the aggregation and texturization of the extrudates.

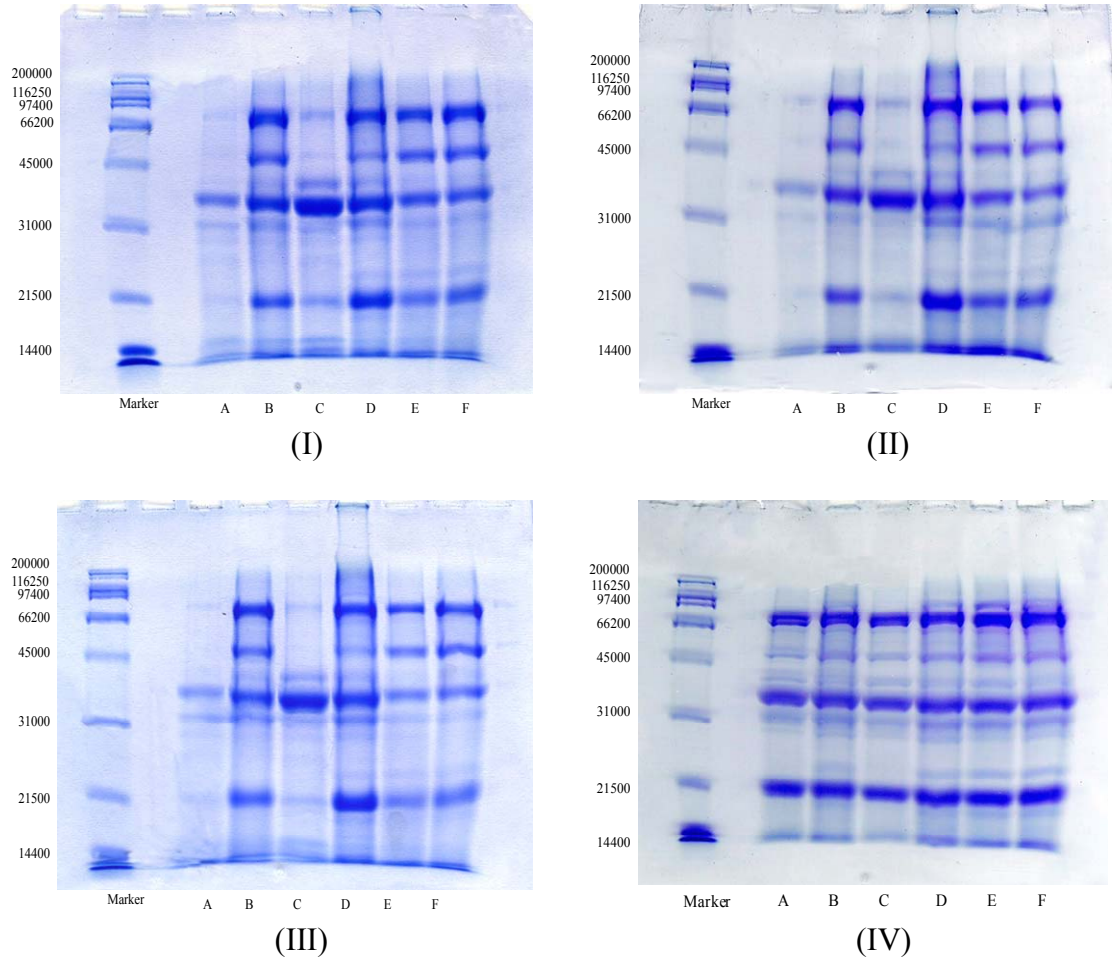


Fig. 3.3.3 SDS-PAGE of samples extruded with 60% moisture content. A-F protein extracted by PBS, Urea, 2-ME, 2ME+Urea, SDS, SDS+Urea, respectively. (I): samples of product temperature 125.3ºC; (II): samples of product temperature 134ºC; (III): samples of product temperature 140.6ºC; (IV): samples of control.

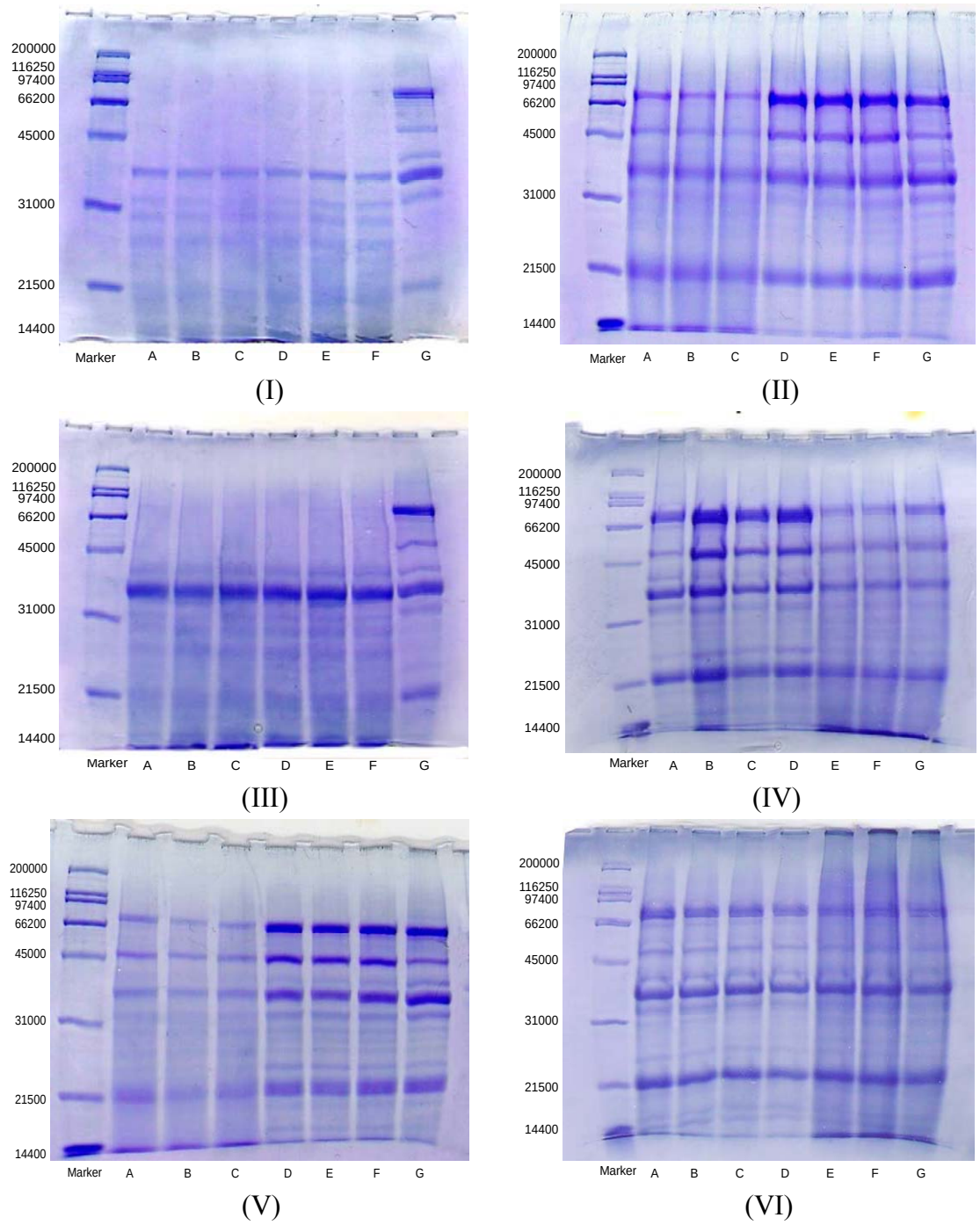


Fig. 3.3.4 SDS-PAGE of samples extruded with 35% and 60% moisture content. (I)-(VI) protein extracted by PBS, Urea, 2-ME, 2ME+Urea, SDS, SDS+Urea, respectively. (A): product temperature 125.3ºC with 35% moisture; (B): product temperature 134ºC with 35% moisture; (B): product temperature 140.6ºC with 35% moisture; (D): product temperature 125.3ºC with 60% moisture; (E): product temperature 134ºC with 60% moisture; (F): product temperature 140.6ºC with 60% moisture; (G): Control

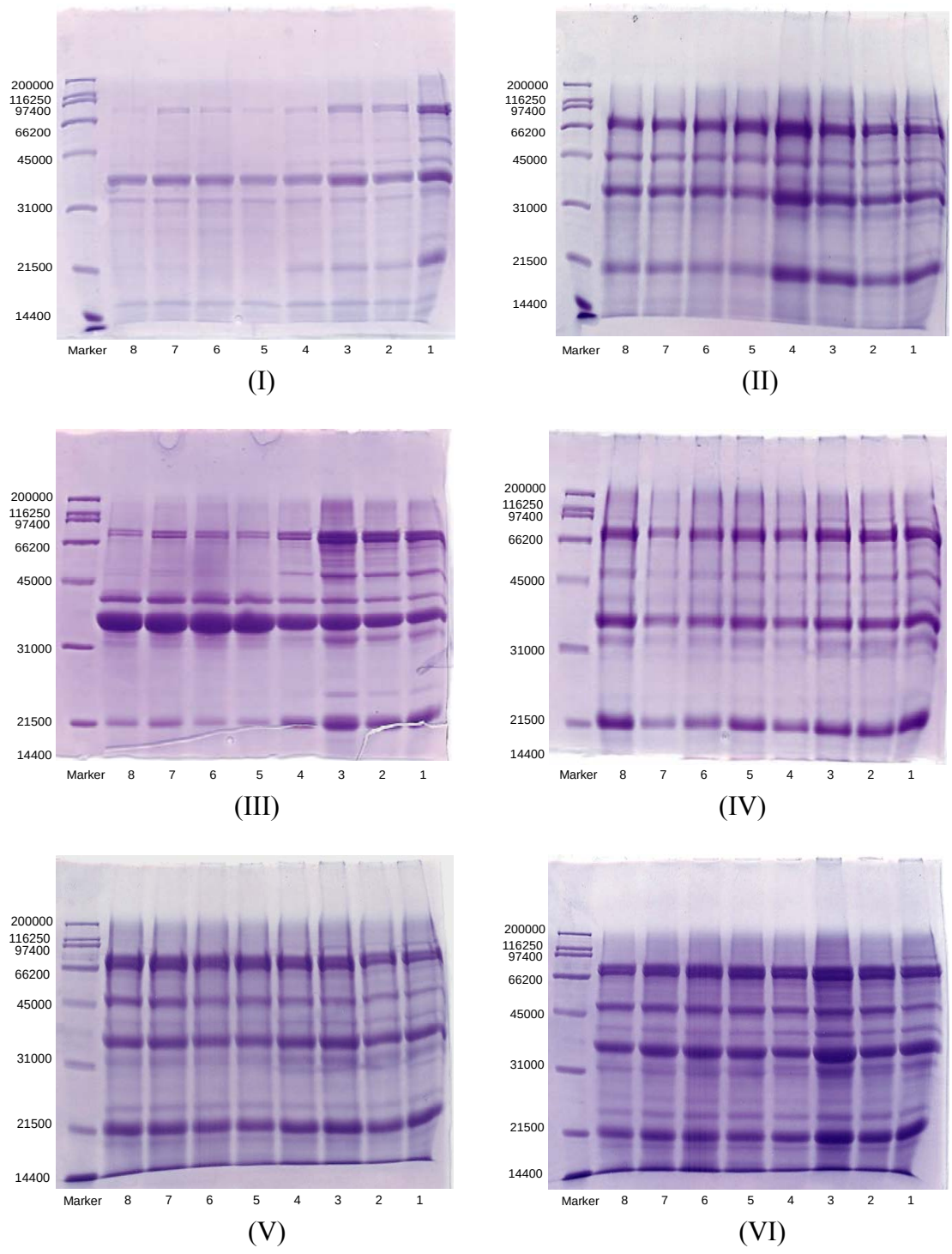


Fig. 3.3.5 SDS-PAGE of samples extruded at 125.3ºC product temperature with 60% moisture content. (I)-(VI) protein extracted by PBS, Urea, 2-ME, 2ME+Urea, SDS, SDS+Urea, respectively. 8-1 are samples from cooling die, samples from zone 6-2 and the control.

3.4 Conclusion

In both low moisture and high moisture extrusion, increasing product temperature from 125.3 to 140.6°C did not have significant difference on proteins that could be extracted by various solvents. The moisture content did have significant affect on the protein solubility, however no difference in protein subunit distribution was observed. In the dead stop experiment, the results suggest that the network of polypeptide chains interfered and lowered the protein solubility were formed in zone 3 and zone 4. This led to little difference in the protein solubility of after zone 4 of extruder barrel to the final product. The chemical bonds that contributed to the texturization of proteins were the same through zone 4 to the product and proteins were aligned by the directional shear force at the cooling die. Therefore, the major drop in protein solubility after low moisture extrusion, high moisture extrusion and that of zone 3 and 4 in the dead stop procedure, was due to the formation of the three dimensional network of soy protein isolate polypeptide chains. These polypeptide chains aggregated together during extrusion and became less accessible to the solvents used to extract the soluble proteins.

Despite differences in low and high moisture extrusions the solvent combination of PBS+2-ME+Urea extracted the proteins most. This was followed by the solvent

combination of PBS+SDS+Urea. In addition, there was no significant change in the molecular weight distribution of protein subunits between the control and extrudates. Based on the results from protein solubility and SDS-PAGE, It appears that the effect of extrusion is to disassemble proteins and then reassemble them together by disulfide bonds, hydrogen bonds and noncovalent interactions forming fibrous structure in extrudates, Despite major difference in appearance, similar protein to protein interactions occurred in both low moisture and high moisture extrusion.

The suggestion by Burgess and Stanley (1976) that intermolecular peptide bonds were the main contributor to protein texturization is questionable since breaking and forming of peptide bonds only occurs at high critical temperatures or very high or low pHs. There was little evidence that peptide bonds could be formed, broken, and formed again during high moisture extrusion cooking at moderate temperatures. If a significant amount of cross-linking exists, the protein subunit of the SDS-PAGE should exhibit a different distribution between the extrudate and the control. Therefore, it appears that the extrusion of soy protein isolates modified very little at the molecular level and that the protein subunits did not have any structural changes.

CHAPTER 4

EFFECT OF REPEATED HIGH MOISTURE EXTRUSION ON PROTEIN-PROTEIN INTERACTIONS OF SOY PROTEIN ISOLATE

4.1 Introduction

Soy protein has been known as an abundant and cost competitive source of protein ever since it was noticed in the 1930s. The steady increase in soybean production and the advances in soy protein process technology give soy protein a broader and more versatile utilization in human foods (Snyder and Kwon 1987; Hettiarachchy and Kalapathy 1997; Liu 1997). In recent years researchers also keep discovering the benefits of consuming soy protein in substitute of animal proteins such as decreasing total serum cholesterol and decreasing the risks for several cancers (Messina and Barnes 1991; Anderson and others 1995; Messina 1997). These advantages let soy protein perform many functions in foods while maintaining their excellent nutritional quality and benefits to human health. Therefore, food industry and researchers have placed increased efforts in the development of foods containing soy proteins that are acceptable to the general public (Faller and others 1999; Drake and others 2000; Friedeck and others 2003). As a result of soy proteins' versatility and abundant advantages, food products incorporated with soy

proteins have been widely used and accepted in virtually every food system. One notable development out of the numerous efforts for soy-based foods acceptable to the western community is the texturization of soy protein into meat analogs (Atkinson 1970; Rhee and others 1981; Snyder and Kwon 1987).

Researchers have been investigating the potential of using high moisture extrusion to improve the texturized vegetable protein for the last 20 years (Noguchi 1989; Cheftel and others 1992; Akdogan 1999). When soy protein is extruded under high pressure and high temperature conditions at a moisture lower than 35%, the sudden release of pressure upon exiting from the die causes instant water evaporation from the extrudates. This creates expanded and spongy structure of common texturized vegetable protein. To reduce extruder die pressure and extrudate expansion, soy protein needs to be extruded at a higher moisture content (>50%). In addition, a cooling die is essential in high moisture extrusion to increase the viscosity of the hot melt and reduce its fluidity so the necessary pressure and temperature can be maintained. When proper cooling is applied, high moisture protein melt forced through the cooling die is the alignment of proteins due to directional shear force producing a fibrous chicken meat like texture (Noguchi 1989).

While texturization of soy proteins is being developed and applied in the food

industries, it is also important to know whether texturized soy protein products could be ground and texturized again. This is because it is very common to blend up to 15% of used ingredients, made of products that do not meet the specifications or that from start-up or shut-down operations, with 85% of new ingredients. The purpose is to lower the ingredient cost. Isobe and Noguchi (1987) extruded defatted soy flour at 60% moisture with the barrel temperature setting at 130, 140 and 150°C. The extrudates were cut into small pieces and extruded two more times. The results suggested that multiple extrusions had little effect on the extrudates. Literature was scarce regarding the effects of multiple extrusions, in particular, their effect on the chemical bonds and texturization of extrudates. Therefore, the objectives of this study were to investigate the effects of repeated high moisture extrusion on extrudate's color, texture, microstructure, and protein to protein interactions.

4.2 Materials and Methods

4.2.1 Materials

Soy protein isolate (SPI) (Profam 974) was obtained from Archer Daniels Midland (Decatur, IL) containing a minimum 90% w/w protein. Starch (Midsol 50) was provided

in gratis by MGP Ingredients, Inc. (Atchison, KS). The ingredients were mixed in 9:1 ratio using a Double Action™ food mixer (Model 100DA70, Leland Southwest, Fort Worth, TX) for 10 min to ensure the uniformity of the feeding material.

4.2.2 High Moisture Extrusion

An MPF 50/25 co-rotation intermeshing twin-screw extruder (APV Baker, Inc., Grand Rapids, MI) was used. The extruder has a screw length to diameter ratio of 15 to 1 and the diameter is 50 mm. A cooling die with dimensions (W × H × L) of 30 × 10 × 300 mm was attached at the end of the extruder with 4.4ºC cold water as the cooling media. The screw profile from feed to exit were 100 mm twin lead feed screws, 50 mm 30º forward paddles, 100 mm single lead feed screws, 87.5 mm 30º forward paddles, 175 mm single lead feed screws, 87.5 mm 30º forward paddles, 50 mm 30º reverse paddles, 100 mm single lead feed screws and finally the cooling die. The barrel was divided into six sections and the barrel temperature settings from zone 1 to zone 6 were 22.9, 24, 42.1, 96.3, 136.1 and 137.8ºC, respectively. Other independent extrusion variables are listed in Table 4.2.1. All samples were sealed and stored at -20ºC for further analysis.

4.2.3 Second High Moisture Extrusion

The second extrusion was performed to investigate its effect on the product properties and on protein solubility. Extrudates were collected from high moisture extrusion conducted at 137.8ºC zone 6 temperature and 60% moisture with variables as shown in Table 4.2.1. About 40 kg of extrudate was collected. The extrudate was ground (FitzMill, The Fitzpatrick Co., Elmhurst, IL) and passed through a No. 28 mesh screen (Tyler Standard Screen Scale, W.S. Tyler, Mentor, OH). The ground extrudates were mixed with starch at a 9 to 1 ratio and extruded again at 137.8ºC and 60% moisture with the same variables. Samples were sealed and stored at -20ºC until further analysis.

Table 4.2.1 Experimental design for high moisture extrusion.

Conditions	Levels
Moisture content	60% w.b.
Screw speed	200 rpm
Water feed rate	12.2 kg/h (26.8 lb/h)
Dry feed rate	9.1 kg/h (20 lb/h)
No. of Replications	2
Formula	90% SPI, 10% wheat starch

4.2.4 Color Analysis

The Konica Minolta chromameter (Model CR-410, Konica Minolta, Inc., Mahwah, NJ) was used to determine the color properties of the extrudates after the first and second extrusion. The chromameter was standardized by a white chromatic reference tile. The values of the reference tile were L*=96.95, a*=0.15, and b*=1.92. The chromameter was placed directly above the samples and 3 measurements were taken. The Konica Minolta chromameter gives three different color units − L*, a* and b*. The L* value is from 0 for darkness to 100 for brightness. The a* represents redness and the b* corresponds to yellowness.

4.2.5 Scanning Electron Microscopy (SEM)

SEM was performed to view the microstructures of the products that have been extruded once and twice with extrusion temperature of 137.8ºC and 60% moisture. Samples were defrosted then stripped into small pieces by hand. Primary fixation, OsO_4 post fixation and dehydration were done by microwave processing (Pelco® EM microwave vacuum chamber, Ted Pella, Inc., Redding, CA). Both primary fixation and OsO_4 post fixation were done by 1 min vacuum without power, 40 s microwave at 120

W, 3 min vacuum without power, 40 s microwave at 350 W and finally three times 5 min buffer rinses at room temperature with the primary fix; and three times 5 min Milli-Q water rinses at room temperature with the OsO_4 post fix. Dehydration was preformed in seven steps of microwave with 20%, 50%, 70%, 90% and 3 times 100% of ethanol, each step for 40 s at 120 W. Critical point drying was operated with Autosamdri®-815 automatic critical point dryer (Tousimis, Rockville, MD). After the samples were mounted on stubs with silver glue they were sputtered coated with Platinum. SEM pictures were taken by a Cold Field Emission Scanning Electron Microscope (S-4700, Hitachi, Schaumburg, IL).

4.2.6 Protein Solubility

Protein solubility was tested on soy protein isolate with 10% starch (control) and extruded products from the first and second extrusion. The following were six solvents used in this study: 1) 0.035 M, pH 7.6 phosphate buffer solution (PBS) (known to extract proteins in their native state); 2) 8 M urea in the phosphate buffer solution (known to dissolve the proteins with hydrogen bonds and hydrophobic interactions); 3) 2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution (known to disrupt the

disulfide bonds); 4) 8 M urea + 2% 2-ME in the phosphate buffer solution; 5) 1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution (used for their ability to interrupt hydrophobic and ionic interactions); and 6) 8 M urea + 1.5% SDS in the phosphate buffer solution. All phosphate buffer solutions from 2 to 6 were the same as the first one. All chemicals were of reagent grade and obtained from Fisher Scientific (Fair Lawn, NJ).

Extruded samples were defrosted and finely chopped with a blender to approximately 3 mm cubes. One gram of chopped sample was weighed in duplicate and dried in a vacuum oven at 103ºC overnight to determine the moisture content. Twenty ml of six different solvents mentioned above were used to extract 1 g of defrosted and chopped sample or 0.5 g of the soy protein isolate and starch mix (control). Each sample in duplicate was slowly added and well-mixed into 20 ml of solvent. Solutions containing the sample were placed into a water bath set at 40ºC and shaked at 100 rpm for 2.5 h. After extraction, the solutions were centrifuged at 12,500 rpm (10000×g) in a centrifuge (Beckman J2-21M/E, Schaumburg, IL) for 30 min. Protein contents of all solutions, except for samples extracted by sodium dodecyl sulphates, were determined with Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL) at 595 nm based on the

Bradford method. The protein contents for samples extracted by sodium dodecyl sulphates were measured with the BCA Protein Assay Kit (Pierce, Rockford, IL) at 560 nm. A microplate reader (Bio-Rad, Hercules, CA) with standard curves that were made for each solvent was used to determine the protein concentration of all solutions.

4.2.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to observe the differences in protein distribution. Samples were obtained from solutions in the protein solubility study. Twelve percent acryl amide gels (Mini-Protean II Ready Gel, Bio-Rad, Hercules, CA) were used to analyze the samples. Samples were prepared by mixing protein extract and sample buffer (Laemmli sample buffer, Bio-Rad, Hercules, CA) at 1:1 ratio, then heated at 95^{o}C for 5 min. Running buffer (Tris/Glycine/SDS, Bio-Rad, Hercules, CA) were added into the inner and outer chambers of the Mini-Protean II Cell (Bio-Rad, Hercules, CA) at approximately 270 ml. Twenty five μL of samples and 5 μL molecular weight marker (SDS-PAGE Standards, Broad Range, Bio-Rad, Hercules, CA) were loaded in each well. The electrophoresis was run for about 1.5 h with the power supplier (1000 V microprocessor power supply, Buchler, Lenexa, KS) set at 100 V, 50 mA, and 10 W.

Gels were stained by Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) for 1 h and destained by a destaining solution (Bio-Rad, Hercules, CA) for 2 h under rotation (Lab-Line, Barnstead International, Dubuque, IA). After destaining, gels were sealed by cellophane (Bio-Rad, Hercules, CA) and dried at room temperature 23ºC overnight. The bands from the samples were compared with the molecular weight markers (GE Healthcare, Piscataway, NJ) of 200, 116, 97.4, 66, 45, 31, 21.5 and 14.5 kD.

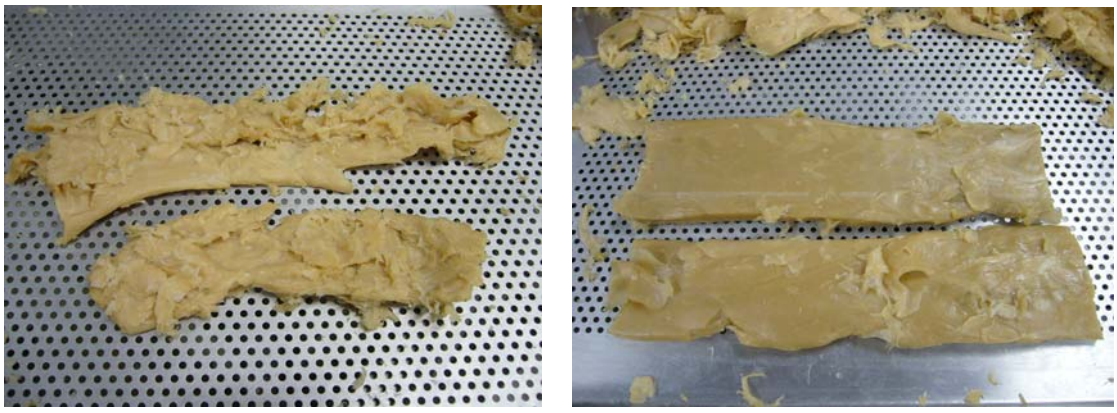
4.3 Results and Discussion

4.3.1 Physical Effects of Repeated High Moisture Extrusion

Fig. 4.3.1 (A) and (B) show the difference in the physical appearance between extrudates that were extruded once and twice. The color properties were determined by the chromameter, where L* being lightness, a* being redness, and b* being yellowness. Effects of repeated extrusion on color could be seen in Table 4.3.1. All three color properties were significantly affected by the second extrusion. It was darker, less redness and yellowness in extrudates that were extruded twice. This was probably due to the nonenzymatic browning (the Maillard reaction) which occurred with the participation of free or protein bound amino groups from soy protein isolate and carboxyl groups of

reducing sugar from wheat starch which was degraded to produce reducing groups during the extrusion process (Pham and Rosario 1984; Phillips 1989). The extrudates that had been extruded twice showed a more uniform outer structure with distinct fibrous layers within than the extrudates that were only extruded once. The latter had scattered layers and less structural fibers. Noguchi (1989) proposed that during extrusion, protein is melted then fused together into an elastic protein mass. When starch together with a large amount of water is incorporated into the feeding material, it will lead to a phase separation where protein domains are interrupted and protein-protein interactions are increased. Such phase separation with the help of shear force at the cooling die would induce the formation of proteinaceous fibrous structures (Harper 1989; Noguchi 1989). Isobe and Noguchi (1987) embedded extrudates that were extruded at 60% moisture and 130, 140 and 150ºC multiple times into a resin. They cut the resin into thin slices and staining them with Coomassie Brilliant Blue, a blue die that was sensitive to proteins, and found the fine structure of the protein matrix in the extrudates were about the same as that extruded only once. They also found a higher extrusion temperature lead to a stronger lengthwise breaking strength, and stated that other physical properties were influenced very little by repeated extrusion.

In this study changes in the microstructure of soy protein extrudates that had been extruded more than once were observed by scanning electron microscopy (SEM). Figs. 4.3.2 (A) and (B) (60× magnification) shows the extrudates that had been extruded once and twice, respectively. Both products had visible directional textures ─ the latter's fibrous structure was more smooth and organized whereas the former seemed rougher and less even. With a magnification of ten thousand (Figs. 3.3.2 (C) and (D)) the fibers that were extruded twice were more visible and distinct; whereas extrudates that were extruded only once had a less obvious fiber formation but more of a layered texture. The difference in texture between these two samples might be due to most protein subunits were broken apart at the first extrusion and realigned due to the directional shear force at the cooling die. When the second extrusion was applied, more protein subunits were broken and the realignment at the cooling die became more complete or easier. Therefore, fibers were more distinct.



(A) (B)

Fig. 4.3.1 Soy protein isolate and wheat starch mix (9:1) extruded with 60% moisture content. (A): Extruded once. (B): Extruded twice.

Table 4.3.1 Effect of repeated extrusion on color.

Solvents	L*	a*	b*
Extruded once	$61.4^{a} \pm 0.4$	$3.8^{a} \pm 4.8$	$15.2^{a} \pm 0.4$
Extruded twice	$57.1^{b} \pm 0.4$	$3.1^{b} \pm 3.7$	$13.5^{b} \pm 0.1$

[a-b]Within each column, values with the same superscript were not significantly different at $p<0.05$.

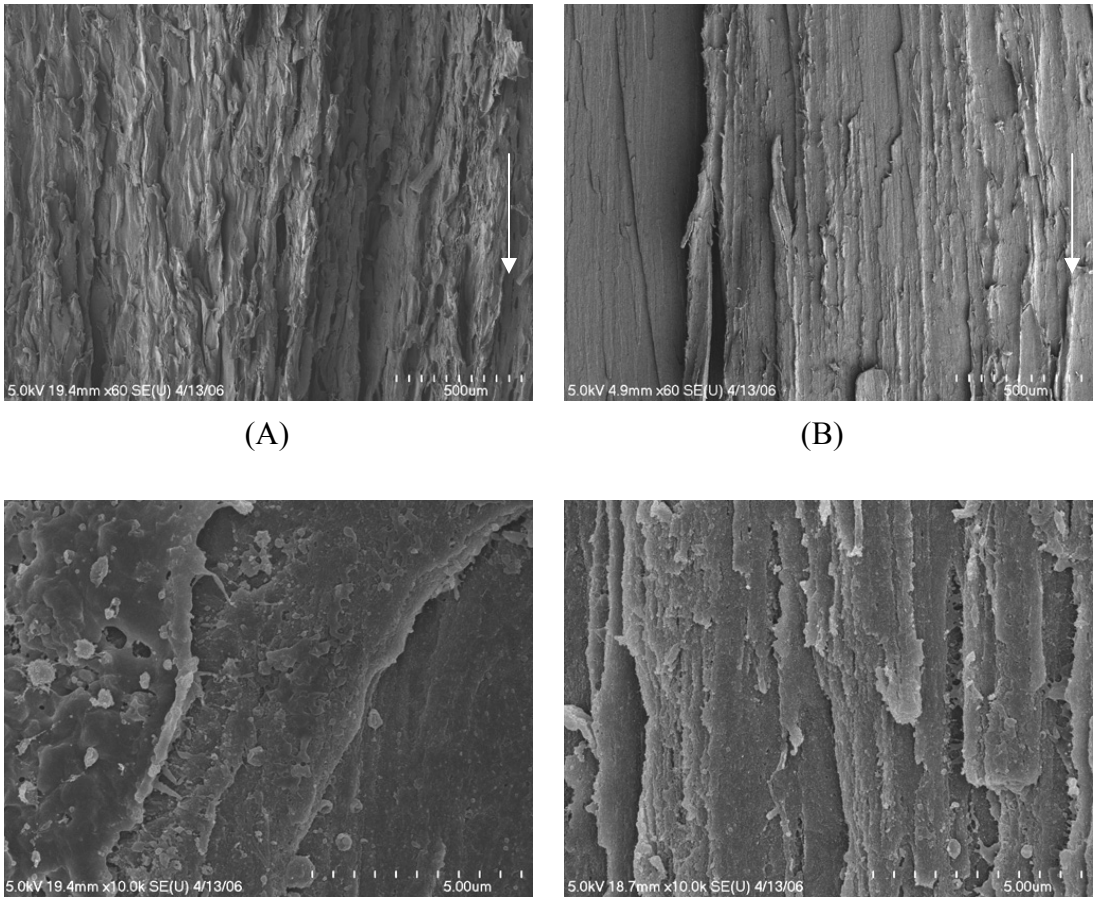


Fig. 4.3.2 Scanning electron micrographs of soy protein isolate and wheat starch mix (9:1) extruded with 60% moisture content. (A) Extruded once at 60× magnification; (B) Extruded twice at 60× magnification; (C) Extruded once at 10000× magnification; (D) Extruded twice at 10000× magnification. Arrows indicate the direction of extrusion.

4.3.2 Protein Solubility of Repeated High Moisture Extrusion

Protein solubility test was performed to investigate the forces responsible for the formation and stabilizing the texturization of extruded soy protein isolate. Table 4.3.2 shows the percentage of proteins extracted by different solvents after the first and second

extrusion at 60% moisture. There was a significant drop in protein solubility after extrusion as shown in Fig. 4.3.3. This might be due to the formation of new chemical bonds or cross-linkages of soy protein subunits which participated in the aggregation forming texture and fibrous structure that were not soluble to the solvents used. Thus, many researchers suggested that these polypeptide chains of soy protein isolate form a three dimensional network during extrusion (Jeunink and Cheftel 1979; Hager 1984; Prudencio-Ferreira and Areas 1993). The phosphate buffer solution (PBS) that was known to extract proteins in their native state had the lowest protein solubility in both control and the extrudates. This was because when soybeans underwent through a series of processing steps to produce soy protein isolates most water soluble proteins and low molecular weight proteins were removed. The proteins that were soluble to PBS from extrudate of first extrusion did not statistically differ from that of second extrusion. This indicates that almost all native proteins had been denatured and participated in the texturization in the first extrusion.

Protein solubility increased with the combinations of PBS and a second solvent, suggesting that more than one kind of chemical bonds presented in the proteins of control and extrudates. Of all six solvents used, the amount of proteins extracted from the

combination of urea and 2-mercaptoethanol was the highest. This suggests that the contribution of disulfide bonds plus hydrogen bonds and hydrophobic interactions for soy protein isolates were most important for both the control and extrudates. The second highest protein solubility for the control was the urea solvent which showed the importance of hydrogen bonds and hydrophobic interactions in the unextruded soy protein. Protein solubility results of first extrusion showed that urea, 2-ME and SDS plus urea had equal importance in the texturization of the product. These observations were similar to the results from many researchers that the forces responsible for the formation of protein network were mainly disulfide bonds, hydrogen bonds and noncovalent bonds (Jeunink and Cheftel 1979; Prudencio-Ferreira and Areas 1993; Lin and others 2000; Liu and Hsieh 2007). After the second extrusion, proteins that are soluble in the 2-ME+Urea and SDS+Urea significantly increased, indicating an increase of disulfide bonds, hydrogen bonds and noncovalent bonds. This increase might be responsible for the difference in texture between the products that were extruded once and twice as previously discussed.

Table 4.3.2 Effect of repeated extrusion at 137.8ºC and 60% moisture content on protein solubility in various solvents.

Solvents	PBS[1]	2ME+Urea[2]	Urea[3]	2-ME[4]	SDS+Urea[5]	SDS[6]
Control	43.9[a] ± 2.2	81.6[a] ± 12.8	71.5[a] ± 13.8	58.5[a] ± 5.8	60.9[a] ± 12.3	63.2[a] ± 13.6
Extruded once	4.8[b] ± 0.5	35.1[b] ± 4.8	20.4[b] ± 4.1	20.1[b] ± 1.3	21.6[b] ± 4.1	15.6[b] ± 2.7
Extruded twice	5.9[b] ± 0.8	49.5[c] ± 3.7	22.4[b] ± 0.9	20.0[b] ± 1.8	31.9[c] ± 3.4	16.2[b] ± 0.4

1. [1]0.035 M, pH 7.6 phosphate buffer solution; [2]8 M urea + 2% 2-ME in the phosphate buffer solution; [3]8 M urea in the phosphate buffer solution; [4]2% 2-mercaptoethanol (2-ME) in the phosphate buffer solution; [5]8 M urea + 1.5% SDS in the phosphate buffer solution; [6]1.5% sodium dodecyl sulphate (SDS) in the phosphate buffer solution.
2. [a-c]Within each column, values with the same superscript were not significantly different at $p<0.05$.

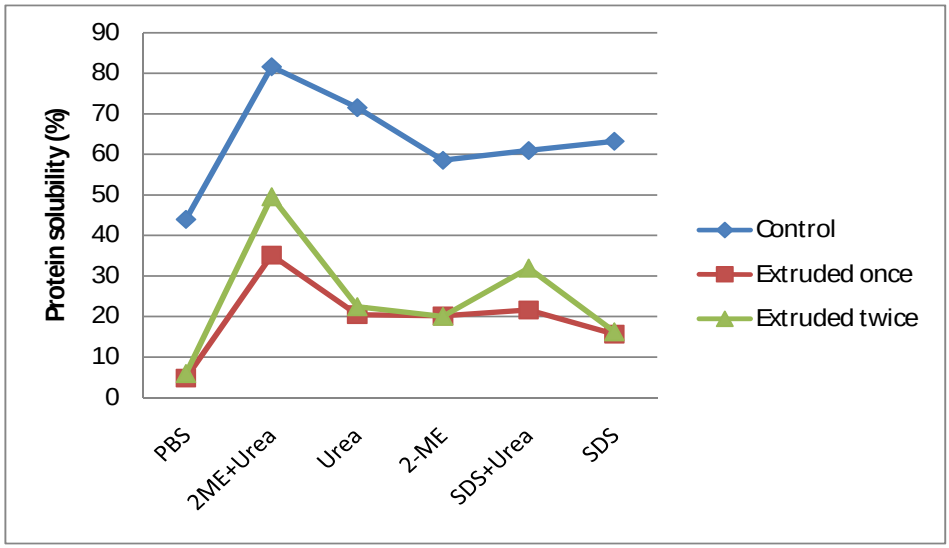


Fig 4.3.3 Effect of repeated extrusion at 137.8ºC and 60% moisture content on protein solubility. PBS= 0.035 M, pH 7.6 phosphate buffer solution; 2-ME+Urea= 8 M urea + 2% 2-ME in the phosphate buffer solution; Urea= 8 M urea in the phosphate buffer solution; 2-ME= 2% 2-mercaptoethanol in the phosphate buffer solution; SDS+Urea = 8 M urea + 1.5% SDS in the phosphate buffer solution; SDS= 1.5% sodium dodecyl sulphate in the phosphate buffer solution.

4.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples in the protein solubility test were used in electrophoresis in the presence of SDS and 2-ME to observe the difference in the molecular weight distribution of the products that were extruded once or twice. The addition of SDS and 2-ME would not affect protein subunits since the proteins that were soluble to solvent without 2-ME were those with non-disulfide linkages and would not be effected by the reducing condition. Preliminary tests showed a sever decrease in soluble protein fractions after the

first extrusion; however, the protein molecular weight distribution did not differ from the control. This result is identical to other research findings (Jeunink and Cheftel 1979; Noguchi and others 1982). Protein subunits of the extrudates extracted by PBS were very low and showed little intensity on the SDS-PAGE (Fig 4.3.4 lane A1 and A2). This result corresponds with the findings of protein solubility test that most of the native protein were denatured and participated in the aggregation and texturization of the extrudates. After the second extrusion molecular weight distribution did not differ from the control or the first extrusion. This result suggests the protein subunits were not modified by repeated extrusion. Isobe and Noguchi (1987) stated that after extrusion soluble protein fractions of the original defatted soy flour decreased or disappeared and all fractions decreased gradually with increased extrusion times. Although extrusion temperature did effect lengthwise breaking strength, there was no affect on the souble protein fractions in the SDS-PAGE. However, in this study, protein fractions that were soluble to the solvents used did not decrease; on the contrary, they increased dramatically especially in the 2ME+urea and SDS+urea solvents, corresponding to the protein solubility tests.

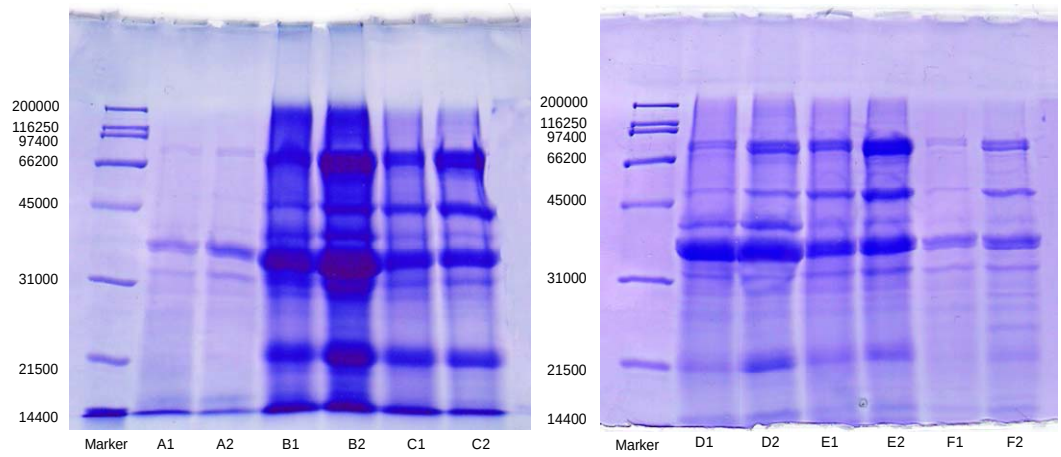


Fig. 4.3.4 SDS-PAGE of samples extruded at 137.8ºC and 60% moisture content. A-F protein extracted by PBS, 2ME+Urea, Urea, 2-ME, SDS+Urea, and SDS, respectively. 1: samples extruded once; 2: samples extruded twice.

4.4 Conclusion

The results of the first and second extrusion suggest that even though the products became slightly darker due to the Maillard reaction, the chemical bonds that contributed to the texturization of the extrudates could be broken apart and reform by the extrusion process. This ensures the possibility of reusing ingredients that do not meet the specifications or that from start-up and shut-down operations in the food industry. Based on the data and micrographic results, multiple extrusions did seem to have some effects on the physical appearances and fiber formation of the product. The second extrusion produced a tougher and more fibrous product. It could be due to the increase of disulfide bonds and noncovalent interactions shown in the protein solubility tests. Although the

concentration of the band in electrophoresis corresponds with the amount of proteins soluble to each solvent, the distribution was not affected by the first extrusion or the second. SDS-PAGE results shows extruded proteins were modified little at the molecular level and that any structure changes in the protein subunits were very small.

CHAPTER 5

RECOMMENDATIONS

One of the objectives of this study was to compare the chemical properties between low moisture extrusion and high moisture extrusion. The effects of repeated extrusion on protein interaction were also investigated. Chemical properties of the extruded samples could be further studied with a more thorough protein solubility test such as different combinations of solvents could be used to provide more detail in the protein reactions that occur after extrusion. Methods such as Raman spectroscopy that are more direct in investigating the chemical bonds responsible to the texturization of soy protein isolates could be used. In this study only the chemical bonds between the proteins isolate polypeptide chains were researched, the secondary structure of the protein could also be investigated.

For repeated extrusion, texture analysis could be done to study the effect of the second extrusion on the texturization of the extrudates. The relationship of the amount of reused ingredients and the texture properties could also be investigated. This may give the food industries a clearer idea of the usage amount of recycled ingredients.

REFERENCES

Akdogan H. 1999. High moisture food extrusion. Int J Food Sci Technol 34(3):195-207.

Allen RC, Budowle B. 1994. Support media. Gel electrophoresis of protein and nucleic acid: selected techniques. Berlin, New York: Walter de Gruyter & Co. p 1-22.

Anderson JW, Johnstone BM, Cook-Newell ME. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. New England J Med 333(5):276-82.

Anonymous. 2006. U.S. Soy: International buyers' guide. ASA-International Marketing.

Atkinson WT, Inventor, Archer Daniels Midland Co., assignee. 1970 Jan. 6. Meat-like protein food products. U.S. patent 3488770.

Berk Z. 1992. Technology of production of edible flours and protein products from soybeans. Rome: Food and Agriculture Organization of the United Nations. 133 p.

Burgess LD, Stanley DW. 1976. A possible mechanism for thermal texturization of soybean protein. J Inst Can Sci Technol Aliment 9(4):228-31.

Cheftel JC, Cuq J-L, Lorient D. 1985. Amino acids, peptides and proteins. In: Fennema OR, editor. Food chemistry. 2nd ed. New York: Marcel Dekker Inc. p 245-369.

Cheftel JC, Kitagawa M, Queguiner C. 1992. New protein texturization processes by extrusion cooking at high moisture levels. Food Rev Int 8(2):235-75.

Cumming DB, Stanley DW, deMan JM. 1973. Fate of water soluble soy protein during thermoplastic extrusion. J Food Sci 38(2):320-3.

Drake MA, Chen XQ, Tamarapu S, Leenanon B. 2000. Soy protein fortification affects sensory, chemical, and microbiological properties of dairy yogurts. J Food Sci 65(7):1244-7.

Dunn MJ. 1993a. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In: Graham JM, Billington D, editors. Gel electrophoresis: proteins. Oxford UK: Bios Scientific Publishers. p 51-64.

Dunn MJ. 1993b. Theory of electrophoresis. In: Graham JM, Billington D, editors. Gel electrophoresis: proteins. Oxford UK: Bios Scientific Publishers. p 7-11.

Faller JY, Klein BP, Faller JF. 1999. Acceptability of extruded corn snacks as affected by inclusion of soy protein. J Food Sci 64(1):185-8.

Friedeck KG, Karagul-Yuceer Y, Drake MA. 2003. Soy protein fortification of a low-fat dairy-based ice cream. J Food Sci 68(9):2651-7.

Hager DF. 1984. Effects of extrusion upon soy concentrate solubility. J Agric Food Chem 32:293-6.

Harper JM. 1989. Food extruders and their applications. In: Mercier C, Linko P, Harper JM, editors. Extrusion cooking. St. Paul, MN: American Association of Cereal Chemists. p 1-15.

Harvath E, Czukor B. 1993. Effect of extrusion temperature and initial moisture content on the protein solubility and distribution in full fat soybean. Acta Alimentaria 22:151-67.

Hayakawa I, Kajiwara Y. 1992. Effect of temperature and pressure on some physicochemical properties of soybean protein. In: Hayakawa I, editor. Food processing by ultra high pressure twin-screw extrusion. Lancaster, PA: Technomic Publishing Company. p 29-38.

Hettiarachchy N, Kalapathy U. 1997. Soybean protein products. In: Liu K, editor. Soybeans: chemistry, technology, and utilization. New York: Chapman & Hall. p 379-411.

Hettiarachchy N, Kalapathy U. 1998. Functional Properties of Soy Proteins. In: Whitaker JR, editor. Functional properties of proteins and lipids. Washington, DC: American Chemical Society. p 80-95.

Hua Y, Cui SW, Wang Q, Mine Y, Poysa V. 2005. Heat induced gelling properties of soy protein isolates prepared from different defatted soybean flours. Food Research Int (38):377-85.

Iosbe S, Noguchi A. 1987. High moisture extrusion with twin screw extruder : Fate of soy protein during the repetition of extrusion cooking. Nippon Shokuhin Kogyo Gakkai Shi 34:456-61.

Jeunink J, Cheftel JC. 1979. Chemical and physicochemical changes in field bean and soybean protein texturized by extrusion. J Food Sci 44(5):1322-5.

Kelley JJ, Pressey R. 1966. Studies with soybean protein and fiber formation. Cereal Chem 43:195-206.

Li M, Lee T-C. 1996. Effect of extrusion temperature on solubility and molecular weight distribution of wheat flour protein. J Agric Food Chem 44:763-8.

Lin S, Huff HE, Hsieh F-H. 2000. Texture and chemical characteristics of soy protein meat analog extruded at high moisture. J Food Sci 65(2):264-9.

Lin S, Huff HE, Hsieh F-H. 2002. Extrusion process parameters, sensory characteristics, and structural properties of a high moisture soy protein meat analog J Food Sci 67(3):1066-72.

Liu K. 1997. Soybeans: chemistry, technology, and utilization. New York: Chapman & Hall. 532 p.

Liu K, Hsieh F-H. 2007. Protein-protein interaction in high moisture-extruded meat analogs and heat-induced soy protein gels. J Amer Oil Chem Soc 84:741-8.

Maurice TJ, Stanley DW. 1978. Texture-structure relationships in texturized sou protein. IV. Influence of processes variables on extrusion texturization. J Inst Can Sci Technol Aliment 11:1-6.

Messina MJ. 1997. Soyfoods: Their role in disease prevention and treatment. In: Liu K, editor. Soybeans: chemistry, technology, and utilization. New York: Chapman & Hall. p 442-66.

Messina MJ, Barnes S. 1991. The role of soy products in reducing risk of cancer. J Natl Cancer Inst 85:541-6.

Ning L, Villota R. 1994. Influence of 7S and 11S globulins on the extrusion performance of soy protein concentrates. J Food Proc Preserv 18:421-36.

Noguchi A. 1989. Extrusion cooking of high-moisture protein foods. In: Mercier C, Linko P, Harper JM, editors. Extrusion cooking. St. Paul, MN: American Association of Cereal Chemists. p 343-70.

Noguchi A, Kugimiya W, Haque Z, Saio K. 1982. Physical and chemical characteristics of extruded rice flour and rice flour fortified with soybean protein isolate. J Food Sci 47(1):240-5.

Pham CB, Rosario RRD. 1984. Studies on the development of texturized vegetable products by the extrusion process. II. Effects of extrusion variables on the available lysine, total and reducing sugars. Int J Food Sci Technol 19(5):549-59.

Phillips RD. 1989. Effect of extrusion cooking on the nutritional quality of plant proteins. In: Phillips RD, Finley JW, editors. Protein quality and the effects of processing. New York: Marcel Dekker, Inc. p 219-46.

Prudencio-Ferreira SH, Areas JG. 1993. Protein-protein interactions in the extrusion of soya at various temperatures and moisture contents. J Food Sci 58(2):378-81.

Rhee KC, Kuo CK, Lusas EW. 1981. Texturization. In: Cherry JP, editor. Protein functionality in foods. Washington, DC: American Chem. Soc. p 51-88.

Shapiro AL, Viñuela E, Maizel JV. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28(5):815-20.

Sheard PR, Mitchell JR, Ledward DA. 1985. Comparison of the extrusion cooking of soya isolate and a soya flour. J Food Technol 20:763-71.

Snyder HE, Kwon TW. 1987. Soybean Utilization. New York: Van Nostrand Reinhold 346 p.

Stanley DW. 1989. Protein reactions during extrusion processing. In: Mercier C, Linko P, Harper JM, editors. Extrusion cooking. St. Paul, MN: American Association of Cereal Chemists. p 321-41.

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

Wolf WJ, Cowan JC. 1975. Soybeans as a food source. Cleveland, OH: CRC Press. 99 p.