THE EFFECT OF EXTRUSION CONDITIONS ON AGGREGATION OF PEANUT PROTEINS

by

ABHAY ARVIND SHAH

(Under the Direction of Robert Dixon Phillips)

ABSTRACT

This study was aimed at understanding a) the major changes occurring to the spectrum of proteins comprised in peanut flour at conformational and aggregation levels as a result of extrusion; b) the influence of extruder operating conditions on the protein scaffold of peanut flour. Protein was isolated from the extrudate using three buffer systems namely phosphate buffer saline (stage 1); phosphate buffer with urea (stage 2) and phosphate buffer with urea and DTT (stage 3). A high protein content in the stage 2 and stage 3 extracts of extruded peanut-flour samples indicated that the process of extrusion results in the formation of aggregates which are stabilized by non-covalent and disulfide linkages. These aggregates are insoluble in non-denaturing buffers. This observation was further confirmed by Agarose gel electrophoreses. SDS-PAGE analyses showed negligible difference in the overall migration patterns of the proteins in the un-extruded and extruded samples. However, there was a variation in the relative amounts of proteins in various samples. Also, size exclusion and ion-exchange chromatography revealed that during extrusion, the proteins undergo various interactions involving non-covalent and disulfide linkages resulting in the formation of aggregates of positive charge or moderately negative charge. Further, these studies showed that formation of these aggregates is more

favored by less severe conditions of extrusion. Extrudates obtained with 25, 30, and 35% feed moisture content at 125, 150, and 175 ºC temperatures were also analyzed similarly. Extrusion conditions of 25% moisture and 175 ºC (most severe condition) had the most profound effect on native peanut proteins. The highest Specific Mechanical Energy (SME) was observed under low temperature and low moisture extrusion condition. The mean residence time peaked during extrusion performed under 175 °C temperature and 35% moisture content.

INDEX WORDS: Extrusion, Peanut Proteins, Protein Aggregation, SDS-Page, Size Exclusion Chromatography, Ion-exchange Chromatography

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ABHAY ARVIND SHAH

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ABHAY ARVIND SHAH

Major Professor: Robert Dixon Phillips

Committee: Manjeet S. Chinnan
Romeo T. Toledo

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To Baa, Mom, Dad, Nikku, Ekta, Kaka, Kaki, Anand, Param, and the rest of my family, for their blessings, love, encouragement, and support.

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INTRODUCTION

The botanical name of peanut, *Arachis hypogaea* L. is derived from Greek word *Arachis* meaning a legume, and *hypogaea* meaning below the ground, referring to the formation of pods in the soil. Peanut is the third important oilseed crop in the world after soybean and cotton. Although it is grown in all the continents, over 75% of the total world peanut production is concentrated in India, China, and the United States of America. Because it is so flavorful while containing only a few antinutritional factors, it is consumed as a snack in a wide variety of ways, the only limitation being one's imagination and inventiveness.

Peanuts are considered as one of the most concentrated foods because of their high oil (45-48%) and protein (28-30%) contents. Oil extracted from peanuts is used in various edible forms, the majority being edible oil. Over 87% of the kernel proteins are globulins distinguished into two types, namely arachin and conarachin.

Extrusion is one of the most dynamic, versatile, and well-established industrial processes used in the food and feed industry today. It is being extensively used worldwide to produce an ever-expanding list of food and feed products including snacks, cereals, pastas, TVPs (texturized vegetable proteins), pet foods, animal feeds, instant beverages, meat analogs, and a range of ethnic foods. Extrusion generally involves the conversion of a "plasticized bio-polymer-based formulation" into a well and uniformly processed viscoelastic mass which is formed, shaped, puffed, and cut as it exists the die.

The effect of extrusion on various components of food like proteins, lipids, carbohydrates, vitamins and minerals, etc. have been well studied and documented. However,

data on peanut protein extrusion is limited and insufficient. Of particular interest in this present work is to gain knowledge about the chemistry of proteins in extruded peanut flour.

The nutritive value of a protein source depends on the relative amounts of constituent amino acids and their digestibility and bioavailability. Various mechanisms are responsible for the change in nutritional quality of proteins, during extrusion, depending on temperature, moisture, pH, shear rate, residence time, nature of proteins, and their interactions. Protein transformation during extrusion takes place by simultaneous thermo-chemical denaturation and cross-linking which is similar to starch degradation resulting from melting and chain-splitting. At lower energy level extrusion, formation of non-covalent and disulfide bonds takes place whereas stronger type of linkages occurs at higher levels of energy. Protein unfolding during extrusion enables reassociation of protein chains. The hydrogen bonds disrupted during the denaturation process, at low water contents, get recreated between protein molecules instead of between water and protein molecules leading to protein aggregation.

The ultimate goal of this research is to understand the effect of extrusion on protein-protein interactions during extrusion of peanut flour. Though there has been a lot of progress to date, protein extrusion is still in its developmental form waiting to be fully explored.

CHAPTER 1

LITERATURE REVIEW

I. Peanuts

A. Botanical

Peanut is a member of the genus *Arachis* in subtribe *Stylosanthinae* of tribe *Aeschynomeneae* of family *Leguminosae* (Moss et al., 1995). The peanut (*Arachis hypogaea* L.) is better known worldwide as groundnut and to much lesser extent as earthnut, monkey, and goobers. *Arachis hypogaea* is an annual herb and is one of the few plant species that forms underground fruits. There are two subspecies of *A. hypogaea,* distinguished primarily on branching pattern and distribution of vegetative and reproductive axes. Subspecies *hypogaea* has two verities (*hypogaea* and *hirsute*), where as subspecies *fastigiata* has four (Krapovickas and Gregory, 1994). It is one of nature's most nutritious seeds and one of the world's most popular crops, cultivated in nearly 100 countries (Nwokolo, 1996) in all the continents. Over 75% of the total world peanut production is concentrated only in India, China, and the United States of America (Salunkhe et al., 1991). The peanut, being a major source of edible oil and protein meals, is considered to be highly valuable in human and animal nutrition (Nwokolo, 1996). Peanut protein content has been reported to range from 16.2 to 36%, with most varieties averaging 25% and the protein content has a significant inverse correlation with oil content (Dwivedi et al., 1990).

B. Production

Peanut is an annual soil-enriching legume native to South America. Peanut plants were carried to Africa and China by early explorers and missionaries and during early colonial days in the United States, peanuts were introduced from Africa (Fletcher et al., 1992). World peanut production has shown a continual increase with a major increase occurring in 1980s (17%

increase over 1970s) mainly attributable to increase in yield, rather than harvested area (Fletcher et al., 1992) attributed to the adoption of available technologies such as crop rotation, improved harvesting practices and weed, insect, and disease control by chemicals (Godoy and Giandana, 1992). The primary peanut producing countries of North America are Mexico and the United States with the majority of peanuts produced in nine states in southeastern and southwestern USA (Isleib and Wynne, 1992). The production, marketing, and processing practices for peanuts in the USA are influenced by the price support program (Davidson et al., 1992). After the peanuts are dried they are graded and a value is assigned to the lot. After shelling, grading, and storage, most peanuts are sold to manufacturers who produce edible products, the largest in the USA being peanut butter (Isleib and Wynne, 1992).While peanuts are used primarily for vegetable oil in most of the world, in the USA they are grown mainly for food including peanut butter, roasted peanuts, candy, and other snack food items (Isleib and Wynne, 1992).

C. Composition

1. Proteins – categories, subunit, and structure

Peanut kernels are considered as one of the most concentrated foods because of their high protein and oil content. The protein content in peanuts ranges from 16 to 36% (Cherry and Ory, 1973; Dwivedi et al., 1990). The storage proteins are broadly classified into albumins (water-soluble), globulins (salt-soluble) and glutelins (acid or alkali-soluble). The majority (over 85%) of the kernel proteins are globulins. These globulins are further divided into two types, namely arachin and conarachin (Cherry and Ory, 1973; Basha and Cherry, 1976). Fontaine et al. (1945) reported that arachin consisted of two components in the ratio 3 to 1 constituting about 63% of the total proteins and conarachin consisted of two components in the ratio 4 to 1 contributing

33% of the total kernel proteins. SDS-PAGE of peanut proteins revealed two fractions, each containing five different subunits having molecular weights between 20,000 and 84,000 D (Basha and Cherry, 1976). In addition, these fractions were found to be glycoproteins containing both neutral and amino sugars (Ahmed and Young, 1982). Arachin was found to be poor in total sulfur (0.4%), lysine and methionine but rich in threonine and proline whereas conarachin was found to be rich in sulfur (1.09%) and poor in phenylalanine and tyrosine (Kaneko and Ishi, 1978). Basha (1979) separated and characterized peanut seed polypeptides and found atleast 74 major and between 100 and 125 minor peptides having molecular weights between 16,000 and 75,000 D, among several peanut cultivars and breeding lines.

2. Lipids

Data indicates that soy beans contain the greatest amount of protein and peanuts provide the greatest percentage of oil amongst legumes. The oil content of peanut seeds ranges from 36 to 56% (Sanders, 1982). Lipids, being the major constituent of peanut kernels, constitute of triglycerides (majority) and some phopshatides, glycolipids, unsaponifiables, phytosterols (β-sitosterol, campesterol, and stigmasterol), free fatty acids, antioxidants, tocopherols, and odor and flavor imparting higher hydrocarbons (Salunkhe et al., 1991). Fatty acid composition of peanut oil is influenced by cultivar, maturity, storage, processing treatments and environmental conditions (Shitole, 1987). Shitole (1987) found peanut oil to contain 17 to 22% saturated and 78 to 83% unsaturated fatty acids. The primary dietary essential fatty acid for man is linoleic but peanut oil contains more of oleic acid (47%) than linoleic acid (33%) (Ahmed and Young, 1982). Peanut oil, is lower in linoleic acid content (33% of total fatty acids) than corn oil (58%), safflower oil (79%) or mixtures of soybean and cottonseed oil (47%) (Carpenter et al., 1974). Peanut oil is used extensively as a salad oil and foods fried in it exhibit an excellent flavor and

keeping quality. It is easily digestible and serves as a good source of linoleic acid and other unsaturated fatty acids necessary for proper growth and nutrition.

3. Carbohydrates

The carbohydrates in peanut kernel and defatted meal have been studied extensively. The total carbohydrates in peanuts amount to 10 to 20% while the defatted meal contains about 38% carbohydrates. Sucrose and starch constitute the major while reducing sugars form the minor portion of peanut carbohydrates (Tharanathan et al., 1975). The reducing sugars are the precursors of compounds imparting flavor characteristic to roasted peanuts. The peanuts are, in general, low in flatulence-causing raffinose family sugars as compared to common food legumes (Salunkhe et al., 1991). While studying the development of starch and sugar during maturation of peanuts, Pattee et al. (1974) found that starch reached a maximum just beyond middle maturity stage of the seed and then remained constant while sugar content increased throughout maturation reaching a maximum at full maturation. Also, the concentration of fructose, arabinose and galactose remained almost unchanged as peanut seeds advanced in maturity. However, glucose content was found to decrease considerably with advanced maturation.

4. Minerals and Vitamins

Peanuts are a rich source of some dietary minerals like calcium (82 mg/100 g peanut), magnesium (174 mg), phosphorous (438 mg), potassium (627 mg), and traces of iron, sodium, zinc, copper and manganese (Ahmed and Young, 1982). The minerals in high quantities are virtually unaffected by heat (Salunkhe et al., 1991). Bioavailability is an important factor in the supplementation of extruded products by nutrients such as iron. Pinto et al (1997) found that bioavailability of lung iron remained unchanged after extrusion of chickpea enriched with bovine

lung under the most deleterious conditions for extrusion. Such products are widely being used in anemia malnutrition programs.

Peanuts are a very poor source of fat-soluble vitamins A, D, and K, and only moderate source of vitamin E (Nwokolo, 1996). Three forms of tocopherol (Vitamin E) are found: α, γ, and δ; with γ –tocopherol as the highest and δ –tocopherol has the lowest concentration (Rao and Rao, 1981). Total tocopherol is in the range 26-59 mg/100 g oil. Among the vitamins, nicotinic acid, thiamin (B_1), choline, inositol, and pantothenic acid are present in relatively higher amounts and B_{12} being practically absent (Dougherty and Cobb, 1970b). The mineral content of raw and roasted peanut kernels does not meet the RDA requirements. However, the vitamine E content of peanuts exceeds the RDA requirements, and there are sufficient amounts of thiamine, niacin, and folic acid in peanuts to approach the recommendations of the RDA (Ahmed and Young, 1982).

5. Other components

Color, texture, flavor (aroma), and antinutritional factors impart a lot of character to peanuts. The typical color of peanuts is attributed to both the testa and the oil and that of roasted peanuts is due to the sugar-amino-acid reactions with subsequent production of brown melanins (Pattee and Young, 1987). Tannins and catechol-type compounds are responsible for the testa color (Ahmed and Young, 1982) and β-carotene and lutein are the major carotenoid pigments present in the oil (Pattee et al., 1969).

The raw peanuts possess slightly sweet, green, beany flavor and roasting of nuts converts this into a flavor that is delicate, pleasant, and uniquely nutty, and thus is widely enjoyed. Amino acids, peptides, and carbohydrates in an oil medium are the precursors of roasted peanut flavor (Rodriguez et al., 1989).

Raw peanuts have very low concentrations of most of the antinutritional factors found in raw soybean. Trypsin inhibitor content was found to be only about 20% of the level in raw soybean. Some other compounds like lectins, goitrogens, phenolic compounds, phytates, flatus sugars, bitter flavor compounds and allergic factors are also responsible for the antinutritional aspect of peanuts (Salunkhe et al., 1991). The best known toxic compounds of peanut are the aflatoxins, metabolic by-products of the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. Toxigenic microorganisms are constantly encountered in the environment, and mould infection of badly harvested/poorly stored peanuts occurs around 20-25 °C. When these moldy peanuts are eaten or processed into food or feed, aflatoxin poisoning occurs. Since aflatoxin can arise from random sources/conditions, extreme care in harvesting and storage of the seeds, and uncompromising quality control practices can be a step towards healthier peanuts.

D. Food uses

There is a growing demand throughout the world for more protein supplies and for a balanced dietary source of protein. Since peanuts are a rich source of proteins, they are often found in foods, snacks, and meal programs. Peanut containing foods such as peanut butter, salted seed, roasted nuts, candies, and snack-type crackers and cookies enjoy widespread popularity because of their unique roasted peanut flavor (McWatters and Cherry, 1982). On a worldwide scale, the majority of peanut production is utilized to produce peanut oil which is favored for cooking and also as salad oil. Oil extraction also produces a protein-rich co-product which may be used for human consumption if processed from edible-grade peanuts and the process conforms to food manufacturing practices for food processing. The defatted peanut meal is available as flakes, grits, or flours and may be further processed to high protein concentrates and isolates. Defatted peanut meal contributes to the physicochemical, functional, and nutritional

characteristics of foods in which they are incorporated. The potential applications of peanuts and peanut products in food systems have been a growing research area. In some countries buffalo milk and cow's milk have been fortified with peanut protein to replace the addition of a costlier product – skim milk powder - to increase milk's availability to a large segment of the population (McWatters and Cherry, 1982). Fermentation of peanut milk with lactic acid bacteria has been shown to yield end products similar to buttermilk and yogurt (Bucker et al., 1979). The utilization of peanuts in beverages and soups, frozen desert and non-fermented cheese analogs has been investigated. Some researchers were successful in replacing wheat flour in breads with peanut and other oilseed flours and concluded that such a replacement increased absorption and decreased mixing tolerance of doughs, thereby improving bread quality (Matthews et at., 1970; Rooney et al., 1972). McWatters (1978) successfully prepared sugar cookies by substituting wheat flour with 10, 20, or 30% defatted peanut flour without adversely affecting dough handling, diameter, height, spread characteristics, and sensory quality attributes. Breakfast cereals with 21% protein have been produced by extrusion processing of defatted peanut flour with corn or oat flours (Ayres and Davenport, 1977). Peanut flour was found to expand easily during extrusion processing, had a low flavor profile, and did not increase the bulk density of the finished product substantially.

1. Peanut-based snacks

Peanuts are consumed in a variety of forms. Cooked/boiled/roasted peanuts, peanut butter, peanut flour, concentrate and isolate, confectionary, and snack products.

Extruded peanut snack consists of a mixture of cereals, peanut and other flours, and ingredients that are mixed together, often with necessary amount of water, and processed through an extruder. After mixing and kneading it is heated above its gelatinization temperature leading

to a cooked product. The product is sometimes enrobed and flavored or sometimes undergoes further processing like frying, roasting, baking or fortification (Cindio et al., 2002a). Roasted peanuts (oil or dry roasted) are widely used snack foods, but the conversion of peanuts to forms such as chips or extruded foods can extend their usefulness to a great extent. Chip-like products can be prepared from peanut meal with or without added binders and these types of products are similar to conventional potato or corn chips but containing higher levels of proteins (McWatters and Cherry, 1982). Extrusion processing of snack foods utilizing high protein ingredients like peanuts depends largely on expansion and puffing characteristics which influence product density and texture. The combination of defatted peanut and cereal/gram flour were found to be suitable for extrusion processing of protein-fortified, ready-to-eat foods (Bongirwar et al., 1979). These authors also described optimum process conditions like extrusion temperature, feed moisture content, screw speed, and retention time necessary for the production of such foods.

II. Extrusion

A. Definitions and overall description of the process

1. Cooking and forming extrusion

Webster's defines the verb to extrude, as "to shape by forcing through a specially designed opening often after a previous heating of the material". Extrusion, therefore, is primarily oriented toward the continuous forming of plastic or soft materials through a die. Extrusion is one of the most dynamic, versatile, and well-established industrial processes used in the food and feed industry today. It is being extensively used worldwide to produce and ever-expanding list of food and feed products including snacks, cereals, pastas, TVP's (texturized

vegetable proteins), pet foods, animal feeds, instant beverages, meat analogs, and a range of ethnic foods. Extrusion generally involves the conversion of a “plasticized bio-polymer-based formulation” into a well and uniformly processed viscoelastic mass which is formed, shaped, puffed, and cut as it exists the die (Rizvi et al., 1995). Product attributes of the extrudates are controlled by varying the mechanical and thermal energy inputs and adjusting the residence time of the feedstock in the extruder, which in turn is achieved by manipulating or varying variables like feed rate, moisture content, barrel temperature and degree of barrel fill, and screw profile and screw speed. Food (biopolymers) is different from chemical polymers due to its composition of lipids, proteins, carbohydrates, fibers, and water, thus making each food material unique with its own viscosity behavior. It is this aspect that makes it difficult to design “all purpose” mixing elements for food materials which makes it necessary to have tailor-made mixing designs to produce certain products (Zuilichem et al., 1999). During extrusion, native starch undergoes changes causing molecular disorganization. From the view point of finished product texture, starch looses its crystalline structure, undergoes molecular fragmentation, and often complexes with other materials in the feed mixture (Gonzalez and Perez, 2002). Hence, wheat products are often used as raw materials because of their low cost, versatile processing performance and blandness, which helps generate and create the desired environment for processing and flavor (Guy et al., 1995). The mechanisms of protein-protein interactions which lead to final product texturization, during extrusion, remain unknown. This has been the main limiting factor for use of extrusion to its full potential in protein processing (Areas, 1992). Single and twin-screw extruders are used widely in the food processing industry for the production of shaped and cooked products.

B. Extruders

1. Their construction, components, and geometry

The feeding, blending, and preconditioning, also known as the premix section, consists of moisturizing and/or heating of ingredients, is an essential part of an extrusion operation. The consistent and uniform feeding of food and other ingredients is necessary for the consistent operation of an extruder. The feeder is a device that provides a uniform delivery of food ingredients which are often preconditioned, thus sticky and non-free-flowing, to the extruder screws. Various types of feeders like vibratory, variable speed; weigh belts etc. are utilized for this purpose.

The screw is one of the most important components of the extruder. It is the central portion of the extruder which accepts the feed from the feeder, conveys, mixes, kneads, and forces the feed through the die restriction at the discharge. According to Harper (1981) the (single) extruder screw is divided into three sections named to correspond with their respective function. (1) Feed Section: the section which accepts the food materials at the feed port. The function of this section is to assure that adequate material is conveyed down the screw. (2) Compression Section: the portion of the screw between the feed and metering section where ingredients are heated and worked into continuous dough mass. The character of the feed changes from a granular to a plasticized dough. Compression is achieved by the gradual decrease in the flight depth in the direction of the discharge or the decrease in pitch of the screw flights. (3) Metering Section: the portion of the screw nearest to the discharge of the extruder which is normally characterized by having very shallow flights. The shallow flights increase the shear rate to maximum level within the screw of the single screw extruder.

The barrel is the cylindrical member which encompasses the rotating extruder screw. The distance from the rear edge of the feed opening to the discharge end of the barrel bore, known as the length (L) of the extruder, and diameter (D) of the barrel play a very important role in the design of the extruders. The requisite amount of heat transfer area is a function of L/D (Mercier et al., 1989).

The die is a major component in the extruder apparatus. Dies are small openings that shape the food material as it flows out of the extruder. Die design plays an important role in controlling the texture of the final product. The stability of the flow coming out of the extruder barrel is a function of the die design and helps shape the extrudates into a desirable product (Akgodan, 1996). Material at the feed end is conveyed, mixed, kneaded, and forced out through the die at the discharge end (Harper, 1981). Typically in a cooker extruder, cooking and starch gelatinization occurs in the end/cooking zone. When the cooked product exits the die, the pressure is quickly released, allowing the vapor to flash off resulting in an expanded (radial, axial, and overall), porous product. The extent of reactions during extrusion directly affects product expansion.

2. Forming extruders

Forming extruders are low shear sanitary and thermally efficient extruders designed for the production of many delicately formed and shaped food products such as snack pellets and half-products, high protein cereal beads, dense textured vegetable protein meat analogs, and semi-moist pet foods.

Forming extruders are also widely used in the plastic industry to form plastic materials. This operation includes progressively heating thermoplastic solid particles simultaneously with

mixing, melting and conveying the particles through the extruder. This molten material is then passed through particular die cavities to obtain the desired shape and size (Thomas, 1996).

Several designs are possible for such extruders. The simplest being a ram or a piston. However, more complex designs are often found having flighted screws or worms rotating within a sleeve or a barrel (Harper, 1981). Forming extruders have found application in extruding precooked doughs to generate special shapes by utilizing high pressure. To retain these shapes it is necessary to control the temperature of the dough while it is being formed so that excessive puffing does not occur once the product leaves the die. For this reason such extruders are usually jacketed and often have hollow screws for cooling water circulation. Forming extruders are often operated in series with cooking extruders.

3. Cooking extruders

a) Single screw extruder

By definition, single screw extruder consists of a single screw encased in the barrel assembly. The feed material on its way through the open channel of the extruder undergoes a combination of various forces like shearing, mixing, and compression (Zuilichem et al., 1999). Single-screw extruders rely on drag flow to move material down the barrel and develop pressure at the die. To be pushed forward, dough should not rotate with the turning screw. A single-screw extruder is a drag flow device, i.e. material is dragged down the barrel. Additionally, the rotational movement of the screw relative to the barrel wall creates another flow, called the cross channel flow. This flow, which recirculates material in the channel between the screw flights, does not contribute to the net forward movement of the material. It does, however, contribute to the mixing of the material passing through the extruder. Finally, there is a third type of flow

called pressure flow and accounts for the movement of material backwards in a negative direction due to the pressure at the die. When all these three flows (drag, cross channel, and pressure) are combined, the net effect is material flow out of the extruder die which has experienced a certain amount of mixing and shearing (Frame, 1994). According to Weert et al. (2001) there are four fundamentally different regions in a single-screw extruder, depending upon the operating conditions and the material being extruded. (1) The feed zone which extends from the hopper to the first screw element; (2) conveying zone, where the feed is compacted and transported; (3) melting zone, where due to temperature and shear the feed begins to melt and form a paste; (4) metering or pumping zone, where complete mixing and cooking of the melted paste takes place and the finished product exits at the end of this zone through a die. Marsman et al. (1995) differentiated the extruder parameters as process, system, and target parameters. Process parameters are those that can be continuously monitored like feed rate, moisture content, screw speed, barrel temperature and screw configuration. System parameters are those that pertain specifically to the extruder, for example, mechanical and thermal energy inputs into the feed and residence time. During extrusion two main energy flows are of primary interest. One is the dissipation of the motor power due to friction of the particles in the extruder and the other is the heat transfer through the barrel wall. Target parameters are a measure of the effect of extrusion on raw materials like solubility and nutritional value (Marsman et al., 1995). These authors found that extrusion of proteinaceous material was highly influenced by the design of the shear heads on the screw.

b) Twin-screw extruder

Twin-screw extruders, as the name suggests, have two screws in various types of configuration inside the extruder barrel. **Figure 1.1** shows a typical line diagram of a twin-screw

extruder system. The relative direction of rotation of the screws, counter or co-rotating, and the degree of screw intermeshing are key points of differentiation. Fully intermeshing counter-rotating screw extruders prevent the cylindering effect and approach positive displacement in pumping materials. Co-rotating screws (both the screws turn in the same direction) are intermeshing and self wiping, and have been the most popular type of screws because of their higher capacity. These types of screws are open length wise and closed cross wise. Co-rotating screw extruders can be operated at a higher screw speed than counter-rotating screws because radial forces are more uniformly distributed. Due to the independent variable screw configuration, twin screw extruders provide greater flexibility of operations to control product characteristics by monitoring time, temperature, pressure, and shear history (Choudhury and Gautam, 1999). They require no or negligible post-extrusion drying and are operational at very low feed moisture (Harper, 1989; Dziezak, 1989). The manipulation of screw configuration by altering the type, location, and length of mixing elements can produce mild to very severe processing conditions which can help achieve desired product expansion and textural attributes (Choudhury and Gautam, 1999). Twin–screw extruders are often operated under starved feeding conditions, i.e. the feed rate is lower than its conveying capacity and the volumetric feed rate is directly proportional to the feed rate, to achieve the desired product attributes (Unlu and Faller, 2002). Though twin screw extruders are more costly and complex than single screw extruders, they have many advantages. Efficient material transport takes place due to the forward conveying action of the twin screws. This minimizes pressure and leakage flows by virtue of the direction of screw rotation and screw configuration. Twin screw extruders are widely used for production of a variety of food products. The extrudates characteristics from proteinaceous and starchy ingredients depend on physiochemical changes occurring during extrusion (Choudhury

and Gautam, 1999). Wet extrusion which was impossible more than a decade ago is now possible due to the developments in twin screw extruders' barrel, screw, and die designs (Akgodan, 1996). **Figure 1.2** shows a typical extruder system used in the industry. The extrusion parameters are constantly controlled by a feed-back mechanism enabling greater automation and monitoring of the process.

c) Single v/s Twin-screw Extruders

The following table (**Table 1.1**) gives a one-on-one comparison between single and twin screw extruders as reported by Harper and Tribelhorn (1992).

Table 1.1: Comparison between single and twin-screw extruders.

Item	**Single Screw**	**Co-rotating Twin Screw**
Capital		
- Extruder	1.0	1.5 – 2.5
- System	1.0	0.9 – 1.3
Relative Maintenance	1.0	1.0 – 2.0
Energy		
- with Preconditioner	Half from steam	Generally not used
- without Preconditioner	Mechanical Energy	Mechanical + Heat
Screw		
- Conveying Angle	≡ 10º	≡ 30º
- Wear	Highest at discharge and transition section	Highest at restrictions and kneading discs
- Positive displacement	No	No
- Self-cleaning	No	Self-wiping
- Variable flight height	Yes	No
- L/D	4 – 25	10 – 25
- Mixing	Poor	Good
- Uniformity of shear rate	Poor	Good
- Relative RTD spread	1.2	1.0
- Venting	Requires two extruders	Yes
Drive		
- Relative screw speed	1.0 – 3.0	1.0
-Relative thrust bearing capacity	Up to 5.0	1.0

- Relative torque/pressure	Up to 5.0	1.0
- Gear reducer	Simple	Complex
Heat transfer	Poor	Good
Operations		
- Moisture	12 – 35	6 to very high
- Ingredients	Flowing granular material	Wide range
- Flexibility	Narrow operating	Greater operating

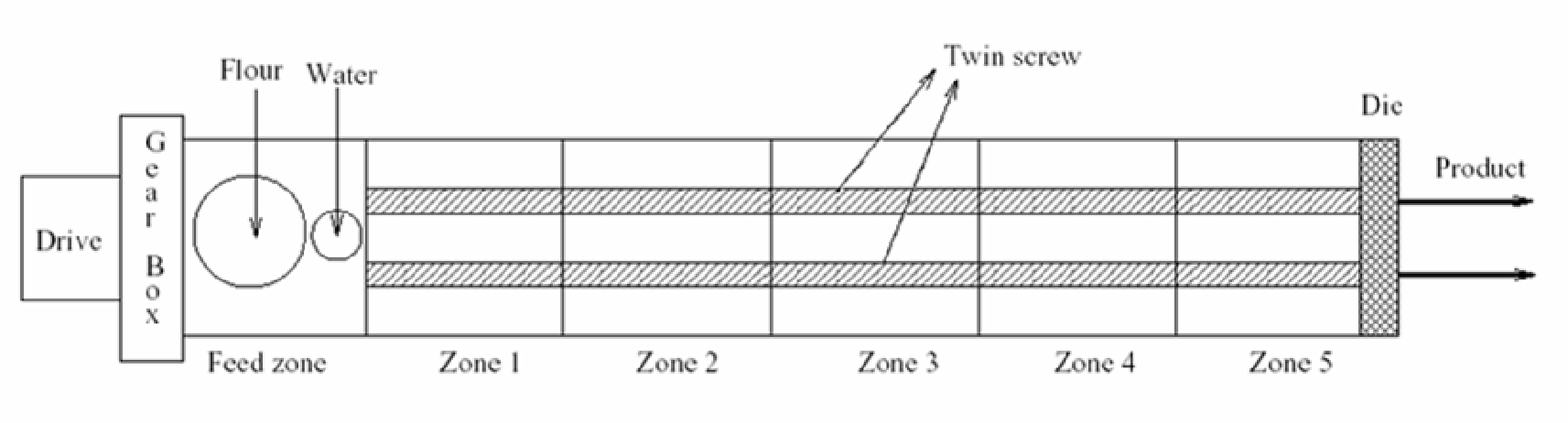

Figure 1.1: Simple outline of a twin-screw extruder system (Taken from Popescus et al., 2002; Reprinted with permission).

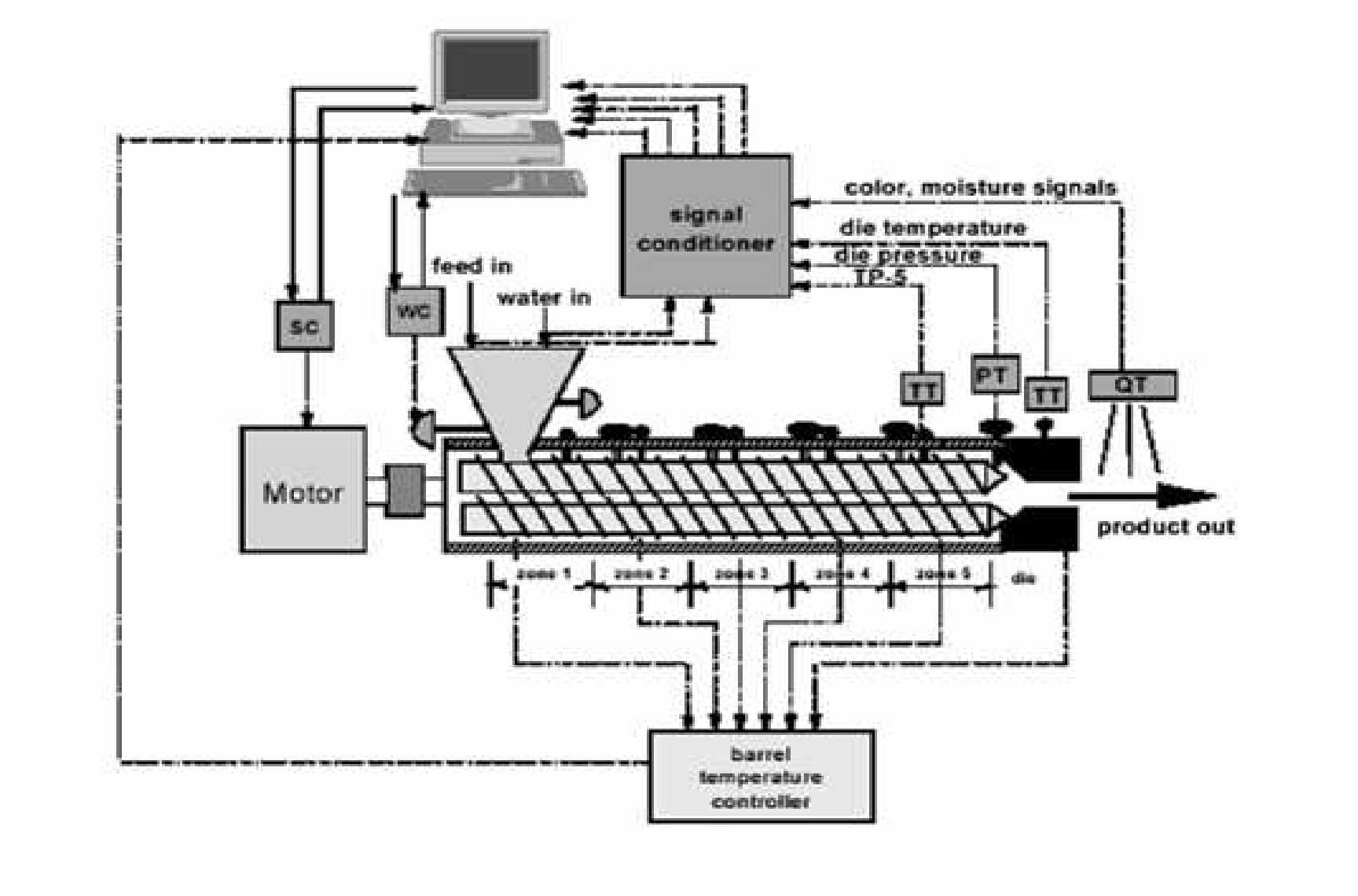

Figure 1.2: A typical twin-screw extruder system setup in the industry.

4. How extruders operate – role of components

Independent and dependent variables govern the processing conditions of an extruder system. Those variables that can be easily controlled and modified like screw speed, moisture content, feed rate, barrel temperature profile, die size, die shape, barrel length, screw profile and cutter speed are known as independent variables, though some of them are not always easily modifiable. These variables determine the product characteristics by generating extruder response variables such as die pressure, motor torque, barrel fill, product temperature, SME, and RTD which are also known as dependent variables (Unlu and Faller, 2002). Lo et al (1998a) concluded that high moisture content resulted in a material with lower viscosity and found the relation between feed moisture content and average apparent viscosity to be exponential which was in accordance with previous studies (Fletcher et al., 1985a, b; Bhattacharya and Hanna, 1987; Senouci and Smith, 1988). A small change in the level of reducing sugar can affect lysine inactivation by browning, changing pH or salt concentration can affect protein denaturation (Harper, 1981). Phillips (1989) concluded that various mechanisms are responsible for the change in nutritional quality of proteins, during extrusion, depending on temperature, moisture, pH, shear rate, residence time, nature of proteins, and their interactions. The relationship among variables that control extruder operation and extrudate characteristics is given in **Figure 1.3** (Phillips, 1989).

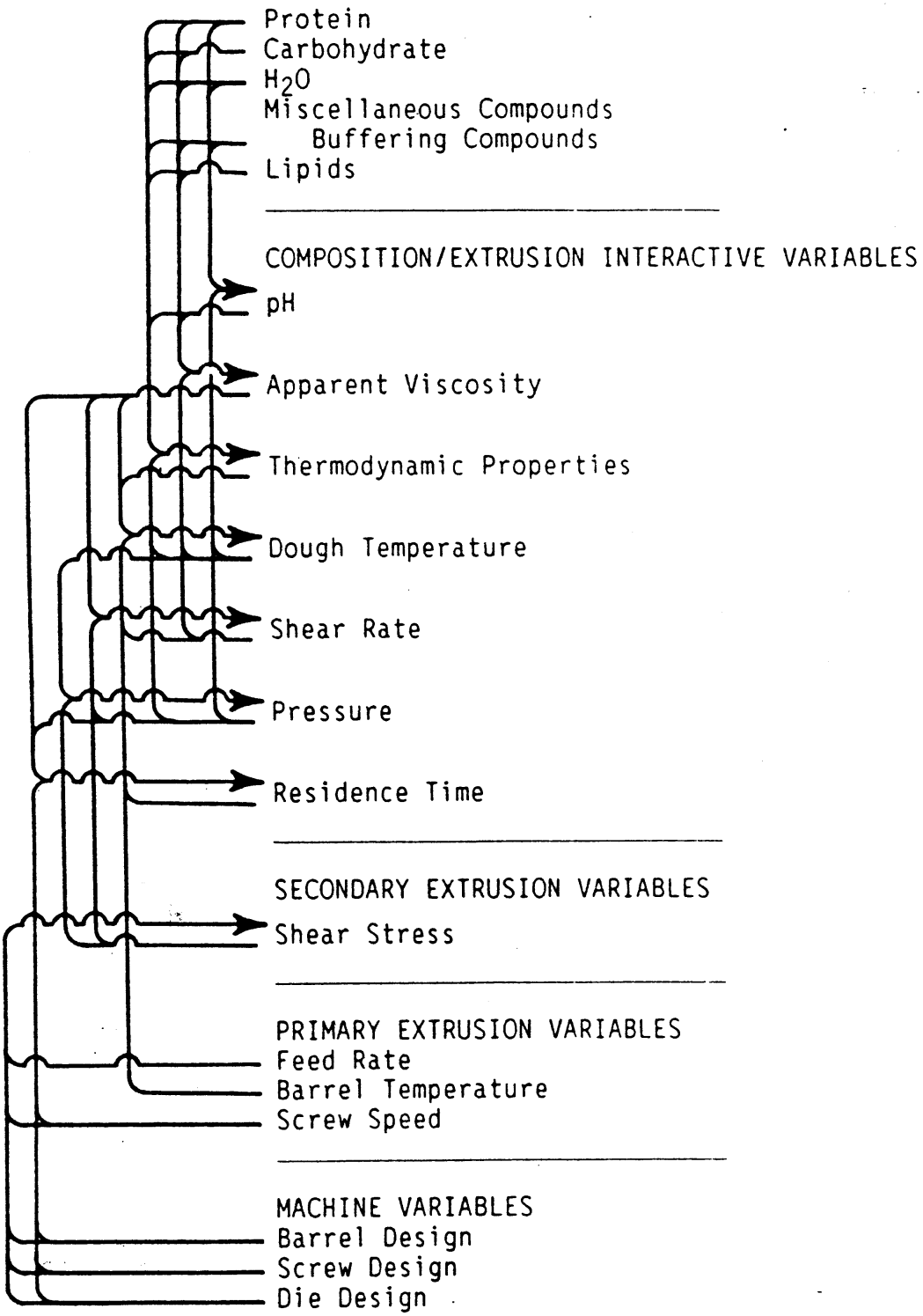

Figure 1.3: Relationship among variables that control extruder operation and extrudate characteristics (Reprinted from Phillips, 1989. p. 224 by courtesy of Marcel Dekker Inc.).

5. Machine variables - how they effect feed components

a) Screw configuration

In food extruders, the screws are built up from a number of pieces attached to a shaft or one another, which gives sufficient amount of variability in the geometrical configuration of the extruder. Each individual screw section is designed to perform specific functions such as conveying, mixing, shearing, or pressure build up duties, thus allowing precise control of conditions along the screw length (Meuser and Wiedmann, 1989). The screw elements differ in pitch, pitch direction, length, and angle of offset. Forward screw elements (FSEs) provide the transportation of the feed. They can be single lead (used for material compression) or double lead (mainly for material conveying). The kneading elements (KEs) are utilized in kneading the feed. They work as mild flow restrictors and can cause static mixing with weak forward or backward conveying. However, individually they have no conveying effect. The reverse screw elements (RSEs) are characterized by a reverse flight that pushes the material backward. Depending on the stagger angle and the length of RSEs the severity of extrusion can be controlled. The pitch, length, and location of such screw elements define a screw profile and influence the product characteristics during extrusion (Choudhury and Gautam, 1999). Changing the screw configuration by adding two extra reverse paddles caused a decrease in fluid consistency coefficient (16.865 vs. 20.130 kPa.s**n) and an increase in shear thinning behavior (0.22 vs. 0.44) of the melt during extrusion of cornmeal (Lo et al., 1998a). The incorporation of kneading paddles/reverse screw elements in the shear section not only reduces pressure and facilitates venting, provides more shear to the feed and enhances mixing, but also helps to form a rapid rise in the back pressure and facilitates cooking (Lo et al., 1998b). Choudhury and Gautam (1999) concluded that screw configuration was a major variable affecting “macroscopic characteristics”

of extrudates. Furthermore, they concluded that the type, length, position, of mixing elements and the spacing between them influenced all the product properties but true density. Barres et al (1990), during extrusion of wheat flour, found that by doubling the number of RSEs, higher filling of the screw, earlier transformation, and an increase in final solubility (24% compared to 19%) could be achieved where as a further intensification of configuration (more severe) increased the final product solubility from 25% to 37%. The absence of a flight element in any section along the screw profile causes the formation of fibrillar structure and provides no conveying capability (Yeh and Huang, 1992).

b) Screw speed

Many extruders have a variable drive which enables changing the screw speed easily. Various studies on screw speed have reported of increased shear rate, increased mechanical disruption, but reduced mean residence time associated with increased screw speeds (Lo et al., 1998b). During extrusion of cornmeal, Lo (1996) found residence time to change from of 55 s at 370 rpm to 47 s at 480 rpm. Residence time is a measure of time that the feed spends in the extruder. Another important phenomenon associated with screw speed is specific mechanical energy (SME). Simply put, SME is a measure of energy absorbed per unit mass of melt. Various authors have reported of observing an increase in the SME by increasing the screw speed and have also observed the relationship between apparent viscosity and screw speed to be exponential (Lo et al., 1998b; Vergnes and Villemarie, 1987; Senouci and Smith, 1988). Higher screw speeds also cause a reduction in the die pressure due to the reduction of viscosity which indicated less resistance for the starchy meal traveling through the extruder. Extrudates produced at high screw speeds have lower bulk density, moisture content, color b index, WAI (Water Absorption Index) and rougher extrudates surface (also known as alligator skin effect) where as

low screw speed extrusion has shown to form cells of bigger size and thicker walls in the extrudates (Lo et al, 1998a). Twin screw extruders are starve-fed, meaning, at steady state the throughput of the extruder is not a function of screw speed. However, as the screw speed increases the degree of fill in the extruder barrel decreases (Akgodan, 1996) due to the proportional increase in forward capacity. A decrease in melt viscosity of a high moisture rice starch system was observed by increasing the screw speed (Akgodan, 1996). Ilo et al. (1999) observed higher puffing during extrusion of rice-amaranth products at higher screw speeds.

c) Feed rate

The feeding, blending, and preconditioning, which consist of moisturizing and/or heating of ingredients, are very important during an extruder operation. The consistent operation of an extruder is highly dependent on consistent and uniform feeding of the feed or other materials (Harper, 1981). The feed rate is controlled by incorporating any of the feeder systems like hoppers or bins, vibratory feeders, variable speed auger, weigh belts, slurry tanks, liquid feeders and pumps, continuous feed systems, etc. which ensure feeding the raw material at the desired uniform rate. During a study on residence time distribution (RTD) in a twin-screw extruder, Apruzzese et al. (2003) found that increasing the feed rate decreased the RTD. They concluded that an increased feed supply resulted in a more complete filling of the extruder screw thereby decreasing the axial mixing and free flow of the product. These finding were in accordance with those of Guha et al. (2003) who also found that increasing the feed rate increased the number of filled channels consequently increasing the torque but had a negative effect on bulk density, peak viscosity, and hot paste viscosity. However, during extrusion of cornmeal, Lo et al. (1998b) found that decreasing the feed rate reduced the average apparent viscosity where as an increase

in the feed rate increased the degree of fill and reduced the mean residence time from 58 s at 45 kg/h to 36 s at 66 kg/h.

d) Barrel Temperature

The extruder barrel is the cylindrical member which fits tightly around the rotating extruder screw. The barrel is often surrounded by hollow jackets or heaters which can be controlled to achieve specific barrel temperatures. These temperatures are easily and most frequently monitored using thermocouples. In order to achieve specific product characteristics the temperature profile across the barrel may be constant or may increase from lower temperatures towards the feeding side to higher temperatures towards the die end. An increase in the barrel temperature causes the viscosity of the melt to decrease when extruding starchy materials. This increase in temperature causes the gelatinization of the starch granules and thus cooking occurs (Lo et al., 1998a). During extrusion of Amaranth blends, Ilo et al. (1999) found that sectional expansion (SE) decreased where as the longitudinal expansion (LE) increased with increasing barrel temperatures due to the dependence of SE and LE on melt elasticity and viscosity respectively. While extruding Sago starch in a twin-screw extruder, Govindasamy et al. (1997) concluded that product temperature was significantly dependent on barrel temperature such that changing the barrel temperature caused a corresponding equidirectional shift in the product temperature and also the barrel temperature exerted an overall positive effect on SME, the intensity of which was dependent on feed moisture content. In general, at constant moisture content, increasing the barrel temperature decreases the SME. While extruding rice flour it was concluded that system parameters as well as the extrudate attributes were mainly dependent on barrel temperature and to a lesser extent on screw speed (Guha et al., 1997). Their findings were: during extrusion of rice flour (14% moisture content) without using a die, low barrel

temperatures (80ºC) yielded extrudates with acceptable cooking and *in vitro* starch digestibility indices, had high bulk density and required high SME (643 kJ/kg) for extrusion and generated high torque (85%). On the other hand, high barrel temperatures (120ºC) required less SME (322-497 kJ/kg) and produced less torque (40%) but had decreased *in vitro* starch digestibility. However, extrusion at 100ºC and 300 rpm (mid-point) needed least SME (317 kJ/kg) and produced least torque (39%) and yielded a product with desirable product profile.

e) Die size and shape

A restriction or die is usually used during extrusion of foods for shaping the final product. But if the product is a modified ingredient, such as, modified starch or cereal powder used in foods like baby foods or weaning foods, the use of die becomes optional. The energy expenditure of an extrusion process highly depends on the die (Guha et al., 2003). When extruded melt exits the die, it suddenly goes from high pressure to atmospheric pressure. This pressure drop causes an extensive flash-off of internal moisture and the water vapor pressure, forming bubbles in the molten extrudate, allowing the expansion of the melt (Arhaliass et al., 2003). The extent of expansion/puffing of the extrudates depends on various parameters, one of the most important being the die. The shape of the dies vary, the simplest being a round hole. Other forms of openings found commonly are annular openings and slits. To improve uniformity of the extrudate, often the feed side of the die is streamlined (Harper, 1981). The use of a die modifies the pressure, shear, and temperature history of the extrudate. However, the die must not be too restrictive to flow, which would create high pressure within the extruder, and thus die design plays an important role in obtaining accurate flow measurements (Drozdek and Faller, 2002). While extruding corn grits, Arhaliass et al. (2003) concluded that the melt expansion at the die

insert was very much affected by the insert diameter: the lower the die insert diameter, the larger the melt growth, and the larger the extent of melt shrinkage.

6. "Intermediate" dependent variables

a) Product temperature

Starch granules undergo swelling as the temperature of the dough rises above the gelatinization temperature. The friction in the extruder is great enough to disrupt these fragile granules into smaller fragments, thereby causing a reduction in viscosity (Lo et al., 1998a). Extrusion of corn meal carried out at higher temperature (172 °C) caused a reduction in product moisture content, viscosity of feed, radial expansion, breaking strength, and WAI whereas WSI and axial expansion of the extrudates were observed to increase, as compared to extrusion at 144 °C (Lo et al, 1998a). Unlu and Faller (2002) found that product temperature increased with increasing screw speed and feed rate due to the fact that with increasing screw speed more SME input to the system happens thereby increasing the product temperature and increasing the feed rate enhances the feed contact with the hot extruder barrel. This was contrary to the finding of Lu et al. (1992) who reported lower product temperatures for higher feed rate, but in accordance with the findings of Huang et al. (1995) who observed the product temperature to decrease with increasing screw speed and moisture content whereas adding ammonia to the feed increased the product temperature, while extruding rapeseed meal. During extrusion of soy proteins, Areas (1992) found that at all tested temperatures, disulfide bridges, followed by non-covalent interactions were the main types of protein-protein interactions in the extrudates. The increase in temperature from 140 to 180 ºC resulted in a proportional reduction in disulfide linkages.

b) Pressure

Pressures are not commonly measured in food extruders although they are very important. Pressure measurements just behind the die are most useful in determining steady flow conditions and the relative magnitude of the pressure to drag flow. Die pressure is dependent on feed rate and viscosity of food dough and is defined by Martelli's (1983a) equation: $P = (FR * \eta)K_f$, where P, FR, η, and K_f are die pressure, feed rate, dough viscosity, and die geometry constant, respectively. Since latter is a constant and assuming viscosity changes are minimal with changing feed rate, the die pressure mainly depends on the feed rate and thus an increase in feed rate increases the die pressure (Unlu and Faller, 2002). They also found the die pressure to decrease with increasing screw speeds which contradicted the findings of Huang et al. (1995) who found an increase in die pressure by increasing the screw speed whereas the pressure decreased with increasing moisture content during extrusion of rapeseed meal. During extrusion of high moisture rice starch system Akdogan (1996) found the die pressure drop to decrease with increase in die temperature at constant moisture content, screw speed, and mass flow rate as a result of decrease in viscosity of the food melt. These finding were in accordance with those of Della Valle et al. (1987). The loss of energy in the form of frictional heat is proportional to the pressure and screw speed (Weert et al., 2001). They observed that the single most important parameter of the system, during extrusion of powders, was back pressure.

c) Shear and Viscosity

Mixing is important within the food extruder to assure uniformity of the extrudate. Mixing is related to the total strain that a material receives and is defined as the relative displacement of a fluid element under deformation and can also be defined as the rate of shear times time. Strain is an indication of the thoroughness of mixing. Shear rate occurs in the screw

channel, clearance, and the die and depends on screw speed, screw geometry, clearance, size and shape of the die, flow rate, and rheological properties of the dough (Harper, 1981). Viscosity is a shear dependent phenomenon and is simply the ratio of shear stress to shear rate (Akdogan et al., 1997). Besides the temperature and excess moisture environment, shear forces also contribute to starch degradation in the extruder. It is also known that the viscosity of starch suspensions decreases with increasing shear. Shear forces can physically disrupt the starch granules, allowing faster transportation of water into the starch molecules. Akdogan et al. (1997) found that the loss of crystallinity during extrusion of rice melt was due to mechanical disruption of molecular bonds by intense shear fields within the extruder rather than by high moisture and temperature gelatinization of granules. They concluded that viscosity substantially decreased as the temperature and moisture content increased due to temperature and moisture induced starch degradation. However, screw speed did not exhibit a major effect on the viscosity as at higher moisture contents, changes in screw speed were not sufficient to induce significant shearing and molecular degradation. Since the shear stress is proportional to torque, an increase in screw speed increased the torque and hence the shear stresses (Huang et al., 1995). This behavior was attributed to an increase in viscosity causing more friction to the flow. The shear stress of the extrudates is often measured with the help of equipments like the Instron Universal Testing Machine, etc.

d) SME (Specific Mechanical Energy)

According to Gilmour and Wang (2002) SME = motor power used/output (kJ/kg)

Or more specifically; SME = (N x T x kW)/R, where; N = percent screw speed; T = percent torque; kW = motor power; and R = feed rate. SME is also known as the ratio of the net mechanical energy input into the extruder to the rate of feed flowing through the extruder

(Sokhey et al., 1994). While extruding starchy materials, various authors have found the SME to decrease with an increase in temperature mainly due to the gelatinization of starch causing a decrease in the apparent viscosity of the mass inside the extruder (Wang et al., 1991; Govindasamy et al., 1997; Guha et al., 1997). SME is also known to be highly influenced by moisture content. For example, Meuser et al. (1989) found that a decrease in product moisture content from 20 to 14% caused an increase in SME from 160 to 200 Wh/kg at a constant barrel temperature (60 °C) and die diameter (2 mm), thus their research concluded that SME could be decreased by increasing product moisture content or barrel temperature or die hole diameter. These findings were in agreement with the findings of their previous work and that of other workers (Meuser et al., 1985, 1987; Senouci and Smith, 1986; Della Valle et al., 1989). SME is a measure of product expansion and is correlated to the melt temperature. An increase in SME and extrudates expansion was observed as a result of reduced moisture due to a change in screw configuration and increased shear (Onwulata et al., 2001a). Incorporation of milk proteins with corn reduced SME input into the extruder significantly and was in the order of corn>casein>whey protein isolates>whey protein concentrates which was also the case with incorporating fiber in the corn product. SME was reduced as fiber increased from 50 g/kg fiber to 125 g/kg (Onwulata et al., 2001b). Unlu and Faller (2002) concluded that SME was directly affected by changes in screw speed and feed rate which also had a significant effect on residence time distribution’s, during extrusion of cornmeal. Also, by increasing the screw speed, a decrease in SME and residence time were observed at constant screw speed due to the fact that SME is a measure of energy absorbed per unit mass of melt (Unlu and Faller, 2002).

e) RTD (Residence Time Distribution)

RTD is a measure of the length of time the feed spends in the extruder. It is a useful means of determining conveying, mixing, cooking, and shearing reactions during the process (Ganjyal and Hanna, 2002). At constant product mass flow, extrusion parameters influence the mean residence time. The influence of these parameters is mainly due to different degrees of filling of the screw profile. Higher amount of filling of the screw chamber increases the "back-mixing" effect which changes the residence time spectrum and also increases the residence time (Meuser et al., 1984). RTD has been commonly used to understand and determine the performance of an extruder. RTD gives and insight about degree of mixing, cooking, and shearing which is helpful in understanding the final product quality (Unlu and Faller, 2002). RTD is usually determined by a stimulus response technique using a tracer. RTD is generally described by E (t) and F (t) diagrams which represent the age distribution of the material in the extruder (Ganjyal and Hanna, 2002). They found that during extrusion of cornmeal, 67% increase in screw speed from 150 to 250 rpm changed the GMRT (geometric mean residence time) from 97.3 to 82.6 s (a 15% decrease) where as increasing the screw speed by 40%, i.e. from 250 to 350 rpm caused a reduction of 11% in the GMRT i.e. from 82.6 to 73.6 s. However, increasing the screw speed by more than 2 times from 150 to 350 rpm the reduction in GMRT was found to be only 24%. Thus it was concluded that the reduction in GMRT did not follow the same ratio as the increase in screw speed (Unlu and Faller, 2002). RTD is characterized by their MRT (mean residence time) and Peclet numbers expressed in the form of distribution curves and is usually determined by a stimulus response technique using a tracer where the stimulus is provided by a color dye (tracer) and the die concentration in the extrudate is measured by spectrophotometry or by reflectant colorimetry (Ganjyal and Hanna, 2002). Peng et al (1994)

found that redness color values significantly overestimated the MRT and RTD spreads and thus concluded that redness color values could not be used to replace the red dye concentrations (converted from redness color values) to determine RTD in twin-screw extruders. Residence time is greatly dependent on variables like screw speed, feed rate, and screw configuration. Residence time is inversely proportional to screw speed, decreases with increasing feed rate, and is influenced by the type, length, and position of mixing element. Residence time was observed to increase as the mixing elements were moved away from the die, as elements were made longer, and the inter spacing between the two elements was increased (Ganjyal and Hanna, 2002).

C. How Machine and Feed variables affect individual components and final product characteristics

1. Gross physical effects on feed

a) Conversion from granular to plasticized mass

Extrusion cooking is characterized by the complex interplay of heat, mass, and momentum transfer in concert with the physical and chemical transformation of the feed material and the conditions that prevail during extrusion (Rizvi et al., 1995). A number of feed materials can be utilized during food extrusion to get desired products. These ingredients undergo various changes including complex physiochemical changes, starch gelatinization and dextrinization, denaturation of proteins, degradation of fats, polymerization of fats/starch/proteins, and many other minor reactions, during extrusion (Lo et al., 1998b). Zuilichem et al. (1999) have defined three different types of mixing operations occurring in cooking extruders having a profound

effect on the ingredients of the feed: [1] coarse or longitudinal/axial mixing; [2] dispersive mixing; [3] distributive or radial mixing.

During extrusion, due to the thermal and mechanical treatment, starch molecules undergo a variety of biochemical reactions such as gelatinization and molecular degradation (Akgodan, 1996). Starch solubilization during extrusion has been reported extensively for both pure starch and cereal products. Starch solubilization is a result of breaking of the granular structure and the large molecular chain splitting (Valle et al., 1994). Guy and Osborne (1996) hypothesized that starch polymers are present in the form of partially crystalline aggregates in cereal derivatives, which during extrusion cooking, are heated and sheared to form new structures and to form a continuous phase due to the unfolding their constituent polymer chains. The precise extent of these physical changes to the native starch is the most important factor influencing the rheology of the material and in turn the structure formation of the product. Gonzalez and Perez (2002) found that extrusion caused several changes in functional, rheological and morphological characteristics of lentil starch. In an experiment to scale up a laboratory extruder, Gonzalez et al. (2001) concluded that the degree of cooking was a good indicator of extruder comparison and rpm. The die l/d ratio or feed moisture level could be easily varied to obtain a particular degree of cooking. Proteins also undergo similar bioconversions aiding plasticization by forming networks and texturized vegetable proteins (TVP's).

b) Puffing/expansion at the die or lack of it

Expansion phenomena are basically dependent on the viscous and elastic properties of the melted dough. The elastic forces attempt to expand the extrudate in the radial direction and contract in the axial direction. While talking about puffing/expansion an important relationship to study these characteristics is the use of expansion ratio (ER). It is defined as the ratio of cross-

sectional areas of the dried extrudates to that of the die (Bhattacharya, 1997). He also defined extrudate density and maximum stress of the dry extrudates. The latter was calculated as the ratio of the maximum force during shearing and the corresponding cross-sectional area of the extrudates. During a study on extrusion of rice and green gram (1:1), Bhattacharya (1997) found a linear positive effect of temperature on the ER followed by a negative linear effect of screw speed. He found that at high barrel temperature (175ºC), an increase in screw speed (250-400 rpm) increased the ER of the extrudates while at low temperatures (100ºC), increasing the screw speed (100-250 rpm) decreased expansion. He also found low density (a desirable attribute of the expanded product) values in extrudates extruded at high temperatures. Moisture level has also been shown to play an important role in ER of the extrudates. Both, the sectional and longitudinal expansion, were found to be negatively affected by feed moisture content (Ilo et al., 1999). They concluded that the increased water content in the melt (rice + amaranth) softened the amylopectin molecular structure and reduced its elastic characteristics decreasing the sectional expansion. High moisture content also decreased the melt viscosity leading to a decrease in the longitudinal expansion. Alvarez-Martinez et al. (1988) and Harper and Tribelhorn (1992) have found similar results while extruding corn meal. However, puffing, during the production of snack food, is desirable but if the product is to be used for developing food for babies or for using the product as the raw material for another product is seldom required (Guha et al., 1997). The above studies conclude that depending on the use of the extrudates, puffing or the lack of it at the die can be achieved by manipulating the temperature, moisture content, and viscosity of the melt. The major effect of increasing moisture is to lower the glass transition temperature so that material exiting the die is able to collapse before it can ‘set’.

c) Texture

During extrusion of low moisture material (rice flour and amaranth blends) Ilo et al. (1999) observed that starch granules were disrupted by mechanical shearing action. The excess fat caused lubrication and reduced the mechanical energy dissipation avoiding dispersal of the starch granules. This action caused the reduction in the amount of gelatinized starch in the melted material and prevented extrudate expansion leading to extrudates having a biscuit-like texture instead of a sponge structure. This problem of excess free fat affecting extrudate texture can be overcome by employing a screw configuration which can provide high shear intensity and also by increasing the residence time in the cooking zone to absorb the free fat. The experimental investigations of Arhalias et al. (2003) have shown that the melt expansion at the exit of the die undergoes two distinct phases: a growth phase followed by a shrinkage phase. The latter was detrimental to the textural quality of the expanded extrudates.

2. Chemical effects

a) On proteins

The nutritive value of a protein depends on the relative amounts of the essential amino acids and the protein's digestibility and bioavailability (Mensa-Wilmot et al., 2001). Protein rich materials behave like non-Newtonian fluids during extrusion (Marsman et al., 1995). Vasanthan et al (2002) reported the interactions between proteins and fiber during extrusion of barley flour, which was observed by analyzing the nitrogen content of the dietary fiber fractions of the extrudates. Proteins undergo cross-linking reactions due to the applied heat and shear that causes denaturation of the proteins. Cross-linking causes the formation of a new molecular aggregate structure (Cindio et al., 2002b). Disulfide cross-linking was found to be the major covalent

binding force for wheat protein aggregation during extrusion processing (Li and Lee, 1997; Rebello and Schaich, 1999). Studies on proteinaceous substrates have focused on the influence of processing conditions on extrudates texture and functional properties. Valle et al (1994) have reported extensively on the effect of extrusion on protein behavior in pea flour. They found that protein transformation during extrusion took place by simultaneous thermo-chemical denaturation and cross-linking which was similar to starch degradation which resulted from melting and chain-splitting. They suggest that at lower energy level extrusion formation of non-covalent and disulfide takes place whereas stronger type of linkages occurs at higher levels of energy. Protein unfolding due to increasing SME enables re-association of protein chains. The hydrogen bonds disrupted during the denaturation process, at low water contents, get recreated between protein molecules instead of between water and protein molecules leading to protein aggregation (Valle et al, 1994). Areas (1992), during extrusion of soy proteins, found that protein extrusion did not necessarily require interactions stronger than the combination of disulfide, hydrophobic, and electrostatic interactions to result in extrudates resistant to retorting at 120 ºC. He argued that if new peptides were formed during extrusion they did not contribute to insolubilization of product, but to the aggregation of protein inside the extruder when the feed was in the melt state. During extrusion of wheat flour, Yeh and Hwang (1992) found that the cooking loss decreased with increase in the protein content. The interactions within the proteins increased gluten networks thereby binding more starch and reducing the cooking loss. Such a loss was mainly attributed to screw profiles mainly having forward elements only causing shorter residence time and low degree of gelatinization which caused release of soluble but ungelatinized starch into the cooking water. During extrusion of soybean meal, shear and heat not only denatured proteins but also enhanced the formation of large protein aggregates and thus

affected the PDI (Protein Digestibility Index) determinations (Marsmen et al., 1995). During extrusion of sorghum-cowpea mixture, Pelembe et al (2002) found that the apparent protein content was not affected by extrusion temperatures but the true protein content (as indicated by nitrogen solubility index) and the quality of the extrudates was affected. The quaternary structure of the proteins unfolds in the hot and moist conditions during HTST (high temperature-short time) extrusion cooking process to produce a viscous plasticized mass. They then undergo polymerization, cross-linking, and reorientation to form fibrous structure (Fellows, 1990). The protein solubility is reduced as a function of temperature due to the thermally induced cross-links among subunits of proteins (Stanley, 1989). Process conditions can change amino acid composition as each amino acid has a specific response to temperature and shear. Although extreme extrusion conditions can reduce protein nutritive value, various researchers have found that nutritional quality only decreased slightly and also found that the iron bioavailability remained unaffected during extrusion of chickpea, corn and bovine lung mix (Cardoso-Santiago et al (2001); Cardoso-Santiago and Areas (2001); Pinto et al. (1997)). The protein quality can be calculated form various indices like: Protein Efficiency Ratio (PER) defined as the weight gain of animals on test diets (g)/ weight protein consumed by animals on the test diets (g), Net Protein Ratio (NPR) defined as (weight gain of test-wt loss by “protein free”) animals/ weight protein consumed by test animals, Relative NPR defined as (mean NPR of test protein) x 100/ mean NPR of reference protein, or True digestibility (% TD) = Nitrogen intake of test animals – (F-Fm) x 100/ Nitrogen intake of test animals where F = fecal nitrogen output by test animals and Fm = fecal nitrogen output by “protein free” animals (Mensa-Wilmot et al., 2001). They emphasized that the most important factor in determining protein quality was the level and proportion of the essential amino acid and next was digestibility of the protein.

The effect of extrusion on the nutritional quality of proteins has been explored in reasonable depth by many researchers (Phillips, 1989; Areas, 1992; Mensa-Wilmot et al., 2001; Marsman et al., 1995).

However, it is difficult, if not impossible, to characterize the spectrum of changes the proteins undergo upon extremely varied natured processing like extrusion. In general, protein bodies may be disrupted or destroyed by all but the mildest extrusion conditions. The native structures of proteins, i.e. the secondary and tertiary configurations, are stabilized by non-covalent, hydrogen, ionic, disulfide, and non-polar bonds as well as weaker, less well-defined forces (Phillips, 1989). The effect of shear and thermal energy input leading to breaking and forming of non-covalent bonds is shown in **Figure 1.4**. Disulfide bonds are the only covalent bonds involved in the stabilization of the native tertiary structure of most proteins. Their disruption helps in protein unfolding and thus digestibility. Various researchers have shown that disulfide bonds contribute to the new and extended protein networks produced by extrusion (Hager, 1984; Phillips, 1989). The process of breaking and forming disulfide bonds during extrusion can be envisaged as shown in **Figure 1.5**. Another important type of linkage susceptible to extrusion is peptide bonds. However, this has been an area of debate amongst researchers. The hydrolysis of peptides does not occur readily even at elevated temperatures, except under extreme pH conditions (Phillips, 1989). However, it has been a strong belief of some authors that peptide bond formation plays a major role in texturization of extruded proteins. **Figure 1.6** shows the possible mechanism by which peptide bonds break and form during extrusion.

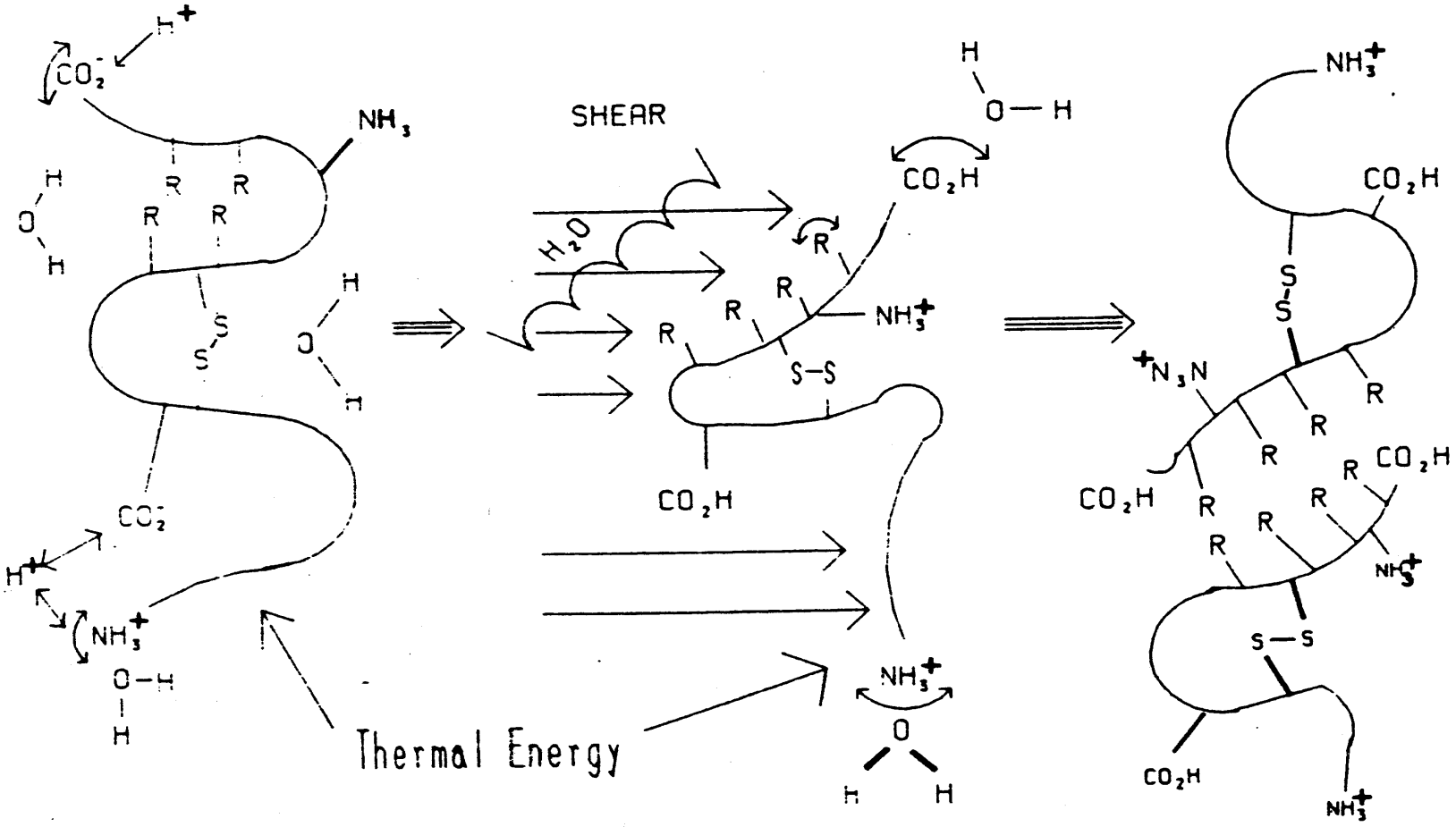

Figure 1.4: Breaking and forming of non-covalent bonds that may occur in extruder (Reprinted from Phillips, 1989. p. 233 by courtesy of Marcel Dekker Inc.).

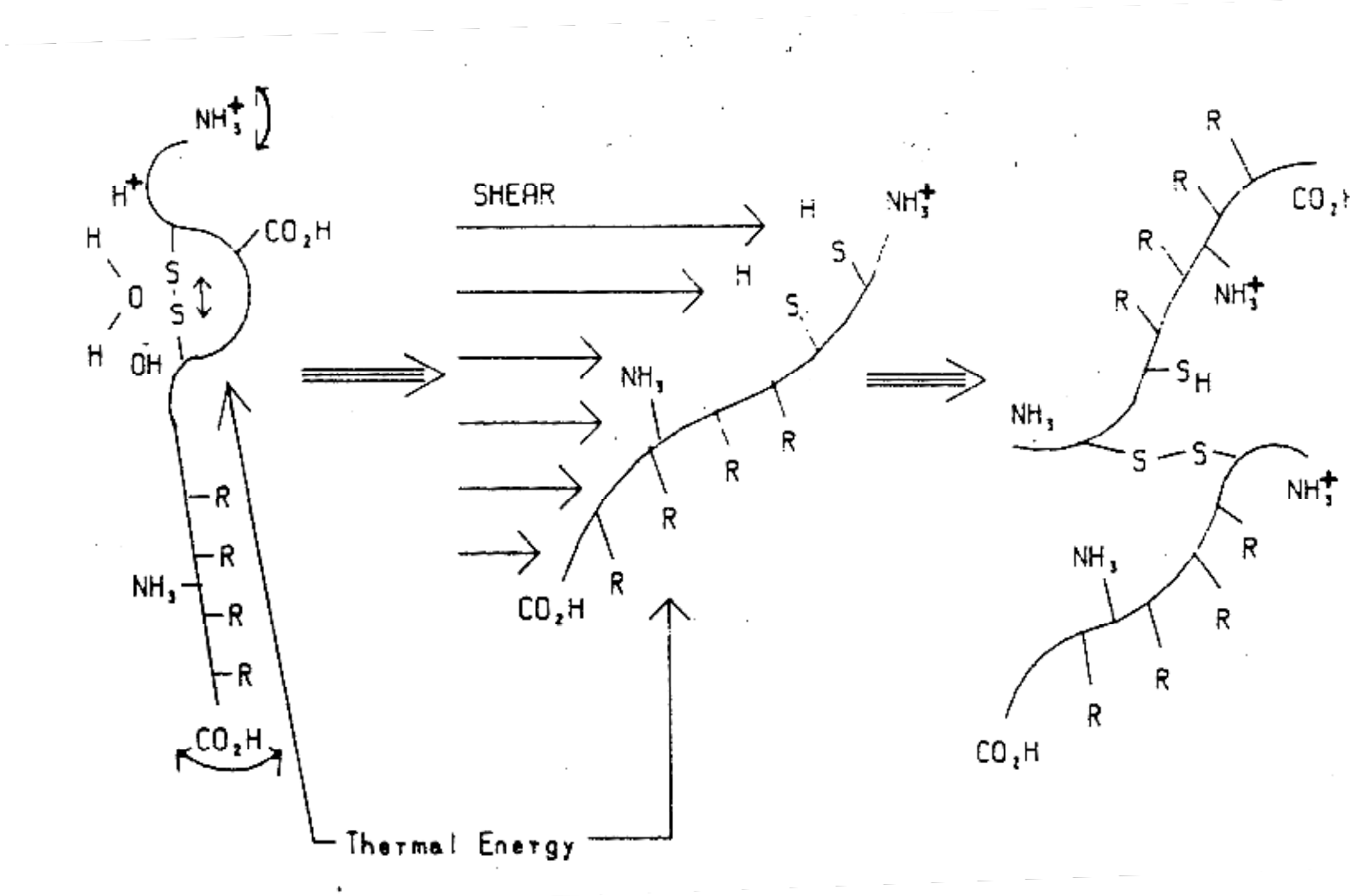

Figure 1.5: Breaking and forming of disulfide bonds that may occur in extruder (Reprinted from Phillips, 1989. p. 236 by courtesy of Marcel Dekker Inc.).

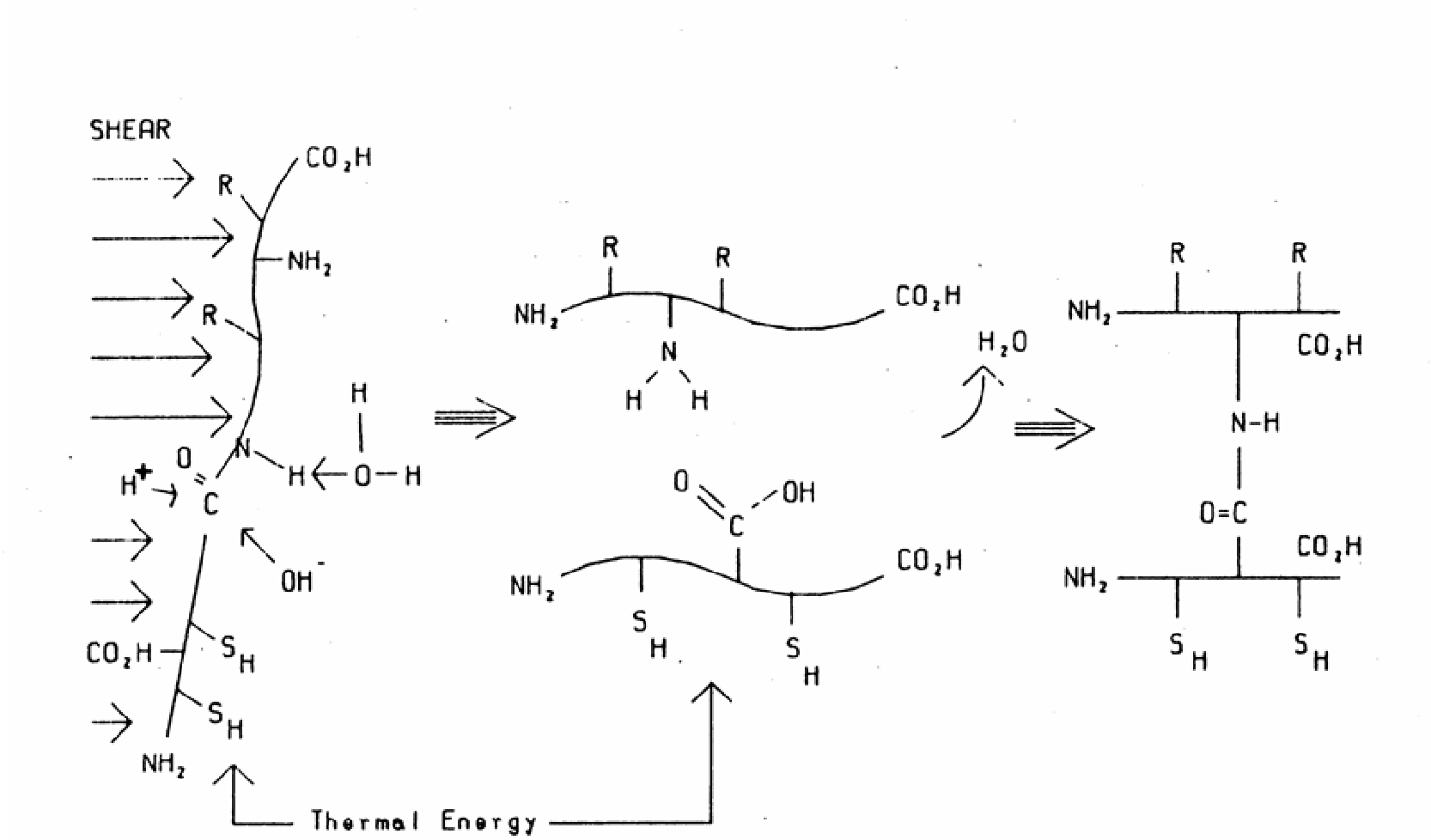

Figure 1.6: Breaking and forming of peptide bonds that may occur in an extruder (Reprinted from Phillips, 1989. p. 237 by courtesy of Marcel Dekker Inc.).

(1) 7S and 11S-type proteins

Plant seeds accumulate massive storage proteins in discrete vesicles termed protein bodies as a source of amino acids for the use during germination and post-germinative growth of seedlings. Based on their solubility in various extraction solvents, seed storage proteins can be classified into four groups: water soluble (albumins), dilute saline soluble (globulins), alcohol/water mixture soluble (prolamins), and dilute acid or alkali soluble (glutelins). The globulins are further divided into two subgroups according to their sedimentation coefficients: 7S vicilin-type and 11S legumin-type globulins (Tai et al, 2001).

Despite the sensitivity of the immunoassay test, the protein fraction has to be present in the material at a concentration high enough to be detected after extrusion. Taking these factors into account, legumin, and more generally the 11S-type plant proteins, appear well suited to such studies: they are the major storage proteins in many seeds; they denature at relatively high temperatures and are oligomeric proteins which can exist in various intermediate forms of denaturation depending on the medium and processing conditions (Valle et al., 1994). There are many differences between the 7S and the 11S (globulins) proteins depending on their different molecular weights, amino acid composition and sequence, carbohydrates content, isoelectric points and their flexibility under the conditions of the system which all play a role in determining their functionality (Molina et al., 2001). Though there is limited knowledge about peanut proteins, soy and other seed proteins have been extensively studied. SDS-Page of the 7S and 11S isolates of soy proteins showed that 7S was comprised of α, ά, and β-7S subunits having molecular weights of 80 kDa (α an ά) and 55 kDa (β) and 11S was comprised of acidic and basic sub-units having molecular weights of 43 kDa (acidic) (A_3), 37 kDa (A1, A2, A4) and 22 kDa (basic) (Molina et al. (2001). In soy proteins the 7S (trimer without any disulfide bonds) was

found to be more pressure liable than 11S as its sub-units are linked by disulfide bonds (Molina et al., 2001). During analysis of extruded coffee grains Rogers et al. (1999) reported that 11S reserve proteins are stored as large complexes of subunits linked by disulfide bonds having molecular weights between 300 and 400 kDa. Under reducing conditions, rupture of these bonds liberates acidic (α) and basic (β) subunits as individual components.

b) On starch

A high fraction of all human food energy is in the form of cereals and starchy foods. In addition to food energy provided by starch, it provides much of the texture, mouth feel, and structure of the foods (Harper, 1981). When starch granules are heated in the presence of water, gelatinization of starch takes place. During gelatinization, starch may undergo hydration, swelling, heat absorption, and loss of crystallinity (Wang et. al, 1991). During extrusion cooking process starchy materials are heated under pressure and adequate moisture contents giving rise to not only starch gelatinization but also melting and also dextrinization under some conditions. To understand starch modification during extrusion in detail, starch transformations must be analyzed at three levels: molecular, crystalline, and granular (Colonna et. al, 1989). During a study on chemical modifications of starch, Colonna et al (1989) found that the monomer D-glucose did not get modified by extrusion. This means that the starch content of extruded products does not change during extrusion, demonstrating that no new linkages are created despite high temperature and pressure conditions. However, substantial research data indicate that starch undergoes degradation giving rise to low molecular weight starch fractions. During extrusion, granular starch is constantly compressed and transformed into a dense, solid, and compact material causing its granular and crystalline structure to disappear. The starch phase becomes homogenous due to the shearing of the molten granules. When the temperature

increases, molecules slide past one another due. Here the polymer becomes viscous, rubbery, and flexible. Under these conditions of moisture, shear, and pressure, the temperature in the extruder is within the range of T_g (glass transition temperature) for starch. Extruded starches appear to be composed of a continuous amorphous phase containing interspaced crystalline regions made from complexes like amylase-lipid. The crystallization of these complexes occurs during the cooling of the melt, during which the motion of the molecular chain freezes in a random conformation making the extruded starches solid and glassy (Colonna et al, 1989). Thus starch undergoes certain degree of fragmentation and modification giving rise to specific extrudate characteristics.

c) On other components and interactions

1. *Nutritional & chemical modification during extrusion*

Chemical constituents of dough are brought into intimate contact in a high shear and high temperature environment, during extrusion. This can sometimes improve or at times damage the nutritional quality of proteins, carbohydrates, lipids, or other reactive/nutritional species (Phillips, 1989). The effect of extrusion on nutritional modification of extrudates has been extensively studied. The incorporation of protein rich materials like whey into extruded products will not only increase the utilization of whey products but also improve the products nutritive value by increasing the protein content (Onwulata et al., 2001a; 2001b). The incorporation of foods with proteins and in some cases fibers into foods provides a nutritional “psycho-social” beneficial product quality which aids in the product’s acceptance by consumers. For example, combining components which would normally reduce expansion like milk proteins and wheat bran fibers enhances the nutritional profile and acceptance of the extruded snack product (Onwulata et al., 2001a). An increase in the total dietary fiber (TDF) was observed in barley and

wheat flour upon extrusion (Vasanthan et al, 2002; Bjorck et al, 1984). Extruded lentil starch exhibited the lowest value of water absorption, solubility, and swelling power (Gonzalez and Perez, 2002). While extruding a mixture of maize, cowpea, and peanut or soybean, Mensa-Wilmot et al. (2001) found that the extrudates were nutritionally adequate and could be utilized for infant weaning food supplementation feeding. The interactions amongst food components, during extrusion processing, can vary depending on the raw material involved and can either have a positive or negative effect on the bioavailability of nutritional species like iron. During extrusion of chickpea, corn, and bovine lung Cardoso-Santiago et al. (2001) found that the iron content was significantly higher in extrudates containing bovine lung in their formulation compared to some commercially available similar type of corn extrudates. Such products can be a good source of iron for malnutrition programs and can have a positive impact on anemia prevalence. The increased iron content of the product after extrusion has been described by various authors. This increase has been attributed to the wear and tear of metallic pieces, mainly screws, of the extruder and the resulting contamination of the product (Hazell and Jolinson, 1989; Pinto et al., 1997; Alonso et al., 2001). A number of substances can reduce the bioavailability of vitamins and minerals due to the formation of extremely insoluble salts or poorly dissociated chelates or destruction by temperature enhanced reactions, including oxidation (Alonso et al., 2001). Thus a heat treatment like extrusion can cause total or partial destruction of antinutritional factors like protease inhibitor, haemagglutinins, tannins and phytates which interfere with absorption of nutrients in legume seeds by polymerization, change in chemical activity, or hydrolysis of these molecules (Alonso et al., 2001). They also found that there was an increase in mineral absorption by rats fed with extruded pea and kidney bean extruded meal which was due to destruction of polyphenols during extrusion.

2. *Maillard reactions*

"Maillard reaction" (MR) is a complex reaction. During heat processing like extrusion, a reducing sugar reacts with a free amino group causing MR to take place. MR products can be classified into two groups: the melanoidins, which are polymeric materials, and the low molecular weight colored compounds, which comprise of two to four linked rings (Ames et al., 1998). This reaction is often desired as it causes the development of the golden brown color and caramel aroma in bakery products and cooked foods, but on the other hand, it is also responsible for the loss of nutritional value of the product (Cindio et al., 2002). Lysine, an essential amino acid found in cereals, undergoes a loss in bioavailability and is involved in Maillard reaction during extrusion (Harper, 1981). During extrusion of sorghum-cowpea mix, Pelembe et al (2002) found that higher temperature extrusion resulted in darker products due to non-enzymatic browning (Maillard reactions) favored by higher temperatures. However, Feillet et al. (2000) concluded that kneading and extrusion had no significant effect on brownness during extrusion of pasta. Prost-extrusion drying was more responsible for the formation of brown melanoidin pigments due to the development of MR related to the reducing-sugar content of pasta.

3. *Cross linkages and Texturization*

High yield and low granule friability occurred during extrusion of α-lactose monohydrate. Due to densification of wet mass in the extruder and its excellent mixing performance, extrusion caused more bonds to form between individual particles by providing a good distribution of granulating liquid (Keleb et al., 2002). The addition of proteins to starchy materials increases the sites for cross-linking, and upon extrusion causes textural quality changes (Onwulata et al., 2001b).

The Texturization of vegetable protein through extrusion cooking is a remarkable example of successful technology allowing about 71 million tons of soy to be transformed and consumed directly or in various forms every year (Areas, 1992). The utilization of soy meal, the most common starting material for the production of meat analogs, is a classic example of extrusion induced texturization (Stanley, 1989). Protein texturization is often achieved in conditions of high temperature and high shear believed to be taking place in the metering section or reactor zone (towards the die end).

D. Extrusion Products

The application of extrusion techniques has had a positive influence, in many respects, on the production of foods. Its advantages are evident, specifically in the simplification of processing techniques for the manufacture of existing products, as well as in development of new and novel type of foods. These advantages are due to the operating mode of the extruder, where the feed conveying through the machine undergoes a short input of mechanical and thermal energy which is sufficient to bring about changes in structure and composition similar to those caused by conventional food-processing operations such as cooking, baking, roasting, and drying (Meuser and Wiedmann, 1989). Extrusion can thus be applied in the production of existing foods whose shape and form can be reproduced at the die where the energy transfer exerts effects on sensory characteristics similar to those about by conventional methods. Extruders can be used to mix, knead, disperse, plasticize, gelatinize, texturize, dissolve, cook, melt, roast, caramelize, sterilize, dry, crystallize, react, and shape. The following table (**Table 1.2**) gives a list of example of products that can be manufactured on an extruder system v/s the conventional processes employed for their production (Meuser and Wiedmann, 1989).

Table 1.2: Example of products made by Extrusion as compared to conventional processes employed for their production.

Product Group	**Product Example**	**Conventional Process**
Modified grain flours	Baby foods	Drum dryer
Chemically modified starches	Phosphate starches	Stirred kettle reactors
Texturized plant proteins	Meat substitutes	Spinning system
Animal feeds	Aquaculture Pet food	Presses Autoclave/Oven
Milk	Caseinate reaction	Stirred kettle reactors
Flavors	Meat flavors, Caramel	Roasting kettle
Baked goods	Flatbreads, Bread crumbs	Baking oven
Breakfast cereals	Puffed grains Cereal flakes	Puffing-gun Cooker
Pasta	Oriental and fish noodles	Cooker
Drinks	Beer Coffee Instant powders Instant cocoa	Kiln Roaster Cooker Alkalizing kettle
Confections	Liquorice, Fruit gums Chocolate Sugar	Cooker/Mogul Press Crystallizer

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CHAPTER 2

THE EFFECT OF EXTRUSION ON PROTEIN AGGREGATION IN MIXTURES OF PARTIALLY DEFATTED PEANUT FLOUR AND CORN STARCH[1]

[1]Shah, A., M. Corredig, L. Chen, and R.D. Phillips, 2003. To be submitted to Journal of Food Science.

ABSTRACT

The present study was conducted to understand the major changes at conformational and aggregation levels on the spectrum of proteins comprised in peanut flour upon the process of extrusion. The studies were conducted with samples subjected to two defined extrusion conditions designated as E1 (severe: high temperature and low moisture) and E2 (less severe: low temperature and high moisture). To thoroughly understand the changes taking place to the component proteins of peanut flour, protein isolation was done using three buffer systems namely phosphate buffer (stage 1); phosphate buffer with urea (stage 2) and phosphate buffer with urea and DTT (stage 3). A high protein content shown by the stage 2 and stage 3 extracts of extruded peanut-flour samples indicated that the process of extrusion results in the formation of aggregates which are stabilized by non-covalent and disulphide linkages. These aggregates are insoluble in non-denaturing buffers. This observation was further confirmed by Agarose gel electrophoretic analyses of stage 1, 2, and 3 samples of peanut flour extrudates. SDS-PAGE analyses showed negligible difference in the overall migration patterns of the proteins in the un-extruded and extruded samples. However, this analysis reflected into a variation in the relative amounts of proteins in various samples. Detailed studies using molecular sieve and ion-exchange chromatography also revealed that during extrusion the proteins belonging to peanut flour undergo various interactions involving non-covalent and disulphide linkages to result in the formation of aggregates which are either highly positively charged or moderately negatively charged. Further these studies showed that formation of these aggregates is more favored by less severe conditions of peanut flour extrusion.

INTRODUCTION

Peanut belongs to the genus *Arachis*, which includes more than 20 described species of tropical and subtropical plants in the legume family. Since peanut has a high oil content (~50%), a large percentage of the world production is used as oil for cooking or salads. About 70% of the production in the U.S. is used in domestic food products, including peanut butter, salted products, confections, and roasted nut (Isleib and Wynne 1992).

Peanut proteins are classified as water soluble (albumins) or saline soluble (globulins). Globulins make up 87% of the total proteins (Basha and Cherry 1976) and are the main constituents of the storage proteins. Globulins like arachin (14S), conarachin II (8S), and conarachin I (2S) make up almost 75% of the peanut total proteins (Prakash and Rao 1986). Yamada and others (1979) conducted SDS-PAGE analyses and found that arachin existed as a large molecule of 600 kDa which can be broken into approximately six subunits ranging in size from 19 to 42 kDa. Conarachin II has a molecular weight of 290 kDa (Prakash and Rao 1986) and was found to contain six to eight subunits with molecular weights ranging from 84 to 23 kDa upon SDS-PAGE (Basha and Cherry 1976; Yamada and others 1979).

Extrusion is one of the most versatile and well established food processes and is widely used in the food and feed industry to make products such as snacks, cereals, pasta, texturized vegetable proteins, pet food and animal feed (Rizvi and others 1995). During extrusion cooking of peanut flour, unique processing features are present because the defatted peanut flour is subjected to high pressure in combination with severe shear forces and high temperature.

During extrusion, proteins are susceptible to both conformational changes and chemical reactions. It has been demonstrated that plant proteins can be texturized into fiber-like bodies (Phillips 1989; Stanley 1989; Araes 1992). The mechanism of protein denaturation was

described by Phillips (1989). As the temperature and water increase, the proteins loose their native globular, three-dimensional shape and unfold. This unfolding allows the relatively linear protein chains to reorient and recombine. Due to the aligning of these protein molecules, reactive sites on adjacent molecules come sufficiently close so that intermolecular bonds and this maintains the denatured fiber state. Chemical reactions involved during extrusion include modification of amino acid side chain, hydrolysis of peptide bonds, and the formation of new covalent cross-links. Cross-linking reactions are either disulfide type, nondisulfide type, or, formation of covalent isopeptides.

Some studies conducted on extrusion of soy and wheat proteins reported that extrusion processing could alter protein structure and solubility by heat, shear force, pressure, and oxygen (Phillips 1989; Ummadi and others 1995; Li and Lee 1996a) and also influence the protein digestibility of the products (Li and Lee 1996b). The type of protein-protein interactions occurring during extrusion processing has been examined by extraction of the extruded material with different solvents (Lee and others 1997; Marsman and others 1998; Rebello and Schaich, 1999). In general, non-covalent interactions are disrupted by using buffers containing urea and sodium dodecyl sulfate (SDS), and Dithiothreitol (DTT) or 2-mercaptoethanol are employed to disrupt disulfide bonds formed during extrusion (Hager 1984; Areas 1992). By treating extruded proteins with various buffers it is possible to extract proteins depending on their interactions formed during extrusion (Li and Lee 1996b).

The aggregation of proteins during extrusion is responsible for the decrease in their solubility, and the formation of new disulfide cross-links during extrusion processing seems to be the major covalent force for protein aggregation (Areas 1992; Strecker and others 1995; Li and Lee 1996a). Hager (1984) showed that disulfide bond formation was the most important

interaction during extrusion cooking of soybean meal concentrates, while Ning and Villota (1994) concluded that noncovalent interactions appeared to be the driving force in soy protein structure formation. Various studies have indicated that that both disulfide and noncovalent interactions are important in protein structure formation during extrusion and upon cooling (Cheftel 1986; Areas and Prudencio 1991; Areas 1992).

The relationship of extrusion parameters such as temperature or moisture content on disulfide bond formation and aggregation of peanut proteins had not been explored in detail. In addition, it is still unclear what the overall mechanism of protein interaction is during the extrusion process. A better understanding of the protein-protein interactions as a function of extrusion parameters would increase the application of extrusion in improving functional and textural properties of protein sources such as peanuts. To date, little results have been reported on peanut protein interactions induced by extrusion processing. This work reports a study of the protein aggregation of defatted peanut flour after extrusion.

Critical transformations of protein molecules takes place in the metering section or reactor zone in an extruder. However, the protein-protein interactions that are responsible for final product texture or texturization of proteins remain unknown and this has been the main limiting factor for the use of extrusion to its full potential in protein production. Addition of proteins to starch-rich flour, above a certain level, has known to change the behavior of transformation into a protein-type extrudate where less expansion occurs and the products are harder and more resistant to water disruption (Areas 1992). Due to the diversity of monomer composition, a wide range of interactions are possible for protein cross-linking with itself or other macromolecules. Thus, hydrophobic, cation-mediated electrostatic interactions and covalent bonds contribute to the stabilization of the three-dimensional network formed after

extrusion of proteins (Areas 1992). The dependence of product variables (e.g. expansion ratio, etc.) on process variables (e.g. temperature, pressure, moisture content, etc.), during the extrusion of proteins, often present a complex behavior. This can only be credited to the diverse and complex molecular interactions that occur during protein extrusion, many of which are understood poorly. Model systems for extrusion, reproducing conditions of particulate matter in a continuous melt containing polymers able to cross-link at the die exit, can be envisaged and would certainly provide a new understanding of the technique. Thermal resistant probes for further spectroscopic detection could be attached on soluble and potentially insoluble aggregates during processing in order to monitor their distribution after extrusion, as well as changes in their aggregation, which could then be related to improved network formation. Better flow can be obtained through careful exploitation of lipid-protein interaction of the raw protein, addition of reducing agents to the feed, and controlling parameters like moisture, pressure, and temperature. Moderate oxidants to favor disulfide cross-linking can be postulated to stabilize the final three-dimensional protein network. However, with the new generations of extruders available today, it is possible to add additives of opposing effects in distinct sections of the extruder to intensify cross-linking in the proteins. Compromise in additive use and extrusion conditions are necessary in order to achieve objectives and allow novel products to be obtained. The enormous potential of extrusion cooking to produce texturized protein of several origins is yet to be fulfilled. This process can improve functional characteristics of the protein source without loosing nutritional quality, provided the right sets of processing variables are used (Phillips 1989). However, the realization and use of this technology will certainly expand after a better understanding of the molecular mechanisms that govern the behavior of proteins in the flow, in the extrusion process itself, and in the final extrudates (Areas 1992).

The extruder is a relatively simple machine in contrast to the complexity of changes the proteinacious feed to be extruded, undergoes. The breaking and forming of intermolecular and intramolecular bonds, aggregates, and protein structure has been most instrumental in final product characteristics and protein texturization (Jeunink and Cheftel 1979; Rhee and others 1981). Cross-linking, aggregation, and de-aggregation of native proteins during extrusion has been influential in improving the digestibility and nutritional quality of proteins (Mensa-Wilmot and others 2001) and may also lead to improved or customary protein texturization (Phillips 1989).

To understand and characterize some of the various changes occurring in native peanut proteins upon extrusion, this study has been carried out. Some of the techniques used to understand peanut proteins before and after extrusion include Agarose gel electrophoresis, SDS-Page, Molecular gel exclusion chromatography, and Ion-exchange chromatography.

MATERIALS AND METHODS

1. Materials

Peanut flour (containing 6% fat) and the two extruded samples were procured form Linyung Chen, Department of Food Science and Technology, The University of Georgia, Griffin, GA.

2. Extrusion

Extrusion was carried out on an APV co-rotating twin-screw extruder (model MPF1700-30, APV Baker Limited, Peterborough, England) having a 1x20mm slit die. The barrel had a length to diameter ratio of 25:1 and consisted of four independent heated/cooled zones and a temperature control zone at the die. Barrel and die temperatures were set at 115 or 135 ºC, except

the zone close to the feeder was 100 ºC. Screw speed was set at 500 rpm. A screw configuration recommended by the manufacturer was used to impart sufficient mechanical energy to the feed material as described in **Table 2.1**. Peanut-cornstarch meal (50:50) was fed into the extruder at the rate of 10 Kg/hr (dry basis). Desired moisture level (20% or 40%) was achieved by pumping a desired amount of water calibrated into the barrel, during extrusion, by a proportioning pump (Bran and Lube, type N-P31, Buffalo Grove, IL). The two extruded products were named E1 and E2 and were a result of the following extrusion conditions: Extruded-1 (E1) - 500 rpm, 20% moisture, 135 °C, 50% Cornstarch, and Extruded-2 (E2) – 500 rpm, 40% moisture, 115 °C, 50% Cornstarch. Cornstarch was used not only as a plasticizing agent to facilitates transfer of shear energy to the peanut proteins, but also for the fact that this peanut-cornstarch mixture led to a model system that examined the effects of extrusion cooking of complex mixtures similar to 'real foods'.

Table 2.1: Screw configuration used in this study.

Screw Element	Length of each element (D)	Number of elements in configuration
2 lead F	1.50	3
2 lead F	1.00	1
30º F	0.25	5
2 lead F	1.50	3
2 lead F	1.00	1
60º F	0.25	5
60º R	0.25	4
2 lead F	1.50	2

2 lead F	1.0	1
60º F	0.25	6
60º R	0.25	4
2 lead F	1.00	2
60º R	0.25	4
1 lead F	1.00	1

F = forwarding screw element (paddles); R = reversing screw element (paddles); D = barrel diameter (as a unit of barrel length).

3. Chemicals

Sodium phosphate, sodium chloride, urea, dithiothreithol (DTT) were obtained from Fisher Chemicals (Fair Lawn, NJ).

4. Sample preparation

The extruded samples were ground in a coffee mill (Philadelphia, PA) and the defatted peanut flour and the ground samples were sieved and samples retained on 50-µm sieve were collected, stored, and used for the experiment.

5. Protein Extraction

Peanut protein from unextruded and extruded samples was extracted in three sequential stages with phosphate buffer, phosphate buffer containing urea and phosphate buffer containing urea and DTT as summarized in Figure 1. Approximately 2.0 g of each ground sample were dispersed in 20 ml of 20 mM sodium phosphate buffer (buffer 1; pH 7.9) containing 0.5 M sodium chloride for a 3-stage sequential extraction procedure. The samples were incubated at room temperature for 1 h and vortexed every 15-min. Samples were then subjected to sonication, to enhance protein solubilization, as described by Li and Lee (1996b) with some modifications.

A high intensity ultrasonic processor (Danbury, CT) was used with a macro tip probe (3 mm diameter). The sample was immersed in ice bath (to prevent heat buildup) and the sonicator probe was inserted into the liquid surface to a depth of approximately 2.5 cm from the surface. 20 sonication cycles, each of 1 min in length, were done with 30 s halt between each cycle. The sonicator was capable of providing 20 kHz and the amplitude control knob was set to 60. The mixture was then centrifuged at 8000 g for 15 min at 10 °C with a Sorvall RC-5B centrifuge (DuPont, CN). The supernatant was filtered through filter paper (#1 Whatman, Kent, England) and the residue was washed with 10 ml of the same buffer. The washed residue was then incubated for 30 min at room temperature and vortexed every 10 min, and then centrifuged at 8000 g for 10 min at 10°C.

The filtered supernatant and the supernatant of the washed residue were combined, extensively dialyzed in deionized water and then freeze-dried (Unitop 600L, Gardiner, NY). These samples, extracted with phosphate/sodium chloride buffer, were termed as stage-1 samples. To the residue of stage-1, 20 ml of 20 mM sodium phosphate buffer (buffer2; pH 7.9) containing 6 M urea was added. This sample was similarly sonicated for 15 cycles. The mixture was then centrifuged at 8000 g for 15 min at 10 °C, and the supernatant was filtered and collected for protein analysis. The residue was washed with 10 ml of the same buffer and centrifuged at 8000 g for 15 min at 10 °C. The two stage-2 supernatants were combined and freeze-dried. These samples, soluble in Urea, were termed as stage-2 samples. To the insoluble residue of stage-2, 20 ml of phosphate buffer (buffer 3; pH 7.9) with 6 M urea and 0.01 M DTT, was added and similarly sonicated for 10 cycles and centrifuged at 8000 g for 15 min at 10 °C. The supernatant was filtered and collected for protein analysis and the residue was washed with 10 ml of the same buffer and similarly centrifuged, supernatant collected, and combined,

dialyzed, and freeze dried. These samples, soluble in Urea and DTT, were termed as stage-3 samples (**Figure 2.1**).

The concentration of protein in the samples, before freeze drying, was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) based on the method of Bradford, by adding the acidic dye to the samples and measuring the absorption at 595 nm in LKB Biochrom Ultrospec 4050 (Cambridge, England).

6. Agarose Gel Electrophoresis

To determine differences in the size of the aggregates extracted with various buffers depending on extrusion conditions, SDS-Agarose homogeneous gel (0.4%) electrophoresis was conducted on the freeze-dried samples. The analysis was performed as described by Alting and others (2000). Briefly, the electrophoresis buffer consisted of 100 mM Tris, 50 mM sodium acetate, 2 mM EDTA, and 0.1% SDS and brought to pH 7.9 using concentrated acetic acid. Two mg of UE, E1, E2, and the buffer soluble extracts (of all three stages) were mixed with 400 µL of a buffer consisting of 20 mM Bis-Tris (pH 7.9) and 5% SDS. After overnight incubation at room temperature each sample was divided into two equal halves (having equal volumes). To one set of samples disulfide reducing agent DTT (0.05%) was added, vigorously vortexed and incubated for 1 hr. Prior to electrophoresis, 5% of a solution containing 60% glycerol and 0.002% bromophenol blue was added to the samples, and 20 µL of the sample were loaded on the Agarose gel. The gel was run with a constant voltage of 30 V for 5 hours in a Midicell Primo EC330 (Holbrook, CITY NY). After the run, the gel was carefully removed and washed three times with demineralized water and stained with Bio-Safe Coomassie (Bio-Rad, CA) for 48 hours and then destained using demineralized water for 48 hours. The gel was then scanned using Imaging Densitometer GS-700 (Bio-Rad, CA).

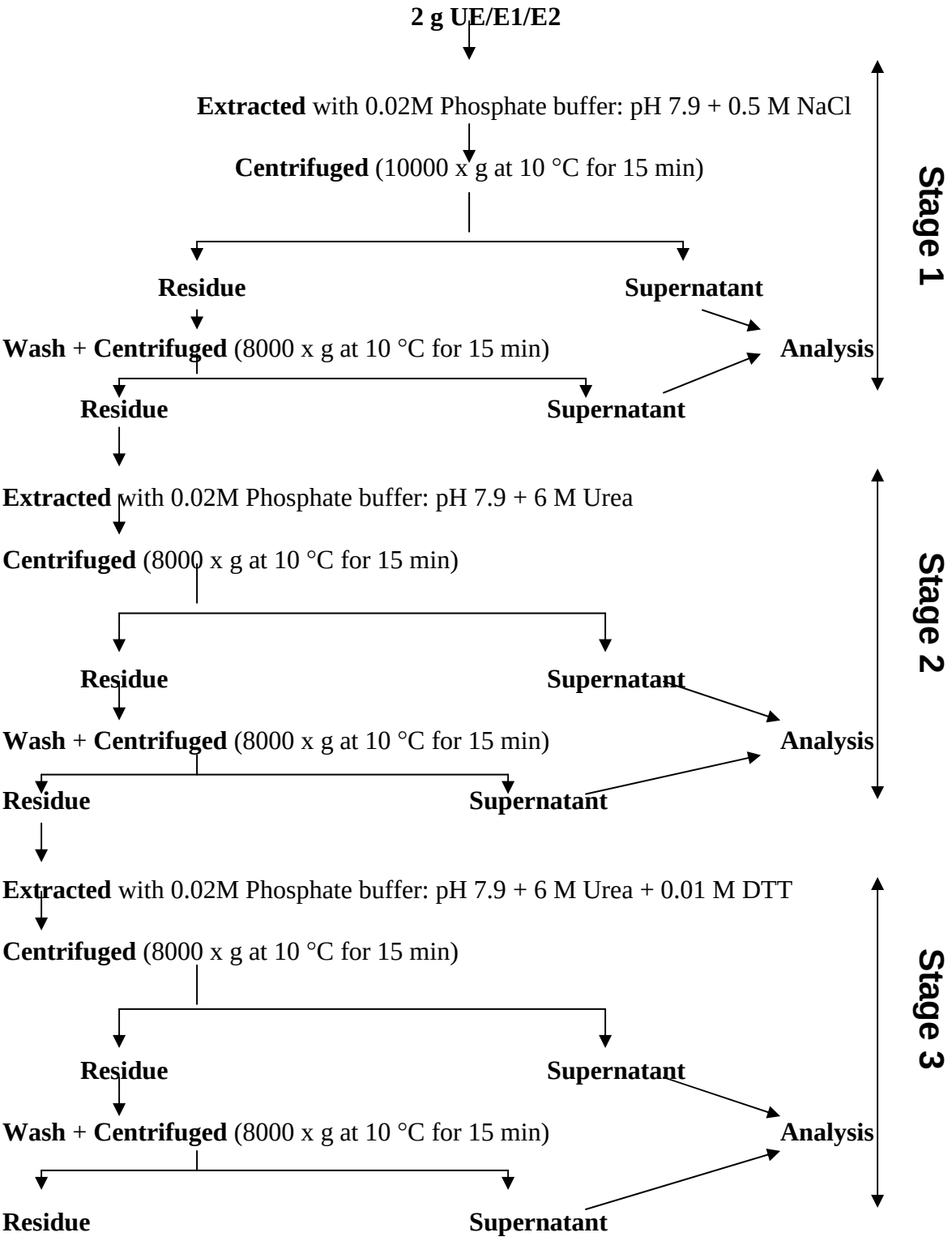


Figure 2.1: Schematic diagram of the sequential extraction process. UE (Control) = unextruded peanut flour – cornstarch (1:1) mixture; E1 = sample extruded at 135 ºC and 20% moisture; E2 = sample extruded at 115 ºC and 40% moisture

7. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Protean II electrophoresis system (Bio-Rad, Hercules CA). To 2 mg of freeze-dried samples, sample buffer (450 µL) (0.5 M Tris-HCl pH 6.8, Glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue) was added. Samples were then boiled for 5 min and then were centrifuged in an eppendorf centrifuge (Brinkmann, City NY) for 2 min. Aliquots (10 µL) were loaded on a 4-20% Tris-HCl gel of 13.3 x 8.7 cm having twelve 20 µL wells, the effective size range of the gel was ~ 10-200 kDa (Bio-Rad, Hercules, CA). Kaleidoscope molecular weight standard (Bio-Rad, CA) was also loaded on the gel along with the other samples. The gel was introduced in the Protean II system with 10x running buffer (Tris, Glycine, and SDS). Upon the completion of run, the gel was removed and stained in a staining solution (40% methanol, 10% acetic acid, 0.1% coommassie blue R-250, 500 ml water) over night. It was then destained for 5-6 hours and dried in drying solution consisting of methanol, ethanol, acetic acid, and water for 30 min. The gel was air dried, and scanned using an imaging densitometer (GS-700, Bio-Rad, CA and Molecular Analyst software, Bio-Rad, CA).

8. Chromatographic analysis

Twenty mg of freeze-dried samples, prepared from the various buffer extractions, were dissolved in 20 ml of 20 mM sodium phosphate buffer (pH 7.9). The samples were filtered through 0.8 µm and 0.45 µm syringe filters (Millipore Corporation, MA). Samples (100 µL injections) were loaded on the a size exclusion column 10 x 300 mm (Superdex 200, Amersham Biosciences, City NJ) column, eluted with 20 mM sodium phosphate buffer (pH 7.9) containing 0.15 M NaCl. The chromatographic column was calibrated using known protein standards.

Samples were also analyzed by ion exchange chromatography using 20 mM sodium phosphate buffer (pH 7.9) containing 6 M urea. 20 mg of freeze dried sample was dissolved in running buffer and then filtered through 0.8 µm and 0.45 µm syringe filters (Millipore Corporation, MA). A HiTrap Q HP (Amersham Biotech, NJ) column was mounted on Akta Purifier (Pharmacia, NJ) and used for the chromatography. The column was equilibrated with 5 column volumes of the buffer before loading the samples. 1 ml samples were loaded on the column for analysis. The samples were eluted with a gradient of 0-1.0 M sodium chloride in the same buffer. Fractions of 1 ml were collected and extensively dialyzed against deionized water.

RESULTS AND DISCUSSION

Protein Content:

The protein content of the buffer soluble extracts was estimated and used to evaluate the protein solubility profile of the samples as shown in **Table 2.2**:

Table 2.2: Protein content of buffer soluble extracts of the three stages in mg/ml.

Sample	Stage 1	Stage 2	Stage 3
UE (Control)	119	96	50
E1	47	102	60
E2	44	91	66

UE (Control) = unextruded peanut flour – cornstarch (1:1) mixture; E1 = sample extruded at 135 ºC and 20% moisture; E2 = sample extruded at 115 ºC and 40% moisture

More protein was solubilization in urea buffer (stage 2) in extruded compared to unextruded samples, showing the predominance of non-covalent bonds in both the extruded products. Thus it can be asserted that extrusion causes the formation of a large number of non-covalent interactions in extrudates. Furthermore, the high protein content of the stage-3 extracts

indicates the presence of disulfide bonds that are broken down by the incorporation of DTT in stage-3 buffer. Thus the increase in the amount of soluble protein in the stage-2 & stage-3 extracts of extruded samples shows the formation of new interactions like non-covalent and disulfide bonds due to extrusion. Amongst the stage-1 samples, UE showed to have the highest protein content, almost three times as that of E1 and E2 indicating the presence of aggregates in the extruded samples, insoluble in sodium phosphate buffer. Protein content was highest for E1 in the stage-2 extracts showing the presence of protein interactions cleaved by urea (present in the stage-2 buffer), namely non-covalent interactions as compared to high protein content of E2 in stage-3 extracts indicating the presence of protein-protein interactions cleaved by the incorporation of DTT in the buffer, namely disulfide bonds. These results highlight the fact that extrusion causes the formation of newer non-covalent and disulfide linkages. However, it was interesting to note that more buffer soluble protein was found in E1 while comparing the stage-2 extracts indicating the prevalence of non-covalent linkages where as the high amount of buffer soluble protein found in E2 while comparing stage-3 extracts indicate more disulfide interactions forming as a result of high moisture-lower temperature extrusion. We cannot rule out the possibility that in E1 (extruded under comparatively severe extrusion conditions) the severity of extrusion may have caused a further disruption of disulfide linkages giving a lesser value of buffer 3 soluble proteins. In a study on extrusion of soy proteins, Areas (1992) found that at all temperatures, disulfide linkages, followed by non-covalent interactions were the prevalent type of protein-protein interactions in the extrudates. They also noted that a further increase in the severity of extrusion resulted in a proportional decrease in disulfide linkages. Our results also show a decrease of disulfide linkages with increase in severity of extrusion conditions. Such

denaturation of proteins and disruption of the food matrix has shown to improve the digestibility of proteins and other nutrients (Mensa-Wilmot and others 2001).

Agarose Gel Electrophoresis:

Studying the molecular mechanisms of protein-protein interactions that take place during extrusion is very difficult. Researchers have documented various techniques like buffer solubilization (Barres and others 1990; Areas 1992), gel filtration & ion-exchange chromatography (Monteiro and Prakash 1994), SDS-Page (Aufrere and others 2001), electron microscopy (Stanley 1989), scanning calorimetry & spectroscopy (Stanley 1989; Areas 1992), etc. to monitor and identify the possible chemical, structural, and physiological changes the proteins undergo upon heat treatment. A protein solubility profile obtained before and after extrusion would thus give an overview of the contributions of each of the most important interactions amongst proteins and protein and other macromolecules (Areas 1992). The difference in mobility of the protein present in defatted peanut flour before (UE) and after extrusion (E1 and E2) and of the protein solubilized with various buffers is illustrated in **Figure 2.2**. It depicts the electrophoretic migration of the samples containing SDS and reducing agent DTT. The presence of SDS and DTT, showed no major difference in the mobility of the samples with the exception of one sample (E1 stage 2; lane 5). While the protein solubilized from the flour (UE) and the extruded (E1 and E2) samples did not show differences in the migration and intensity of staining (**Figure 2.2A**), samples extracted with phosphate buffer (stage-1), phosphate buffer containing urea (stage-2) and phosphate buffer containing urea and DTT (stage-3) appeared different depending on the extrusion conditions (**Figure 2.2B**). Migration of UE, E1 and E2 extracted with phosphate buffer (stage-1) indicated that while unextruded peanut flour contained large aggregates, both the extruded samples did not contain soluble material, and very

little sample was observed in lanes 2 and 3, **Figure 2.2B**. The absence of phosphate buffer-soluble protein after extrusion confirmed what already was determined by protein analysis of the extracts. Comparing the stage-2 extracts of buffer containing urea, differences in the migration in agarose gel were shown not only between control (UE) and extruded samples, but also between E1 and E2. Vertical streaking was seen (lane 5) in the sample extruded at low moisture-high temperature (E1) indicating the formation of different type of aggregates having different mobility due to more severe extrusion conditions. These samples may contain different type of peptides or other bonds resistant to SDS and DTT. In addition, E2 showed the darkest intensity of spot in the samples of stage-3 (extracted with buffer containing urea and DTT) indicating the presence of a higher amount of disulfide interactions in E2 than E1. E2 was extruded at a higher moisture content and lower temperature than E1. Studies have shown the prevalence of disulfide bonds in extrudates extruded under less severe (high moisture, low shear, low temperature) conditions compared to more severe (low moisture, high shear, high temperature) extrusion (Areas and Prudencio 1991; Areas 1992).

SDS-Poly Acrylamide Gel Electrophoresis (SDS-PAGE):

Figure 2.3 illustrates the electrophoretic migration of protein samples from the various stages of the extraction as depicted by SDS-PAGE. Most peanut protein showed peptides of molecular weight less than 83 kDa as previously shown by other authors (Monterio and Prakash 1994) wherein eight subunits for total protein of molecular weights between 15.8 and 66.1 kDa were found. Samples solubilized in phosphate buffer (stage-1) showed differences in solubility and the amount of protein present. Both, E1 and E2 stage-1, showed very little protein present, both samples contained the same type of protein, with a band at about 30 kDa being the most abundant in the soluble phase. This could be due to the formation of protein aggregates insoluble

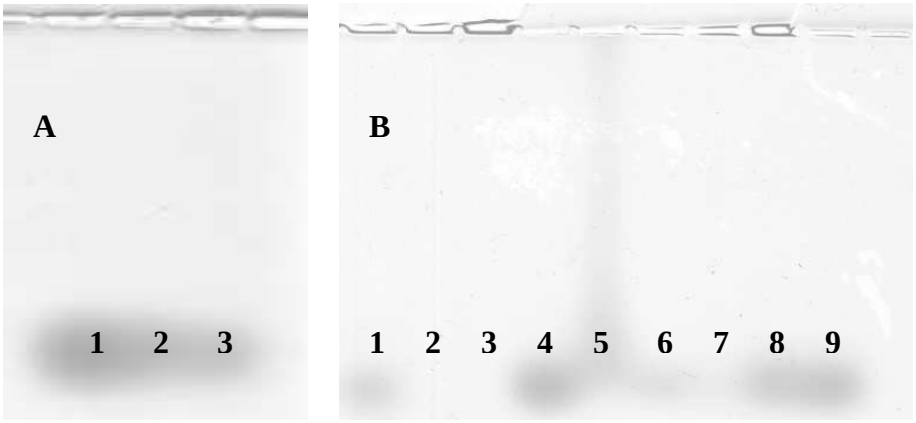


Figure 2.2: (A) Agarose Gel Electrophoresis of UE, E1, and E2 in the presence of DTT - electrophoretic mobility and the analysis of aggregates present in the unextruded control (UE) and extruded samples (E1 and E2). (B) Agarose gel of buffer soluble proteins of all three stages - Lane 1-3 represent the migration of protein aggregates of UE, E1, and E2 extracted in sodium phosphate buffer (stage 1 samples); Lane 4-6 represent the migration of protein aggregates of UE, E1, and E2 extracted in sodium phosphate buffer containing urea (stage 2 samples); Lane 7-9 represent the migration of protein aggregates of UE, E1, and E2 extracted in sodium phosphate buffer containing both urea and DTT (stage 3 samples).

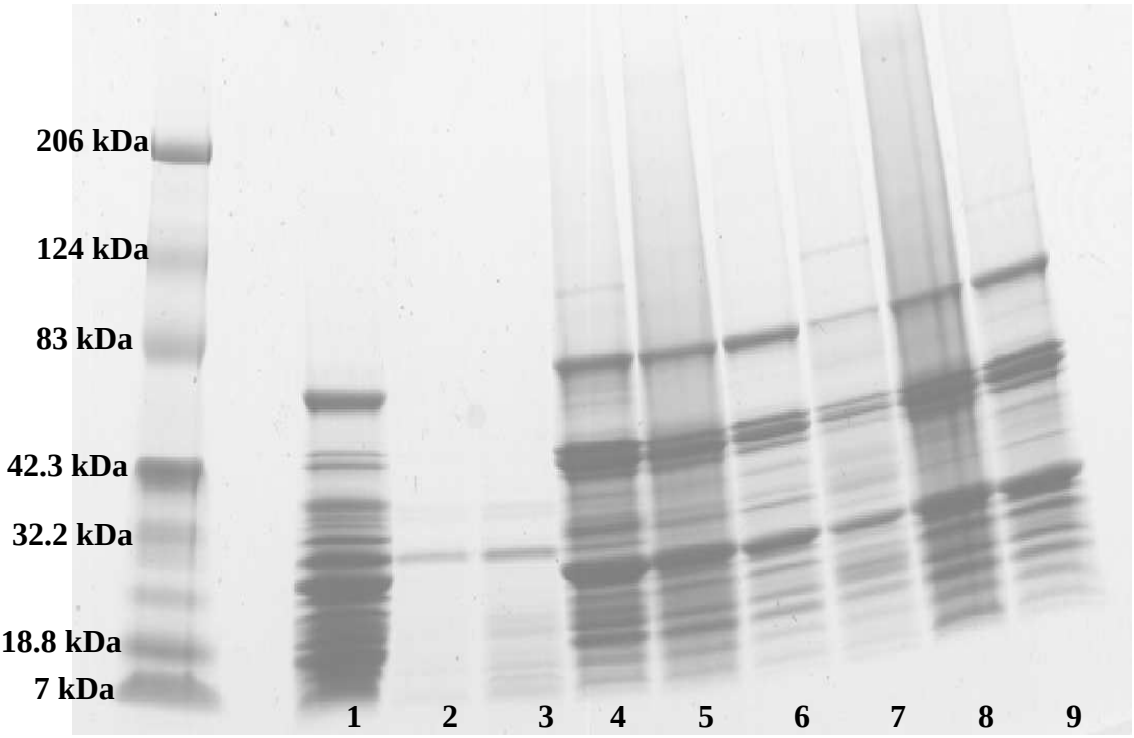


Figure 2.3: SDS-PAGE of buffer soluble extract of the peanut protein fractions from control (UE) and extruded (E1 and E2) samples: Lanes 1-3 represent UE, E1, and E2 of stage 1 extracted using sodium phosphate buffer (pH 7.9). Lanes 4-6 represent UE, E1, and E2 of stage 2 extracted using sodium phosphate buffer containing urea. Lanes 7-9 represent UE, E1, and E2 of stage 3 extracted using sodium phosphate buffer containing urea and disulfide reducing agent DTT.

in sodium phosphate buffer (Li and Lee 1996b). Though the protein solubilized in the electrophoresis buffer is assumed to be on equal weight basis, there may have been large molecular weight complexes that were not resolubilized. However, there were also some light bands seen in E2 stage 1 sample, which were absent in E1 stage 1 sample. This could be due to the effect of severity of extrusion on peanut proteins. Eight major and several minor subunits of peanut protein, ranging from 7 to 70 kDa were shown in the samples dissolved with urea buffer (stage-2) and, as already indicated in the agarose gel, there were differences in the samples from E1 and E2. However, the overall migration pattern was similar in the extruded and unextruded samples with prominent bands at 70, 43, 42, 40, and 25 kDa. Protein complexes solubilized by urea and urea + DTT were comprised mainly by subunits in the ~40 kDa and ~30 kDa ranges and contained much less of the high molecular weight subunit. No difference was shown in the type of protein present, although more band streaking was shown in E1 samples, indicating a higher amount of insoluble material and polydisperse components. These results confirmed that not only in soybean protein (Marsman and others 1998) but also in peanut protein, extrusion causes changes in protein-protein interactions, with the formation of non-covalent and disulfide interactions, depending on extrusion conditions as also found in the wheat proteins (Li and Lee 1996a, 1996b; Rebello and Schaich 1999). A higher amount of protein was also present in the unextruded sample extracted with buffer containing urea. Similar band pattern was seen in the composition of polypeptide amongst stage-2 samples. Non-covalent bonds, between the protein aggregates in the buffer extractable portion, were disrupted by incorporating urea in the sodium phosphate buffer (Marsham and others 1998). SDS-PAGE of samples extracted with DTT (stage-3) revealed the important role of disulfide groups in extrusion of peanut proteins. Hager (1984) defined four states of proteins (1) proteins soluble in simple buffers; (2) proteins insoluble

due to non-covalent forces; (3) proteins insoluble due to disulfide covalent bonds; and (4) proteins insoluble due to a combination of both disulfide bonds and non-covalent interaction. Buffer 3 soluble proteins belonging to the extrudates were seen to contain lot of lower molecular weight subunits compared to stage 2 which suggests that these low molecular weight subunits comprise disulfide bonded proteins. Stage 3 extruded samples (E1 & E2) also showed small amount of unresolved proteins soluble in buffer 2 and 3, that failed to enter the gel (result not shown in the gel). This indicates the presence of protein subunits linked neither by non-covalent nor disulfide linkages, but by other stronger linkages such as iso-peptide bonds (Stanley 1989).

Chromatography:

To determine if differences occurred in the soluble proteins/aggregates present in the various extracts, chromatographic measurements were carried out, to look at size and charge differences. Size and charge data is important to understand the effect of extrusion on macromolecular proteins. However, the published work on these aspects is limited.

a) Size Exclusion Chromatography:

After sequential extraction, freeze dried samples were solubilized in a phosphate buffer (mobile phase) and injected in a size exclusion column. Size exclusion chromatography of the three unextruded samples indicated 3 major peaks, in agreement with what was reported by Monterio and Prakash (1994) (**Figure 2.4**). The three distinct peaks were identified by Monterio and Prakash (1994) as arachin, conarachin II, and conarachin I in order of elution. Using the superdex 200 column peanut protein showed 2 distinct peaks at large molecular weight elution. No difference in the elution pattern was shown for protein soluble in the unextruded extract UE at stage-1 and stage-2. Very negligible amount of protein aggregate peaks were seen to elute in the extract prepared from UE stage-3.

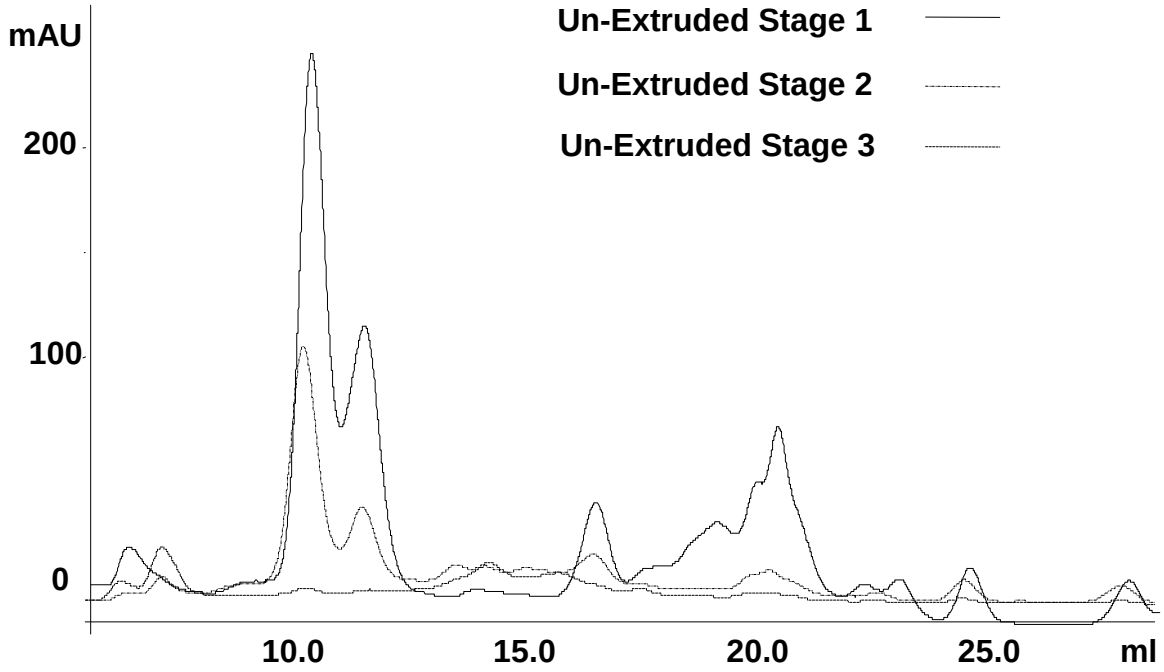


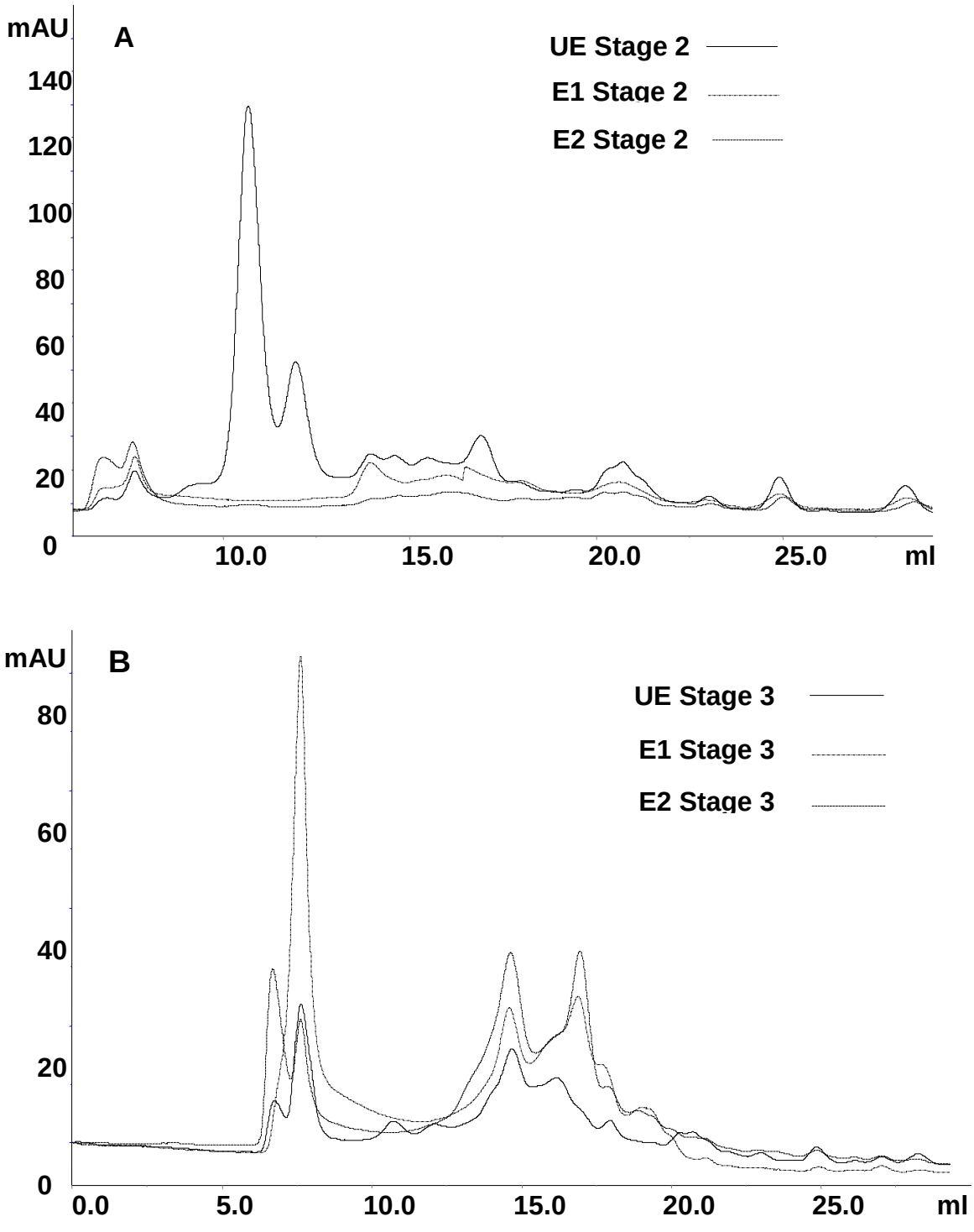
Figure 2.4: Size exclusion chromatography of unextruded sample extracted sequentially with phosphate buffer (stage 1), phosphate buffer containing urea (stage 2), and phosphate buffer containing urea and DTT (stage 3).

The effect of extrusion on protein aggregation was evident when comparing size exclusion profiles of soluble fractions of UE, E1 and E2 of stage-2 and stage-3 (**Figure 2.5**). The first two peaks eluting at 10 and 12 ml in the unextruded sample were not present in the extruded samples, but were replaced by high molecular weight peaks that eluted near the void volume (**Figure 2.5A**). Higher molecular aggregates are better visualized in **figure 2.5B**, where samples showed higher intensity peaks near the excluded volume (elution at about 6 ml). These peaks are found to be enhanced by extrusion and probably were comprised of arachin and conarachin II subunits. Simultaneously, species eluting near conarachin I (~36-38 kDa.) were also enhanced by extrusion. This indicates that extrusion may not only cause the formation of higher molecular weight aggregates, but may also lead to the formation of intermediate and smaller species. Also, E1 is found to be comprised mainly of high molecular weight species, formerly held together by disulfide bonds, while E2 is comprised mainly of intermediate-sized species. This suggests that the more severe extrusion condition favored the formation of larger aggregates and milder conditions favored production of smaller components held together by disulfide bonds.

b) Ion Exchange Chromatography:

Charge distribution differences in the soluble aggregates were determined by ion exchange chromatography after dissolving samples in phosphate and phosphate/urea buffers. Ion-exchange chromatography of the 3 unextruded sample extracted with various buffers, and eluted in sodium phosphate buffer is shown in **Figure 2.6**. The analysis showed the presence of 3 distinct peaks eluting at different gradient concentration, showing the presence of different amounts of charged species between elution patterns of the three stages. The UE Stage 1 and 2 samples mainly comprised of negatively charged complexes. The large unbound peak in UE

Figure 2.5: Size exclusion chromatograms: (A) represents the chromatogram of UE, E1, and E2 extracted in the presence of sodium phosphate buffer containing urea (stage 2 samples); (B) represents the chromatogram of UE, E1, and E2 extracted in the presence of sodium phosphate buffer containing urea and DTT (stage 3 samples).


A
mAU
140
120
100
80
60
40
20
0
10.0
15.0
20.0
25.0
ml
UE Stage 2
E1 Stage 2
E2 Stage 2
B
mAU
80
60
40
20
0
0.0
5.0
10.0
15.0
20.0
25.0
ml
UE Stage 3
E1 Stage 3
E2 Stage 3

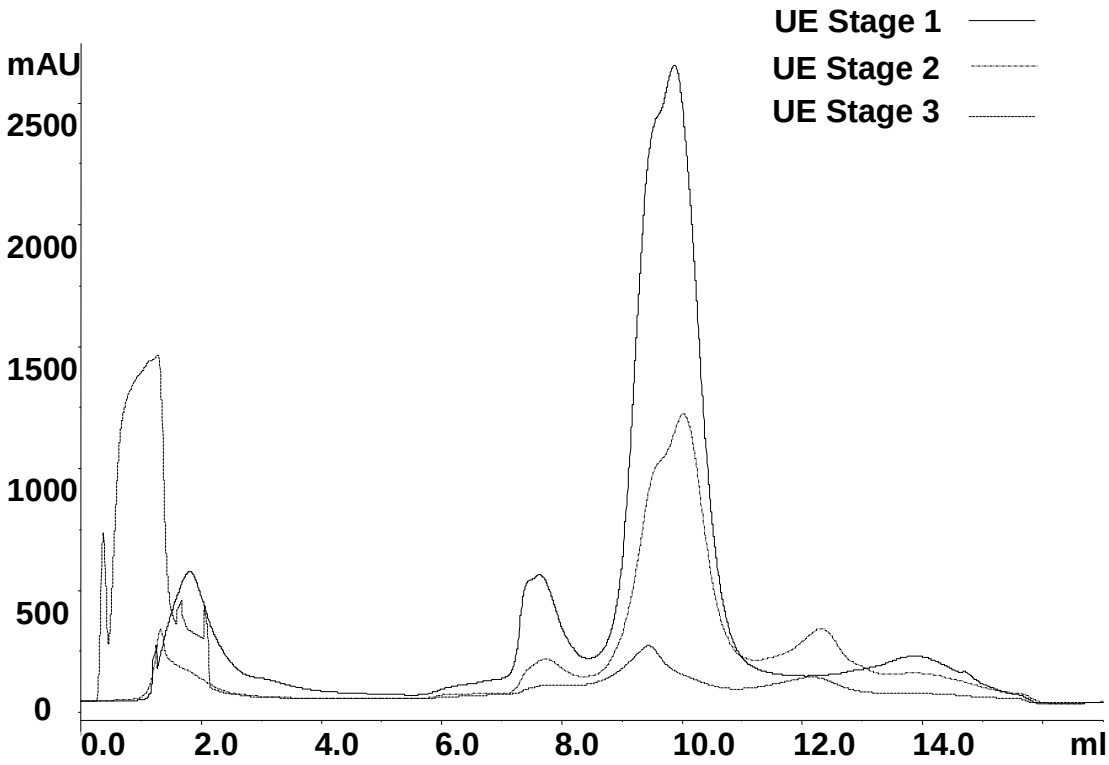


Fig. 2.6: Ion exchange chromatography elution of unextruded samples of all three stages in sodium phosphate buffer (pH 7.9).

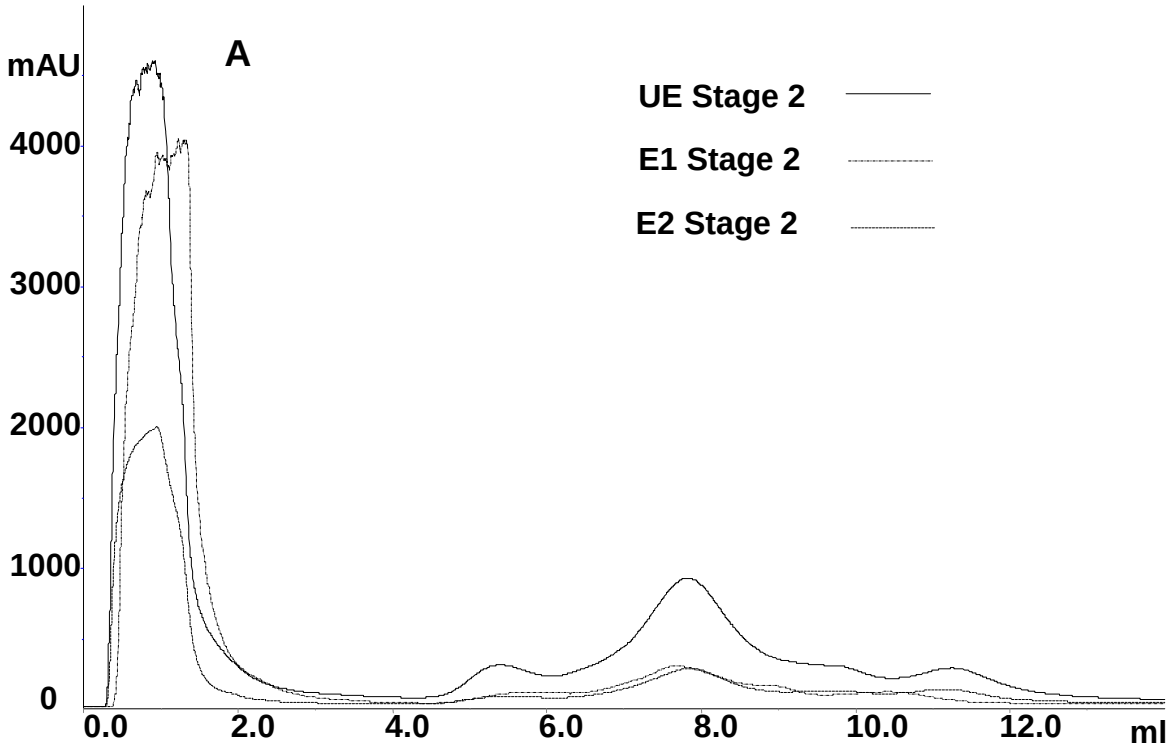
Stage-3 suggests that there might be species in which basic (positively charged) amino acid residues were confined to the interior due to proximal disulfide bonds. These positive groups were then apparently exposed due to cleavage of disulfide bonds by DTT. Similar elution profile was also seen by Monterio and Prakash (1994) while analyzing peanut protein complexes by DEAE-Cellulose chromatography.

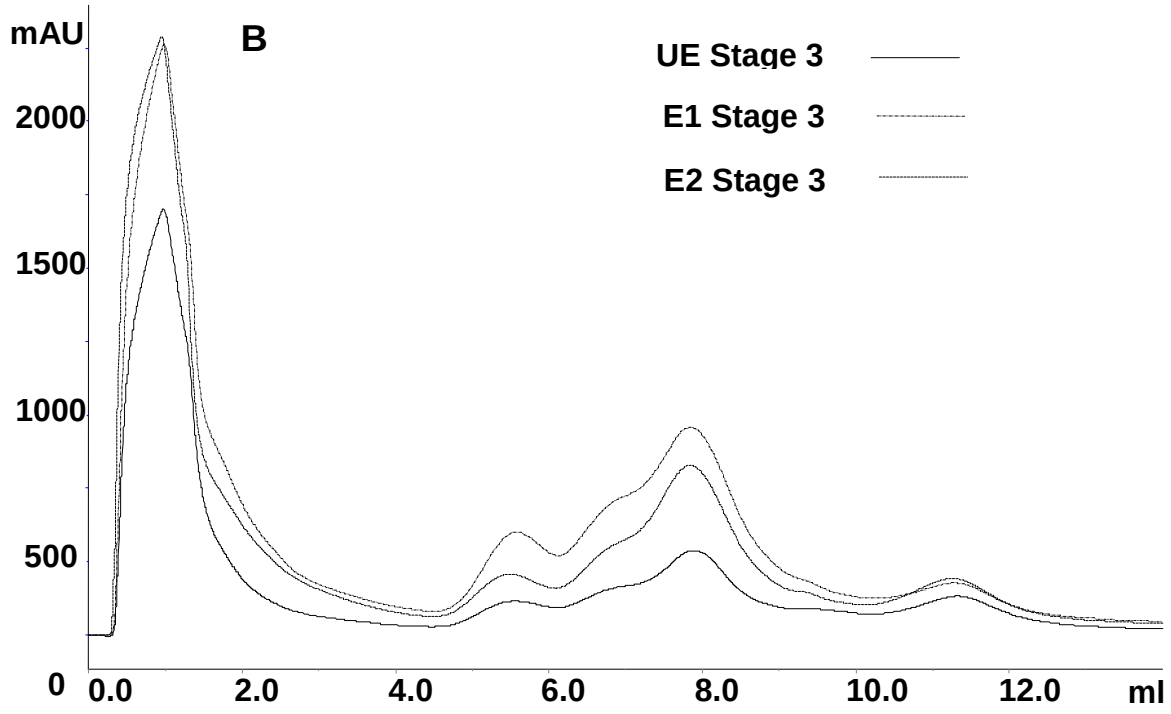
To enhance the solubility of the samples to be separated in the ion exchange column, and to assess differences in charge of samples extracted from extruded peanut flour, a buffer containing 20 mM sodium phosphate and 6 M Urea at pH 7.9 was also used as mobile phase in the ion exchange chromatography analysis. Ion exchange chromatograms of stage-2 and stage-3 extracts revealed differences amongst the samples (**Figure 2.7A** and **2.7B**). A high amount of unbound protein was observed in all the samples of soluble protein injected into the ion exchange column. The column (HiTrap Q, Amersham Biotech, NJ) was a strong anion exchange column and thus the protein injected in the column had a positive charge at pH 7.9 as most of it was seen to elute in the void volume. Similar behavior was shown by other authors employing DEAE-cellulose chromatography (Monteiro and Prakash 1994). Ion exchange chromatography of stage 2 and 3 samples showed similar a pattern. While comparing UE, E1 and E2 after stage 2 extraction, the soluble aggregates showed minor differences depending on the extrusion conditions. Also, minor differences were observed in stage 3 samples.

SDS-PAGE of Peaks:

SDS-Page electrophoresis was performed on the bound and unbound (< 2 ml point) samples separated by ion exchange chromatography to observe differences in the protein composition (**Figure 2.8**). The unbound fractions of stage 2 samples consisted of subunits ranging from 70 to 10 kDa. Some differences were found in the polypeptide pattern between UE,

Figure 2.7: Elution chromatography by ion exchange of 3 different samples UE, E1 and E2, extracted with urea buffer (A) and urea and DTT (B).


A
mAU
4000
3000
2000
1000
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
ml
UE Stage 2
E1 Stage 2
E2 Stage 2


B
mAU
2000
1500
1000
500
0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
ml
UE Stage 3
E1 Stage 3
E2 Stage 3

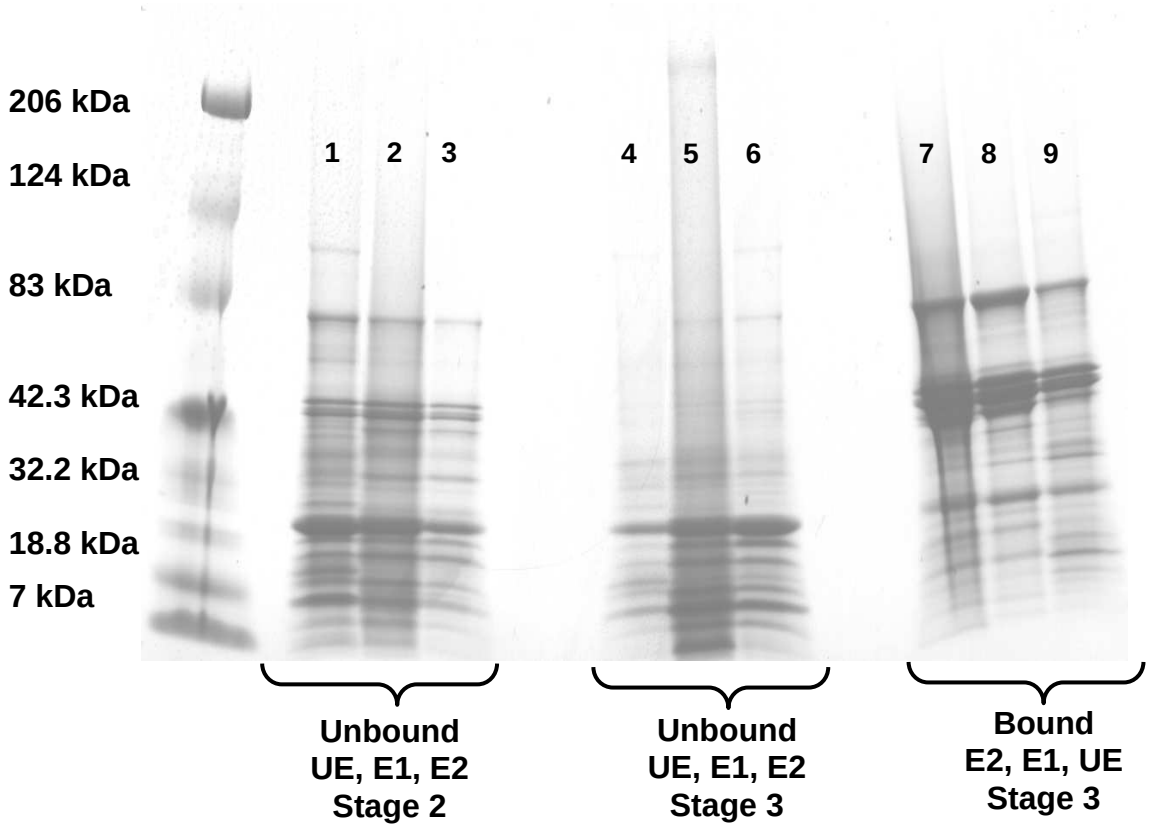


Fig 2.8: SDS-PAGE of peaks of figure 2.7 (A) & (B): Lanes 1-3 represent the unbound (<2ml mark) of stage 2 samples eluting in the void volume. Lanes 4-6 represent the unbound (<2ml mark) and lanes 7-9 represent the portion eluting after 6ml of stage 3 samples.

E1 and E2. These large, positively charged aggregates contained most of the peanut protein subunits. The amount of proteins was highest for UE followed by E1 and E2, which is in accordance with the ion-exchange chromatograph. Comparing E1 and E2, more positively charged aggregates were held together by non-covalent bonds in E1 than E2 as shown by the higher band intensity of proteins in the SDS-Page of the peaks (**Fig. 8**: lane 2 & 3). The electrophoresis of the unbound fraction of stage 3 samples was also in concordance with the chromatograph with UE having the least and the lightest amount of bands compared to E1 and E2. Theses species were comprised of subunits of less than 32.2 kDa MW. The unbound proteins of stage 2 and 3 showed marked differences in the distribution of subunits. Samples were also collected belonging to the peaks eluting in the column after 5 ml of column volume. These peaks contained larger subunits having average molecular weights between 70 and 32.2 kDa (lane 7-9). It is important to note that the protein subunits found in lanes 7, 8, and 9 belong to E2, E1, and unextruded peaks respectively. The band pattern of E1 and E2 was found to be similar and E2 was observed to have the highest amount of aggregated subunits; this could be because of the prevalence disulfide interactions cleaved by the presence of DTT in the buffer. In general, the large amounts of positively charged aggregates in the unextruded and extruded samples comprised of smaller molecular weight subunits, where as the negatively charged aggregates comprised of higher molecular weight subunits. Extrusion was also responsible for an increase in positively charged aggregates and negatively charged aggregates as seen by an increased amount of subunits belonging to these species, while comparing lane 5 & 6 with lane 4 and lane 7 & 8 with lane 9 respectively (**Fig. 2.8**).

CONCLUSIONS

Extrusion of peanut flour-cornstarch mixture has been shown to modify the structural and functional properties of proteins. Extrusion reduced the solubility of peanut proteins in sodium phosphate buffer but the resulting aggregates were soluble in buffer containing urea and urea-DTT. Extrusion caused the formation of not only disulfide bonds, but also non-covalent bonds. Though the molecular size increase seems to follow a similar pattern, but unique differences were observed in the amount and type of protein aggregates after extrusion. Extrusion carried out under lower moisture and higher temperature (severe conditions) facilitated the formation of non-covalent interactions and that carried out under higher moisture and lower temperature (milder conditions) enhanced the formation of disulfide bonds. Extrusion under severe conditions also promoted formation of other stronger covalent bonds. Various analysis conducted in the present study showed that the process of extrusion enhances non-covalent interactions, disulfide linkages, and the enhancement in these interactions further stimulate the formation of new aggregates which are either highly positively charged or moderately negatively charged.

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CHAPTER 3

THE EFFECT OF EXTRUSION CONDITIONS ON SOLUBILITY, BONDING, AND AGGREGATION OF PROTEIN IN DEFATTED PEANUT FLOUR[1]

[1]Shah, A., M. Corredig, L. Chen, and R.D. Phillips, 2003. To be submitted to Journal of Food Science.

ABSTRACT

The effects of extruder operating conditions (moisture content of the feed and temperature of extrusion) on the spectrum of changes taking place in native proteins of peanut flour, specific mechanical energy (SME), and residence time distribution (RTD) were studied. Defatted peanut flour (~1.5% fat) was extruded on an APV-Baker co-rotating twin screw extruder to obtain the different extrudates. A three stage sequential extraction procedure was employed to solubilize the proteins belonging to the control (defatted peanut flour) and the extrudates. Extrusion was carried out under 25, 30, and 35% feed moisture content at 125, 150, and 175 ºC temperatures. The buffer soluble fractions of the samples were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page), size exclusion chromatography, and ion-exchange chromatography. Analyses revealed that extrusion causes the enhancement of non-covalent and disulfide linkages in peanut proteins. During the process of extrusion the native proteins in peanut flour undergo disintegration from high molecular weight to low molecular weight forms, which subsequently reform into very large networks. Temperature of extrusion followed by feed moisture content was found to be the most significant parameter influencing peanut proteins. Extrusion carried out at 25% moisture and 175 ºC (most severe condition) had the most profound effect on native peanut proteins. The highest SME (485.6 KJ/kg) was observed under low temperature and low moisture extrusion condition. The mean residence time was observed to be the highest (88.8 s) during extrusion performed under 175 °C temperature and 35% moisture content.

INTRODUCTION

Peanut is one of the five most important oilseeds produced in the world (Carley and Fletcher 1995). Peanut containing foods such as peanut butter, salted seed, candies, and snack-type crackers and cookies are widely enjoyed because of their unique roasted peanut flavor (McWatters and Cherry 1982). Peanuts are considered as one of the most concentrated foods because of their high oil (45-48%) and protein (28-30%) contents. Oil extracted from peanuts is used in various edible forms, the majority being edible oil. Over 87% of the kernel proteins are globulins distinguished into two types, namely arachin and conarachin.

Extruded peanut snack consists of a mixture of cereals, peanut and other flours, and ingredients that are mixed together, often with necessary amount of water, and processed through an extruder. After mixing and kneading it is heated above its gelatinization temperature leading to a cooked product. The product is sometimes enrobed and flavored or sometimes undergoes further processing like frying, roasting, baking or fortification (Cindio and others 2002). Roasted peanuts (oil or dry roasted) are widely used snack foods, but the conversion of peanuts to forms such as chips or extruded foods can extend their usefulness to a great extent. Chip-like products can be prepared from peanut meal with or without added binders and these types of products are similar to conventional potato or corn chips but containing higher levels of proteins (McWatters and Cherry 1982). Extrusion processing of snack foods utilizing high protein ingredients like peanuts depends largely on expansion and puffing characteristics which influence product density and texture. The combination of defatted peanut and cereal/gram flour were found to be suitable for extrusion processing of protein-fortified, ready-to-eat foods (Bongirwar and others 1979).

Extrusion cooking is characterized by the complex interplay of heat, mass, and momentum transfer in concert with the physical and chemical transformation of the feed material and the conditions that prevail during extrusion (Rizvi and others 1995). During extrusion, due to the thermal and mechanical treatment, starch molecules undergo a variety of biochemical reactions such as gelatinization and molecular degradation (Akgodan 1996). A number of feed materials can be utilized during food extrusion to get desired products. These ingredients undergo various changes including complex physiochemical changes, starch gelatinization and dextrinization, denaturation of proteins, degradation of fats, polymerization of fats/starch/proteins, and many other minor reactions, during extrusion (Lo and others 1998).

The nutritive value of a protein source depends on the relative amounts of constituent amino acids and their digestibility and bioavailability (Mensa-Wilmot and others 2001). Protein rich materials behave like non-Newtonian fluids during extrusion (Marsman and others 1995). Vasanthan and others (2002) have reported of formation of interactions between proteins and fiber during extrusion of barley flour, which was concluded by analyzing the nitrogen content of the dietary fiber fractions of the extrudates. Proteins undergo cross-linking reactions due to the heat and shear induced denaturation of the proteins. This causes the formation of a new molecular aggregate structure (Cindio and others 2002). Studies on proteinaceous substrates have focused on the influence of processing conditions on extrudates texture and functional properties. Valle and others (1994) found that protein transformation during extrusion took place by simultaneous thermo-chemical denaturation and cross-linking which was similar to starch degradation, which resulted from melting and chain splitting. They suggest that at lower energy level extrusion formation of non-covalent and disulfide takes place whereas stronger type of linkages occurs at higher levels of energy. Protein unfolding due to increasing SME enables re-

association of protein chains. The hydrogen bonds disrupted during the denaturation process, at low water contents, get recreated between protein molecules instead of between water and protein molecules leading to protein aggregation (Valle and others 1994). Areas (1992), during extrusion of soy proteins, found that protein extrusion did not necessarily require interactions stronger than the combination of disulfide, hydrophobic, and electrostatic interactions to result in extrudates resistant to retorting at 120 ºC. He argued that if new peptides were formed during extrusion they did not contribute to insolubilization of product, but to the aggregation of protein inside the extruder when the feed was in the melt state. During extrusion of wheat flour, Yeh and Hwang (1992) found that the cooking loss decreased with increase in the protein content. The interactions within the proteins increased gluten networks thereby binding more starch and reducing the cooking loss. Such a loss was mainly attributed to screw profiles mainly having forward elements only causing shorter residence time and low degree of gelatinization which caused release of soluble but ungelatinized starch into the cooking water. During extrusion of soybean meal, shear and heat not only denatured proteins but also enhanced the formation of large protein aggregates and thus affected the PDI (Protein Digestibility Index) determinations (Marsmen and others 1995). During extrusion of sorghum-cowpea mixture, Pelembe and others (2002) found that the apparent protein content was not affected by extrusion temperatures but the true protein content and the quality of the extrudates was affected. The quaternary structure of the proteins unfolds in the hot and moist conditions during HTST (high temperature-short time) extrusion cooking process to produce a viscous plasticized mass. They then undergo polymerization, cross-linking, and reorientation to form fibrous structure (Fellows 1990).

The effects of high and low shear extrusion conditions on peanut proteins have been explored Shah and others (2003) (manuscript to be submitted to Journal of Food Science).

Peanut protein analysis techniques have been fine-tuned to get better protein yields and improve data collection techniques qualitatively and quantitatively by Shah and others (2003) (manuscript to be submitted to Journal of Food Science). The present study is an attempt to understand the changes in the protein composition/aggregation of the peanut flour upon the process of extrusion employing a wider range of extrusion parameter settings. The process of extrusion exerts a high pressure and high temperature environment on the peanut flour. Extrusion conditions employed in the present study were also varied with respect to the moisture conditions to which the peanut flour was exposed. It is quiet obvious that under these varying conditions of extrusion, the protein components/aggregates of the peanut flour must undergo characteristic changes in their natural properties like non-covalent interactions, folding and there by overall quaternary structure. In the current study we have employed various techniques like SDS-PAGE, molecular gel exclusion chromatography and ion-exchange chromatography in order to thoroughly understand various alterations taking place in the physical properties of peanut flour proteins.

MATERIALS AND METHODS

1. Raw Materials

Raw blanched peanuts were obtained from Seabrook Enterprise (Edenton, NC).

2. Raw Material Preparation

In order to reduce the oil content of the raw peanuts, a solvent extraction method was employed. The raw blanched peanuts were first passed through an oil expeller (Hander Oil Machinery Corporation, Osaka, Japan). To avoid protein denaturation due to heat, the expeller barrel was not heated. The expeller did not remove any oil from the peanuts but reduced the particle size. A solvent extraction method was employed to reduce the oil content of these

crushed peanuts by treating them with hexane. Four passes with hexane were performed to reduce the oil content. For the first two passes a 1:2 (w/v) ratio of peanut to hexane was performed and for the next two passes a 1:1 (w/v) ratio of peanut to hexane was employed. In each pass hexane was added to the crushed peanuts and mixed thoroughly by manual agitation. This mixture was then filtered under vacuum using a Buckner funnel with Whatman filter # 4 filter paper. The same extraction procedure was performed on the residue (cake left behind on the filter paper) using appropriate amount of hexane depending on the pass. The filtrate was removed and passed to the solvent recovery system. The solvent recovery system consisted of a jacketed glass beaker connected to a condensation trap. The condensed solvent was collected and reused. After the four passes the defatted peanut cake was air-dried under the hood. The oil content of the defatted peanut flour was measure by Goldfisch apparatus (model 35001, Laboratory Construction Co., Kansas City, MO), drying and weighing was done according to AACC method No. 30-20 (1995). The moisture content of the flour was determined by AOAC Vacuum Oven method (AOAC 1990). The flour was then crushed to fine uniform particle size in a Hammer Mill MP849 equipped with a 2 mm screen (Magnetic Products Inc., Walled Lake, MI) and stored.

3. Extrusion

Extrusion was carried out on an APV co-rotating twin-screw extruder (model MPF1700-30, APV Baker Limited, Peterborough, England) having a conical die with constant taper. The length of the die was 43mm and tapered from a diameter of 30mm to 3mm at the exit point. The barrel had an inside diameter of 30mm and a length of 750mm, thereby having a length to diameter (L/D) ratio of 25:1 and consisted of four independent heated/cooled zones and a temperature control zone at the die. The zone close to the feeder (zone 1) was maintained at 100

ºC, zone 2 at 125 °C and the remaining zones (3, 4, and 5) were set at 125 or 175 ºC. In order to validate the consistency of the runs at different parameter settings a run was also performed at the mid-point (150 °C). Screw speed was set to 300 rpm. A screw configuration was so chosen as to impart maximum mechanical energy and supply enough shear to the feed material as described in **Table 3.1**. It consisted of four sets of reversing paddles along with other forward screw elements. Defatted peanut flour was fed into the extruder at the rate of 10 Kg/hr (dry basis) by a K-Tron volumetric feeder (Model K2VT20, K-Tron, Pitman, NJ). Desired moisture level (25% or 35%) was achieved by pumping the required amount of water into the barrel, during extrusion, by a proportioning pump (Bran and Lube, type N-P31, Buffalo Grove, IL). A run was also performed at the mid-point (30% moisture). The five extruded products were named E1, E2, E3, and E4 and E5 and were a result of the following extrusion conditions: Extruded-1 (E1) - 35% moisture and 125 °C, Extruded-2 (E2) – 25% moisture and 125 °C, Extruded-3 (E3) - 35% moisture and 175 °C, Extruded-4 (E4) - 25% moisture and 175 °C, and Extruded-mid-point (E5) – 30% moisture and 150 °C.

Table 3.1: Screw configuration used in this study.

Screw Element	Length of each element (D)	Number of elements in configuration
2 lead F	1.50	3
2 lead F	1.00	1
30º F	0.25	5
2 lead F	1.50	2
30º R	0.25	5
60º F	0.25	6

2 lead F	1.50	2
30º R	0.25	7
2 lead F	1.5	2
30º R	0.25	5
2 lead F	1.50	1
2 lead F	1.00	1
60º R	0.25	6
1 lead F	1.00	1

F = forwarding screw element (paddles); R = reversing screw element (paddles); D = barrel diameter (as a unit of barrel length).

3.1 Steady-state Operation

Figure 3.1 gives the overall methodology adopted for the extrusion of the defatted peanut flour leading to the formation of the different extrudates.

Data were obtained under varied operating variables of barrel temperature and feed moisture content while keeping the feed rate, screw speed and screw profile constant. Responses of torque, die pressure and temperature at the die to these changes were then recorded. Values for torque, in % torque, were read off the extruder panel and utilized to compute the specific mechanical energies. Extrudates were collected after equilibrium was obtained as evidence by negligible fluctuation in the values of torque, die pressure and die temperature, termed as steady-state operation.

4. Residence Time Distribution (RTD)

The RTD was determined for all treatments but one. It was difficult to maintain steady state conditions for extrusion performed under most severe conditions of 25% moisture and

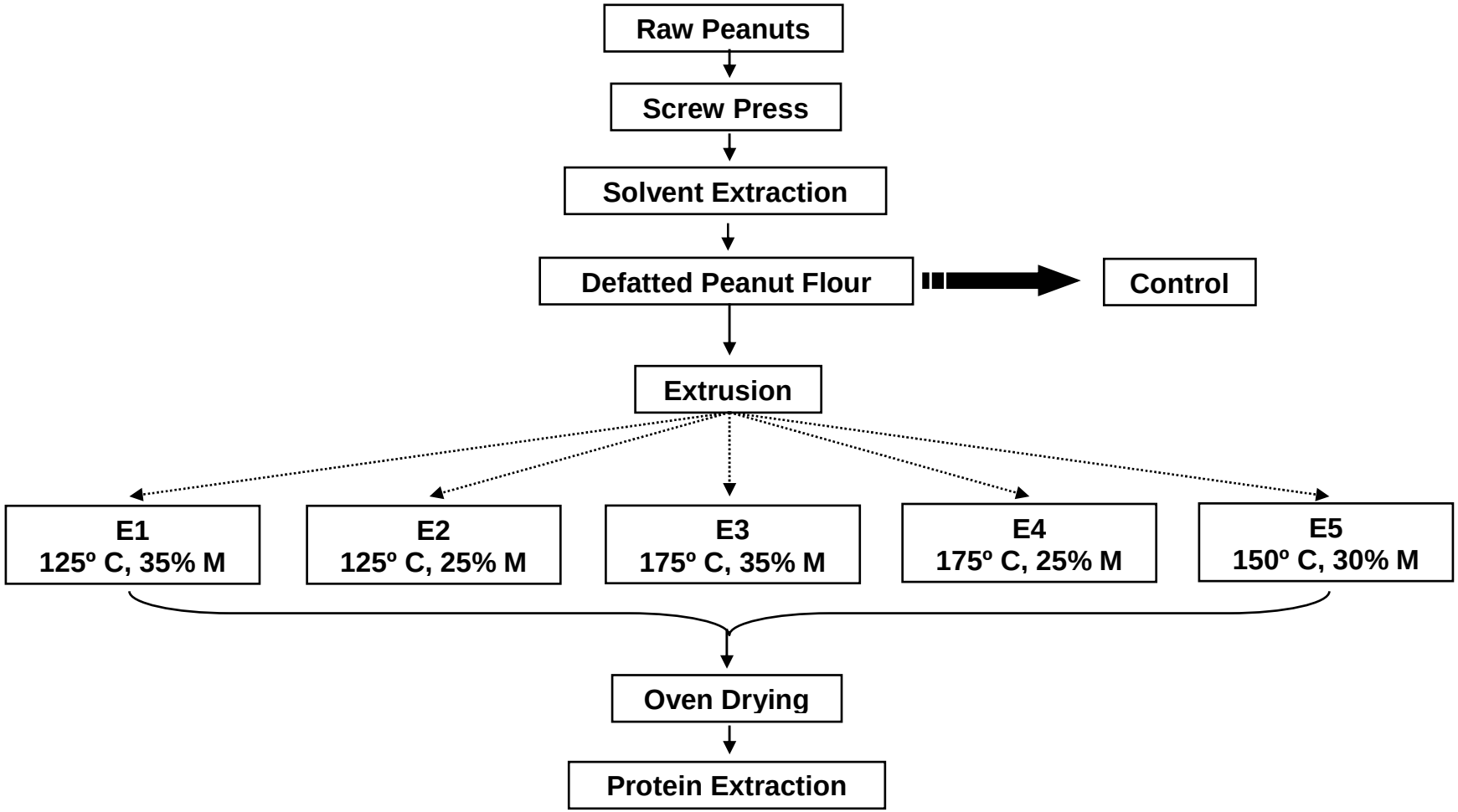


Figure 3.1: Schematic diagram of the preparation of various extrudates from raw peanuts.

175 °C temperature. This RTD method was based on the color values of the extrudates directly from a colorimeter (Likimani and others 1990). RTD was determined for each of the treatments using the methods of Levenspeil (1972) and Bounie (1988) with food dye (FD&C Red #40, Warner Jenkinson Company, Inc, St. Louis MO) as tracer. 5 g peanut feed was mixed with 1 mL of the food dye and dropped into the feed port after steady state conditions had been attained. A continuous sequence of extrudate segments, each representing 10 sec of time, was collected from the instant the timer was started until the red dye was no longer visible in the extrudates. Samples were dried in a forced air Thelco Laboratory Oven (Jouan Inc., Precision, VA) maintained at 75 °C over night, milled into fine powder in a coffee mill (Philadelphia, PA), and transferred to transparent Ziploc bags. The intensity of the red dye in the ground extrudates was measured using a hand held Minolta Color instrument (Chroma meter CR-200, Minolta, Japan). For each sample the red color component (a* values) was recorded. Three determinations were made on each sample. These values were used to plot E (t) and F (t) curves and the mean residence time calculated according to Levenspiel (1972).

5. Specific Mechanical Energy (SME)

SME was determined from the torque measurements on the drive motor at constant screw speed and constant mass flow rate according to Gogoi and others (1996). In the extruder, this energy is supplied by a 7.5 KW electric motor, which is coupled to the twin screws through a gear drive assembly. The equation used to compute SME was:

$$SME = (n \text{ (actual)} / n \text{ (rated)}) \times (\% \tau / 100) \times (P \text{ (rated)} / m);$$

where n is the screw speed (rpm), τ is the net torque (measured torque – lost frictional torque due to bearings and screw drive assembly), P is the motor power (kJ s^{-1}), and m is the mass flow rate

or the feed rate (kg/s). The calculated SME is in KJ/kg. n and P for this machine are 500 rpm and 7.5 kW respectively.

6. Chemicals

As given in Chapter 2 (Shah and others 2003) (manuscript to be submitted to Journal of Food Science).

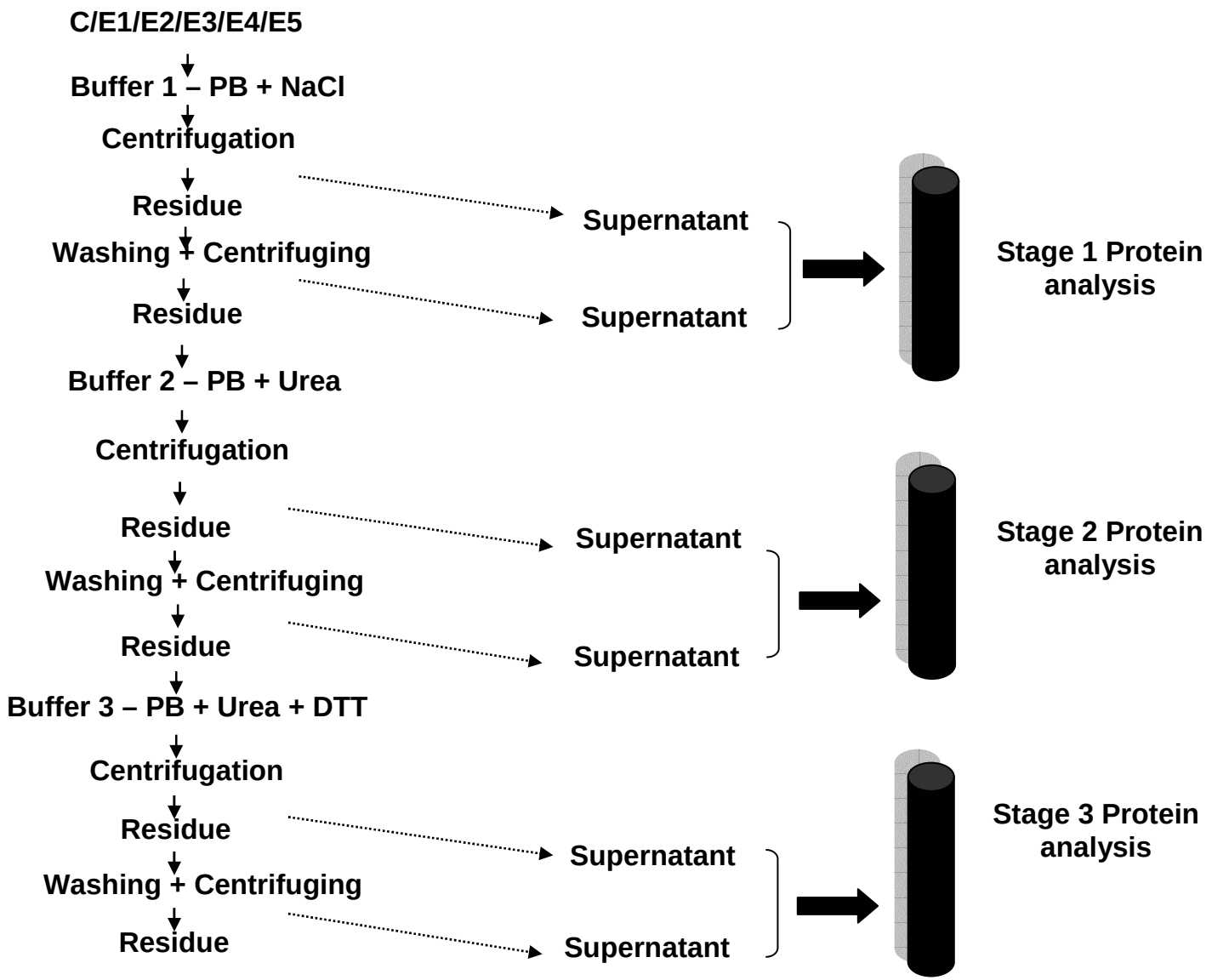
7. Sample preparation

The extruded samples were ground in a coffee grinder mill (Philadelphia, PA) and the control and ground samples were collected, stored at 4 °C, and used for the experiment.

8. Protein Extraction

Peanut protein from unextruded and extruded samples was extracted in three sequential stages with phosphate buffer, phosphate buffer containing urea and phosphate buffer containing urea and DTT as summarized in **Figure 3.2**. Approximately 2.0 g of each ground sample were dispersed in 20 ml of 20 mM sodium phosphate buffer (pH 7.9) containing 0.5 M sodium chloride (1:10). The samples were incubated at room temperature for 1.5 h and vortexed every 15-min. To enhance protein solubilization samples were subjected to sonication, as described by Li and Lee (1996) with some modifications. A high intensity ultrasonic processor (Danbury, CT) was used with a macro tip probe (3 mm diameter). While sonication, the sample was immersed in an ice bath (to prevent heat buildup) and the sonicator probe was immersed into the liquid surface to a depth of approximately 2.5 cm from the surface. 20 sonication cycles, each of 1 min, were done with 30 s pause between cycles. The sonicator was capable of providing 20 kHz and the amplitude control knob was set to 60. The samples were then centrifuged at 9300 g for 15 min at 10 °C in a Beckman Allegra 21R centrifuge (Beckman, Germany). The supernatant was filtered through Miracloth (Calbiochem, Biosciences, La Jolla, CA) and the residue was washed

Figure 3.2: Schematic diagram representing the three stages of protein extraction from unextruded (Control – C) and extruded peanut flour samples (E1 – E5) using three buffer systems: Buffer 1 – 20 mM sodium phosphate (pH 7.9) with 0.5 M NaCl (Stage 1); Buffer 2 – 20 mM sodium phosphate (pH 7.9) with 6 M Urea (Stage 2); Buffer 3 – 20 mM sodium phosphate (pH 7.9) with 6 M Urea and 0.01 M DTT (Stage 3).


C/E1/E2/E3/E4/E5
Buffer 1 – PB + NaCl
Centrifugation
Residue
Washing + Centrifuging
Residue
Supernatant
Supernatant
Stage 1 Protein analysis
Buffer 2 – PB + Urea
Centrifugation
Residue
Washing + Centrifuging
Residue
Supernatant
Supernatant
Stage 2 Protein analysis
Buffer 3 – PB + Urea + DTT
Centrifugation
Residue
Washing + Centrifuging
Residue
Supernatant
Supernatant
Stage 3 Protein analysis

with 10 ml of the same buffer. The washed residue was then incubated for 30 min at room temperature and vortexed every 10 min, and then centrifuged at 9300 g for 15 min at 10°C.

The filtrates and the supernatant of the washed residue were combined and extensively dialyzed using dialysis tubing, having a nominal molecular weight cut out of 6000-8000 Da (# 21-152-4, Fisher Scientific Inc., Pittsburg, PA), in deionized water by replacing the water at least 3 times and then freeze-dried (Unitop 600L, Gardiner, NY). To the residue of stage-1, 20 ml of 20 mM sodium phosphate buffer (pH 7.9) containing 6 M urea (buffer 2) was added. The proteins belonging to these samples were extracted similar to S1 samples. To the insoluble residue of stage-2, 20 ml of phosphate buffer (pH 7.9) with 6 M urea and 0.01 M DTT was added and extraction similar to S1 and S2 was performed. These samples, soluble in Urea and DTT, were termed as stage-3 samples (**Figure 3.2**). All the extractions were performed in duplicate.

9. Polyacrylamide Gel Electrophoresis (SDS-PAGE)

As given in Chapter 2 (Shah and others 2003) (manuscript to be submitted to Journal of Food Science).

10. Chromatographic analysis

10.1 Size Exclusion Chromatography

Freeze-dried samples, prepared from the various buffer extractions, were dissolved in 20 mM sodium phosphate buffer (pH 7.9) containing 6 M Urea such that the final sample concentration was 5 mg/ml. The samples were vortexed thoroughly and filtered through non-pyrogenic Millex-AA 0.8 µm syringe filters (Millipore Corporation, MA). Samples (100 µL injections) were loaded on a size exclusion column (Superdex 200, Amersham Biosciences, City NJ) and eluted with 20 mM sodium phosphate buffer (pH 7.9) containing 6 M Urea as the running buffer. The eluents corresponding to the peaks were collected and stored for further

analysis These eluents were desalted using disposable desalting columns (Econopac 10 DG, Bio-Rad, CA) using deionized water as the buffer. These desalted samples were then freeze-dried and analyzed by SDS-PAGE.

10.2 Ion Exchange Chromatography

Freeze dried samples were dissolved in 20 mM sodium phosphate buffer (pH 7.9) containing 6 M urea such that the final sample concentration was 10 mg/ml. These were then thoroughly vortexed and filtered through non-pyrogenic Millex-AA 0.8 µm syringe filters (Millipore Corporation, MA). A HiTrap Q HP (Amersham Biotech, NJ) column was mounted on an Akta Purifier (Pharmacia, NJ) and used for the chromatography. The column was equilibrated with 5 column volumes of 20 mM sodium phosphate buffer (pH 7.9) containing 6 M urea before loading the samples. One ml samples were loaded on the column for analysis. The samples were eluted with a gradient of 0-1.0 M sodium chloride in the same buffer. Fractions of eluting peaks were collected and extensively dialyzed against deionized water using dialysis tubing. The dialyzed samples were then freeze dried and analyzed using SDS-PAGE.

RESULTS AND DISCUSSION

RTD

Analysis of residence time distribution (RTD) provides information about the degree of mixing, cooking and shearing (Lee and McCarthy 1996), which plays a significant role in the final product quality. The residence time distributions include the exit age (differential) distribution function, E(t), and the cumulative distribution function, F(t). The mean residence times (t_m) and the relative dispersion by rank (σ^2) for the extrudates under different extrusion conditions are given in **Table 3.1**.

Table 3.2: The mean residence time (t_m) and the spread of distribution (σ^2) for the peanut flour samples extruded under different extrusion conditions.

Extrusion Condition (T °C-%M)	Product	Mean Time (t_m) (seconds)	Relative dispersion by rank (σ^2)
125 °C – 35%	E1	83.50	1047.63 (1)
125 °C – 25%	E2	86.62	1238.60 (2)
175 °C – 35%	E3	88.82	2139.40 (4)
175 °C – 25%	E4	-	-
150 °C – 30 %	E5	84.05	1289.76 (3)

Longer average residence time (t_m) and broad RTD spreads were observed due to the incorporation of a severe screw profile during this study, which is in accordance with the findings of Altomare and Ghossi (1986). It was difficult to maintain steady state conditions under the most severe conditions of temperature (175 °C) and moisture (25%). Surging of the product took place making it difficult to conduct RTD study under these most severe extrusion conditions. The mean time was observed to be the highest (88.8 s) during extrusion performed under 175 °C temperature and 35% moisture. This was due to the decrease in the dough viscosity as a result of high temperature and high moisture extrusion. Also the die pressure was found to be the lowest (120 psi) under these conditions. According to Martelli (1983a) the die pressure is directly proportional to the viscosity and is given by;

$$P = (FR * \eta)K_f$$

where P, FR, η, and K_f are the die pressure, feed rate, dough viscosity and die geometry constant. Under the conditions described in this study, i.e. constant feed rate, all the other terms of the equation were constant other than viscosity. Thus, the lowest die pressure suggested the viscosity

to be the least thereby increasing the mean time spent by the feed in the extruder (high t_m). The net material flow in a co-rotating twin screw extruder is a combination of drag flow and pressure flow (White and others 1987; Frame 1994; Brouwer and others 1999) and is given by:

$$Q = \alpha N - [\beta/\eta]\,[\Delta P/L]$$

where Q is the net material flow, N is the screw speed, αN represents the drag flow component and $[\beta/\eta]\,[\Delta P/L]$ represents the pressure flow component (Frame 1994). The forward conveying of the feed is a result of the difference between the drag flow and the pressure flow (which tends to push the feed backwards). The reduction in viscosity might be due to the increase of temperature, increase in moisture or molecular breakdown which is in accordance with Unlu and Faller (2002) who suggested shear thinning, temperature increase, or molecular breakdown during extrusion of yellow corn meal responsible for a reduction in viscosity by 50%.

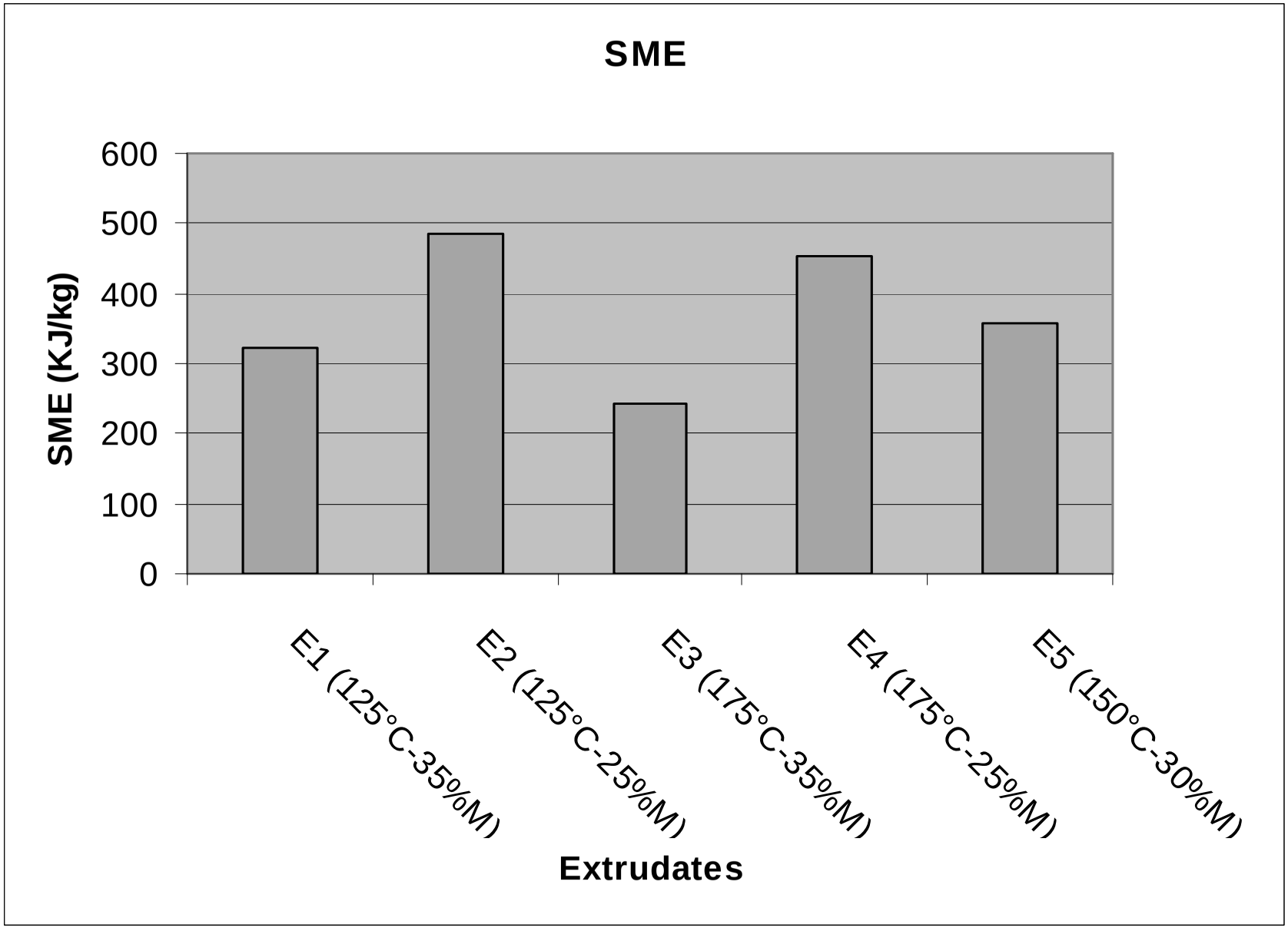
SME (Specific Mechanical Energy)

The motor torque measured off extruder panel is a result of net torque and no-load torque (Unlu and Faller 2002). In an ideal extruder where screw elements have a perfect symmetry and the axis of the twin screws are perfectly parallel, and there is no contact between the screw surface and the barrel inner surface no frictional losses occur. However, in practice, it is difficult to maintain ideality and thus some amount of torque is lost to overcome such frictional losses and also some amount of torque is lost due to bearings and screw drive assembly. The torque necessary to work the product is known as net torque while the torque utilized to turn the empty screw is known as no-load torque (Akdogan 1996). The torque is read off extruder panel. The motor torque varies with the changes in melt viscosity during extrusion as a result of changes in other extruder parameters such as screw speed, feed rate, temperature profile, and moisture (Unlu and Faller 2002). The motor torque is a measure of the energy absorbed by the feed as a result of

the shear exerted by the screws. The mixing, kneading, compression and cooking taking place in the extruder barrel transfers mechanical energy to the melt through friction. SME is this mechanical energy per unit mass provided by the motor drive to the melt in the extruder (Fichtali and van de Voort 1989).

Figure 3.3 shows the SME values of the extrudates extruded under different extrusion conditions. The highest SME (485.6 KJ/kg) was observed under low temperature and low moisture extrusion condition. As the temperature and moisture decrease the melt viscosity increases and more energy is required to work the melt. An increase in SME was observed with decrease in moisture level, which was in accordance with the findings of other workers (Senouci and Smith 1986; Della Valle and others 1989; Govindasamy and others 1997). The SME was found to be 485.6 KJ/kg and 453.2 KJ/kg (high) during extrusion at 25% moisture content compared to 323.7 KJ/kg and 242.8 KJ/kg (low) at 35% moisture content. This could be due to an increased melt viscosity at lower moisture content. As the temperature and moisture content increase the melt viscosity decreases due to shear thinning effect and molecular breakdown (Unlu and Faller 2002) causing the SME to reduce. Thus, the least SME (242.8 KJ.kg) was observed during extrusion at 175 °C and 35% moisture (high temperature-high moisture). Comparing SME at low and high temperatures with moisture level being constant (i.e. E1 v/s E3 and E2 v/s E4) a higher SME was observed among extrudates extruded at lower temperature as compared to higher temperature due to the fact that lower the temperature of extrusion higher the viscosity of the melt (Govindasamy and others 1997; Unlu and Faller 2002). While comparing

Figure 3.3: SME values computed for the extrudates at different extrusion conditions.


SME
SME (KJ/kg)
600
500
400
300
200
100
0
E1 (125°C-35%M)
E2 (125°C-25%M)
E3 (175°C-35%M)
E4 (175°C-25%M)
E5 (150°C-30%M)
Extrudates

all the treatments, SME increased in the order of E3<E1<E5<E4<E2. This further reinstates the fact that E5 is the mid-point as far as extrusion conditions are concerned. This trend also shows that SME increases as the % moisture in the feed decreases from 35% to 30% to 25%.

SDS-PAGE Analysis of Stage 1 (S1) samples:

Figure 3.4 shows the SDS-Page depicting the protein profile of S1 samples. Monteiro and Prakash (1994a) have shown that 95% of the proteins from the defatted peanut meal were soluble in 10 mM phosphate buffer, pH 7.9, containing 0.5 M NaCl. The S1 buffer contained 20 mM sodium phosphate, pH 7.9, having 0.5 M NaCl. Seventy-five % of the total peanut proteins comprise of arachin, conarachin II, and conarachin I (Prakash and Rao, 1986). Monteiro and Prakash (1994b) have reported subunits for total proteins of peanuts corresponding to molecular weights between 15.8 kDa to 66.1 kDa. They also reported the following proteins having different molecular weight bands: arachin – seven subunits of molecular weights from 15.8 to 72.4 kDa; conarachin II – eight subunits having molecular weights of 15.8 to 72.4 kDa; conarachin I – three subunits having molecular weights from 12 to 18.2 kDa. The subunit composition of the peanut protein fraction analyzed by SDS-Page indicated numerous bands for total proteins ranging from molecular weights between ~70 kDa to ~10 kDa. The predominant subunits had molecular weights of approximately 70, 50, 43, 40, 35, 30, 25, 19, 16, 12, and 10 kDa. The figure clearly shows the abundance of a high molecular weight subunit of ~70 kDa in the un-extruded peanut flour (**C**). During the process of extrusion, irrespective of various conditions employed, it was observed that the presence of this protein was negligible. This subunit is greatly reduced in Stage 1 extracts of extrudates, irrespective of extrusion conditions. Similar results were observed by Chen (2001) who extruded peanut flour containing ~6% fat under various conditions of temperature (115 or 135 ^{o}C), moisture content (20 or 40%), and

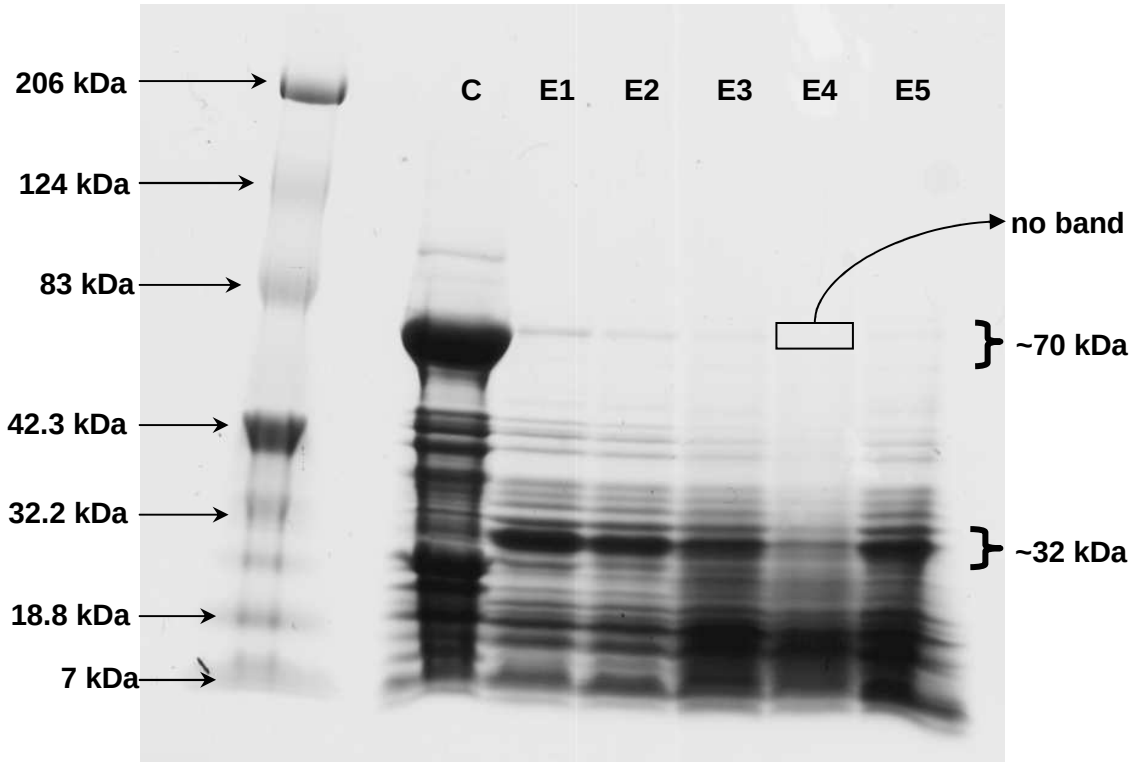


Figure 3.4: SDS-Page of buffer soluble extract of the peanut protein fractions from defatted peanut flour (control) and the five extruded samples (E1 to E5) – stage 1. The samples were extracted using 20 mM sodium phosphate buffer (buffer 1) (pH 7.9) containing 0.5 M NaCl.

screw speed (300 or 500 rpm). Further it was also observed that under the most severe conditions of low moisture (25%) and high temperature (175 ºC) extrusion the level of this high molecular weight protein was found to be almost nil (**lane 6; E4**). Again under the conditions of relatively higher moisture and low temperature (**lane 3; E1**) the residual level of this protein was found to be the highest amongst the various extrudates. The present analysis also shows that in general there is a relative abundance of high molecular weight subunits (>35 kDa) in the control as compared to the samples subjected to different extrusion condition. These observations indicate that during the process of extrusion the native proteins in peanut flour undergo disintegration from high molecular weight to low molecular weight subunits as observed by an increase in the number and intensity of the latter in the Stage 1 extracts of the extrudates. Supporting this, an important correlation can be observed while comparing ~70 kDa band in the control and ~32 kDa protein band in the extrudate lanes. Concomitant with the reduction in the level of 70 kDa protein in extrudates there was a significant enhancement in the level of the 32 kDa proteins. This suggests that during the process of extrusion the 70 kDa protein might be getting disintegrated into approximately half of its size (32 kDa). Alternatively, the 70 kDa subunit may have been incorporated into species insoluble in stage 1 buffer and the low molecular weight subunits were simply concentrated in the soluble fraction. The other higher molecular weight subunits are probably also undergoing similar behavior during the process of extrusion.

Molecular gel exclusion chromatographic analysis of S1 samples:

Using stage 1 buffer soluble extracts (buffer 1) from unextruded (control) as well as extruded peanut flour protein samples molecular gel exclusion chromatographic (MGEC) study was conducted. MGEC gives direct evidence pertaining to the exact molecular weight of various proteins/aggregates present in a mixture. Preliminarily the MGE chromatographic column was

calibrated using a known protein standard. **Figure 3.5** shows the elution profile of protein extracts (solubilized in buffer 1) from both the control (**C S1**) and various extruded (**E1 S1 to E5 S1**) peanut flour samples. As can be seen from **Figure C S1** the protein extract from control clearly resolved into three distinct peaks representing two high molecular weight peaks and one low molecular weight peak eluting at around 7 ml, 10ml, and 20 ml respectively. These three peaks correspond to arachin, conarachin II, and conarachin I. Similar elution pattern was observed by Monteiro and Prakash (1994b). As a major variation from the SDS-Page analysis 6 M urea was used in the solubilizing buffer as well as in the elution buffer. Urea is a known denaturizing agent for proteins. Urea has been shown to disintegrate high molecular weight protein complexes/aggregates to low molecular weight products (Dunn 1993). Our study clearly demonstrated that the peanut flour proteins extruded under various conditions and solubilized in buffer 1 are comprised of f low molecular weight protein molecules/subunits. This is clear from the results depicted in **Figure E1 S1 to E5 S1**. It was also noted that a characteristic broad peak elutes between 10 and 17 ml, representing higher molecular weight species. This was especially noticeable in severe extrusion conditions of 175 ºC temperature and a moisture level of 25% (**Figure E4 S1**). This is simultaneous with a reduction in the level of the low molecular weight protein peak. It is speculated that under these severe conditions a re-aggregation of low molecular weight proteins may occurs. Furthermore, the highest peak of low molecular weight proteins was observed at a condition of high temperature and high moisture content (**Figure E3 S1**).

Ion exchange chromatographic analysis of S1 samples:

Ion exchange chromatographic technique directly indicates the net charge distribution among variously sized protein molecules/aggregates. **Figure 3.6** shows the elution profile of the

Figure 3.5: Molecular gel exclusion chromatographic analysis of unextruded (control) and extruded peanut flour samples of Stage 1. The vertical arrows shown in figure C S1 correspond to the calibrated molecular weights using standards.

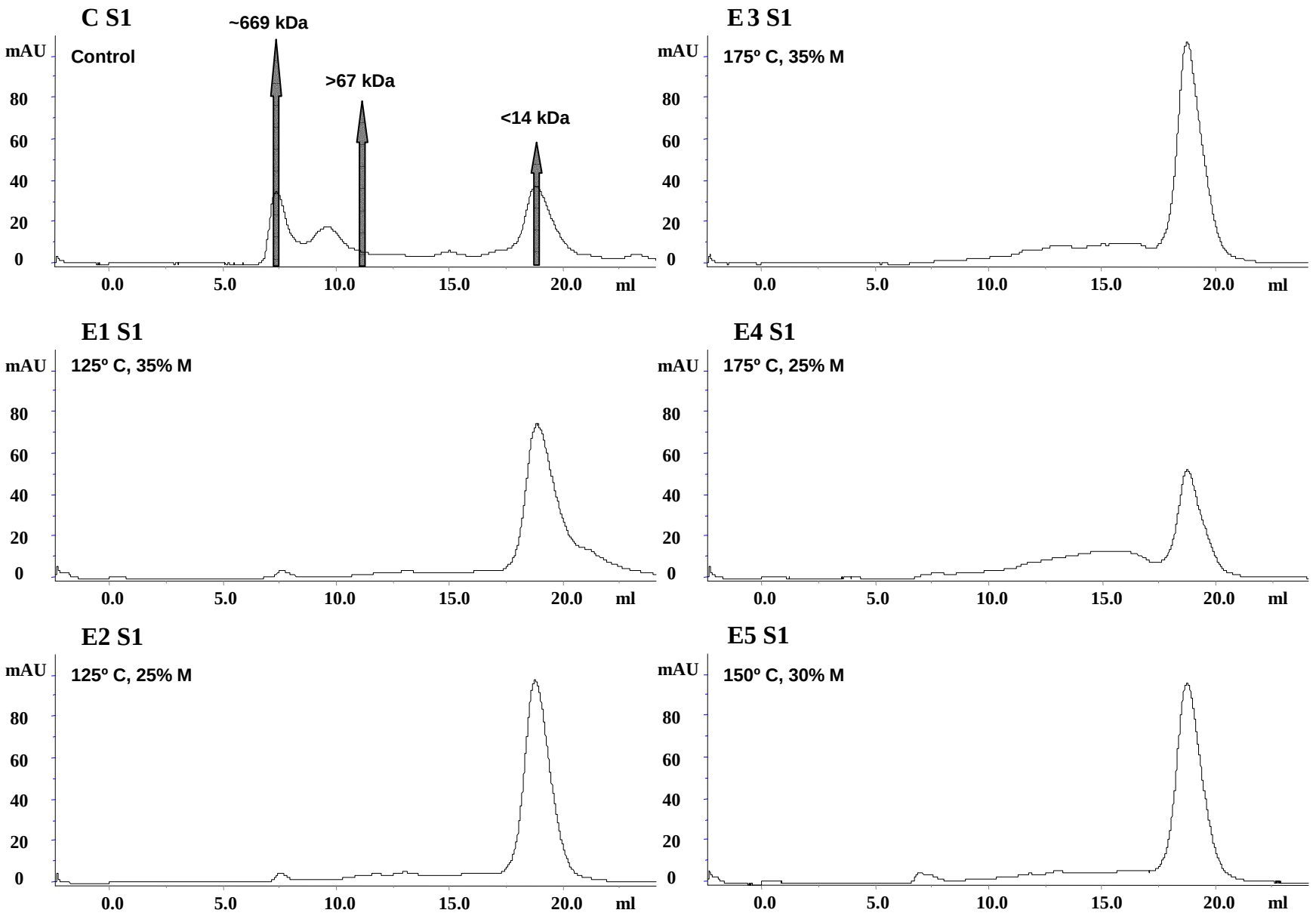
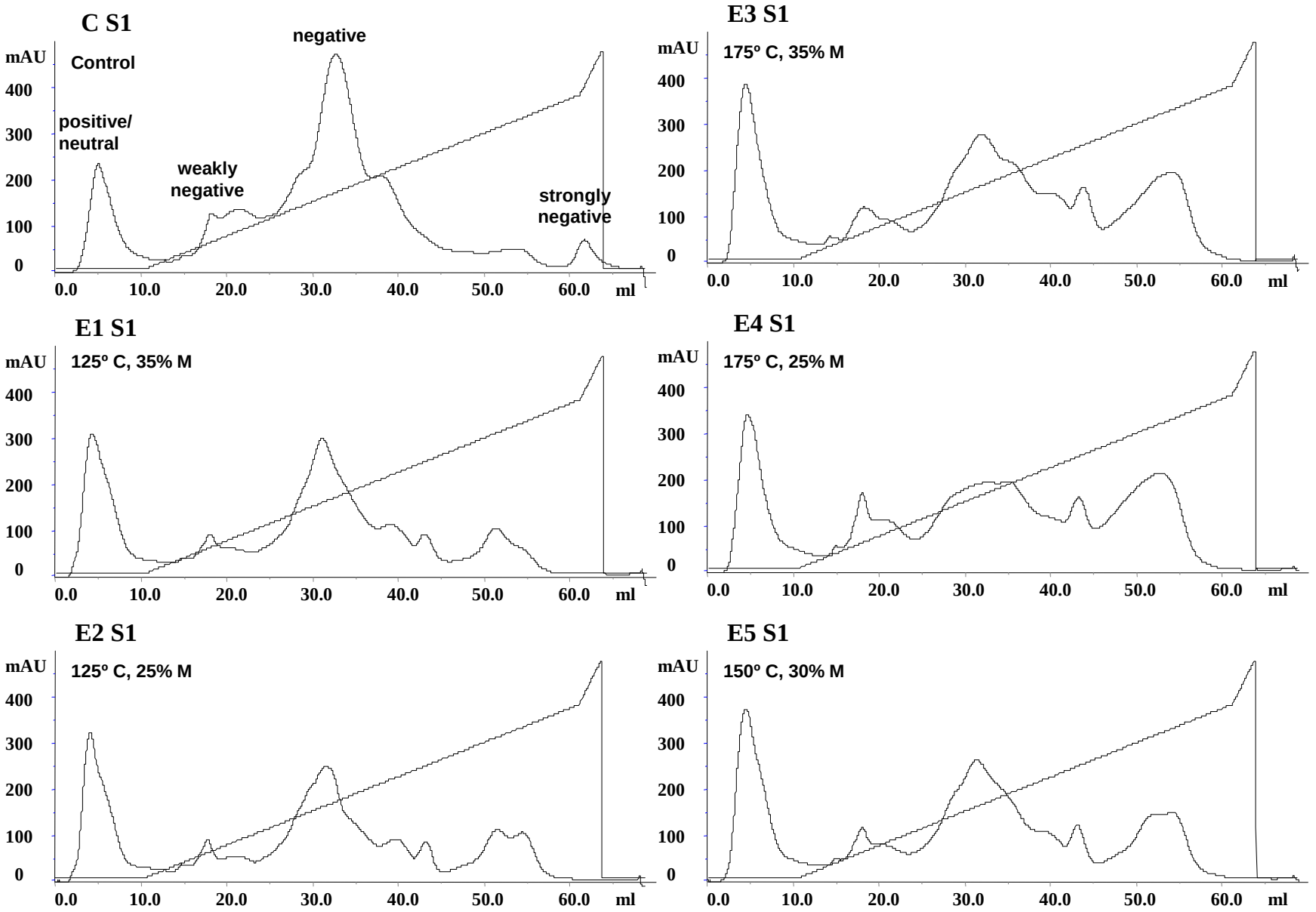

C S1
Control
~669 kDa
>67 kDa
<14 kDa
E 3 S1
175° C, 35% M
E1 S1
125° C, 35% M
E4 S1
175° C, 25% M
E2 S1
125° C, 25% M
E5 S1
150° C, 30% M
mAU
0
20
40
60
80
0.0
5.0
10.0
15.0
20.0
ml

Figure 3.6: Ion-exchange chromatographic analysis of control and extruded peanut flour protein samples of Stage 1.


C S1
Control
negative
positive/
neutral
weakly
negative
strongly
negative
E3 S1
175° C, 35% M
E1 S1
125° C, 35% M
E4 S1
175° C, 25% M
E2 S1
125° C, 25% M
E5 S1
150° C, 30% M
mAU
0
100
200
300
400
0.0
10.0
20.0
30.0
40.0
50.0
60.0
ml

control and extruded samples based on the charge distribution of the aggregates. Five distinct peaks were observed in ion exchange chromatograms regardless of any extrusion activity. However, 3 of these 5 peaks were chosen for further analysis as they were found to be in most significant amounts. These peaks were named **A**, **B**, and **C** as shown in **Figure 3.7** and corresponded to the peaks of **Figure 3.6: C S1 to E5 S1**. This classification was based on neutral or positive charge distribution (**Peak A**), intermediately negative charge distribution (**Peak B**) and strongly negative charge distribution (**Peak C**) of protein aggregates. **Peak A** proteins showed a marginal increase in their levels after extrusion when compared to the control (as shown by the level of **Peak A** in **Figure 3.6: C S1 to E5 S1**). However, there were marked variation in the levels of **Peak B** and **Peak C** while comparing the samples. In general the extruded samples exhibited a marked reduction in the levels of **Peak B** species. Contrastingly, the **Peak C** species increased in their levels upon extrusion.

Earlier, the gel exclusion and SDS-Page analysis showed that upon extrusion the proteins undergo disintegration/de-aggregation forming excess of low molecular weight products. Again the ion exchange chromatography data pointed out that upon extrusion the level of proteins either with weak negative charge or strong negative charge distribution increases. Thus the results demonstrated that the extrusion process leads to de-aggregation of protein complexes forming low molecular weight products which are either weakly negatively or strongly negatively charged (**Peak A** and **Peak C**). In order to understand the exact molecular composition of proteins/aggregates belonging to **Peak A**, **B**, and **C**, freeze dried samples from these peaks were analyzed by SDS-Page. The results of these experiments are shown in **Figure 3.8** and **3.9**. The SDS-Page analysis of **Peak A** clearly showed an abundance of low molecular weight subunits (below 32 kDa) in the case of all extruded protein samples. However, it was observed that a

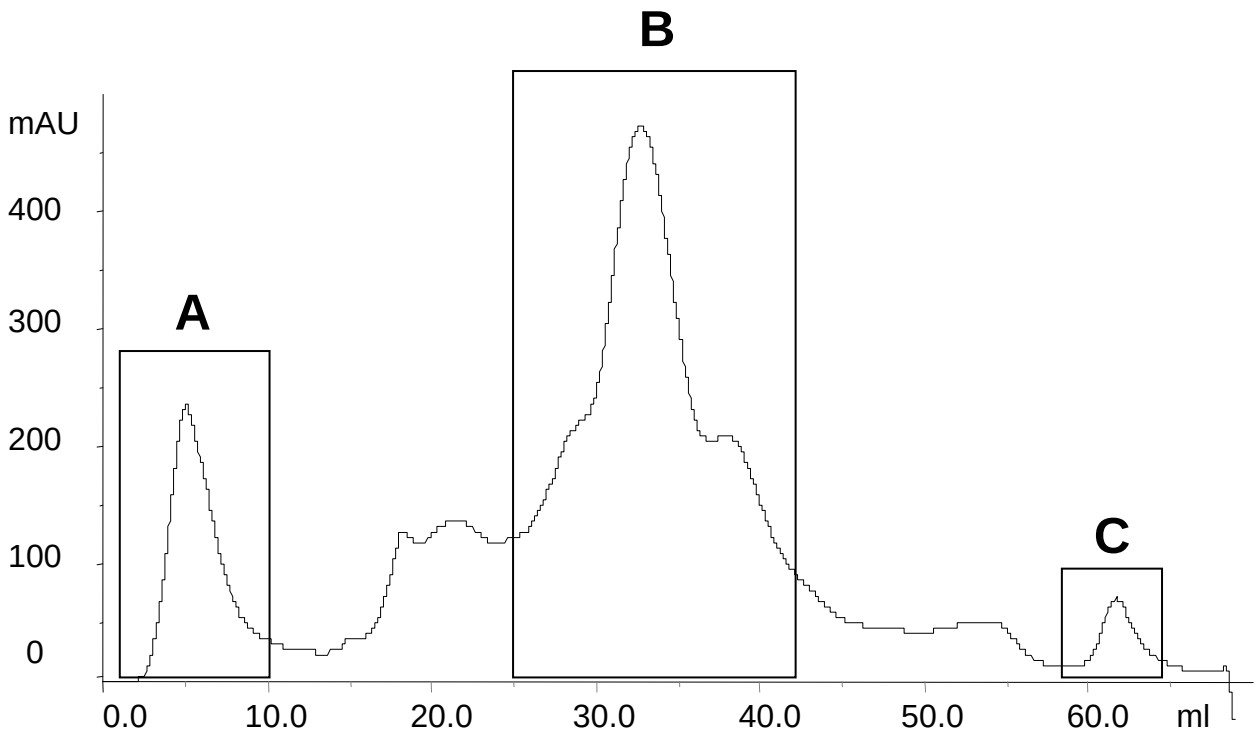


Figure 3.7: Peak fractions (as highlighted by boxes A, B, and C) selected for further analysis of the charged aggregates eluting in the ion-exchange chromatographic analyses of Stage 1 buffer soluble protein extracts. SDS-Page was employed to understand the protein composition of each fraction (A, B, and C) for the unextruded and extruded Stage 1 samples.

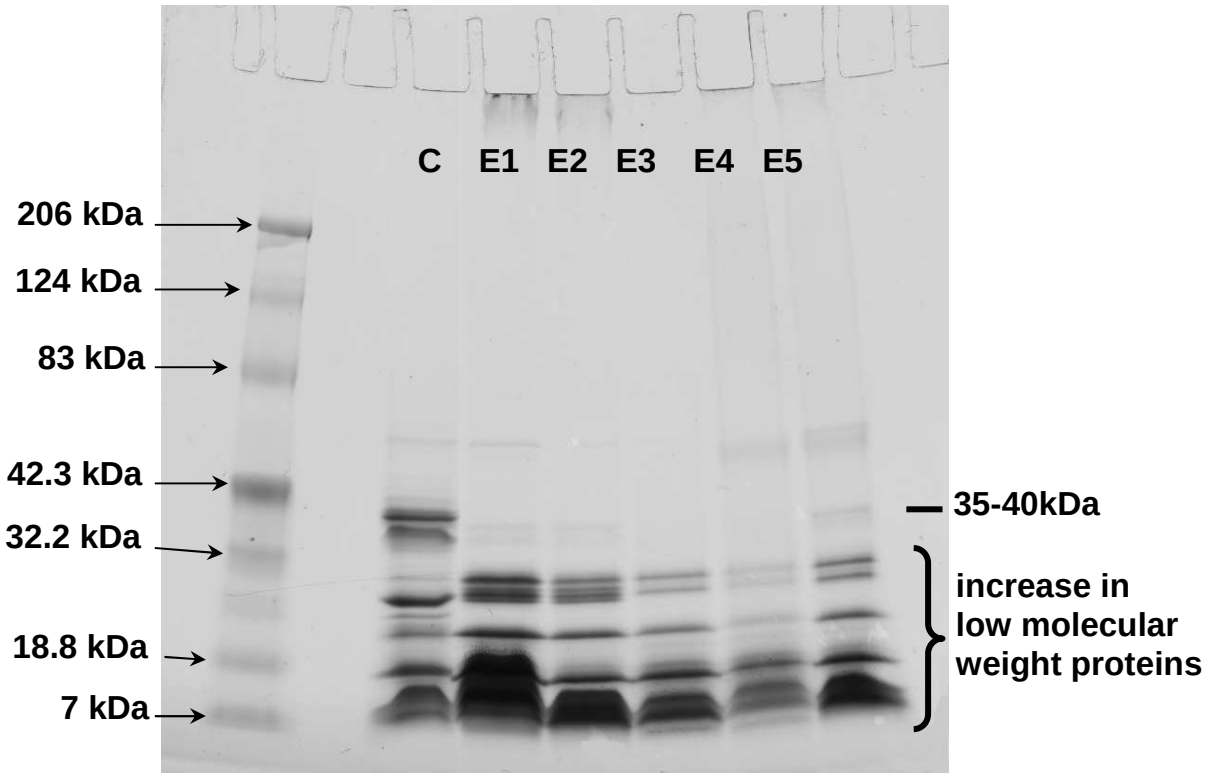


Figure 3.8: SDS-Page of proteins belonging to the aggregates corresponding to fraction A as shown in Figure 3.7 (Peak A).

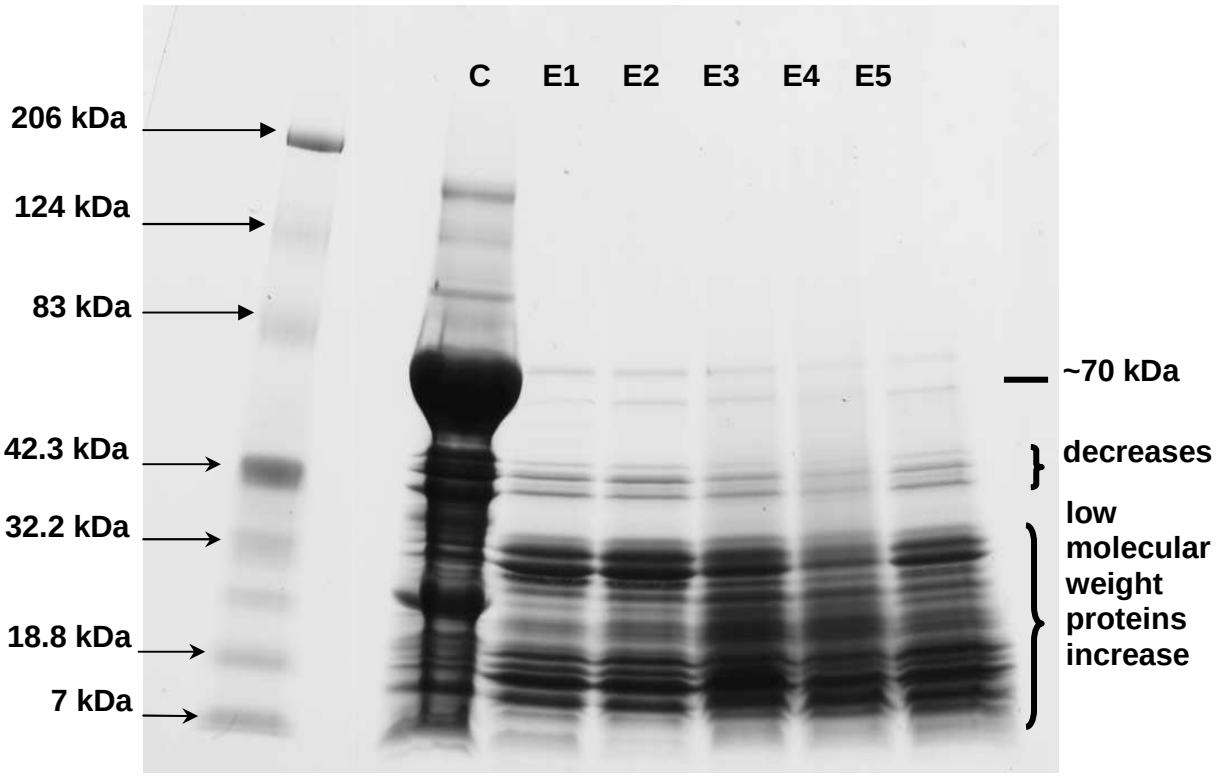


Figure 3.9: SDS-Page of proteins belonging to the aggregates corresponding to fraction B as shown in Figure 3.7 (Peak B).

relative abundance of some high molecular weight subunits, 35-40 kDa, occurred in the control. These results indicate that the positive/neutral charged proteins are mainly low molecular weight ones. Similarly, protein samples belonging to **Peak B** were analyzed by SDS-Page. The protein profile obtained in this study was identical to the results of SDS-Page analysis of S1 samples. Here also it was observed that a high level ~70 kDa subunit occurred in the control. Further it was also observed that upon extrusion the abundance of low molecular weight subunits increased with a marked increase in the 30-32 and 10-20 kDa ranges. Amongst the various extrusion conditions employed, the extrusion at 175 ºC and 25% moisture content (most severe) resulted in a lower yield of all characteristic low molecular weight subunits found in the extrudates. However, in all the other extrusion conditions the level of these subunits was identical. In summary, these results indicate that the majority of buffer 1 solubilized protein extracts from control and extruded samples have an intermediate negative charge distribution. Proteins belonging to **Peak C** of ion exchange chromatogram were also analyzed by SDS-Page. Due to very low recovery of proteins in this peak, only scanty band formation was observed (data not shown).

SDS-PAGE Analysis of S2 samples

Extracts were made from the residual precipitate of S1 by solubilizing the residue in a buffer containing 20 mM Sodium Phosphate and 6 M Urea (pH 7.9). The buffer 2 solubilized material contained a comparatively higher yield/amount of proteins than the S1 extract of extrudates.

In contrast to the SDS-Page analysis of S1 protein extracts, the proteins belonging to the S2 extracts contained the same subunits in both control and extruded samples as shown in **Figure 3.10**. Compared to S1 samples, S2 samples contained predominant subunits in the 70,

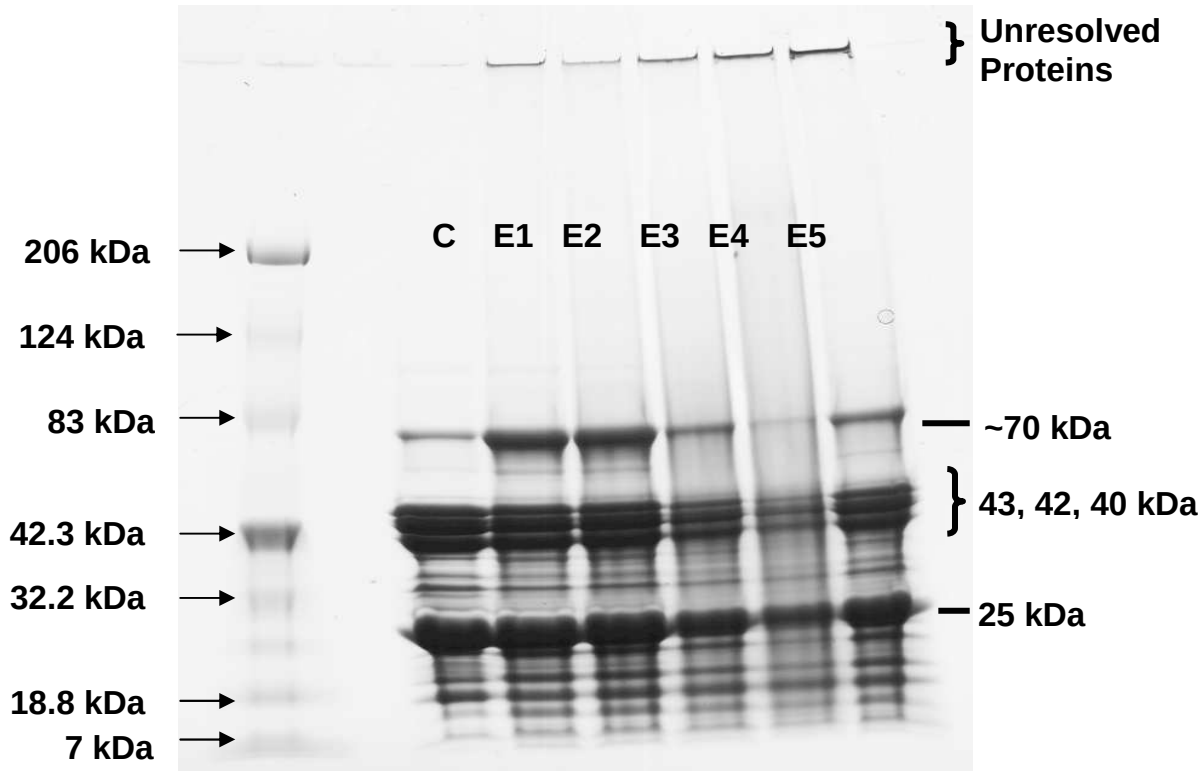
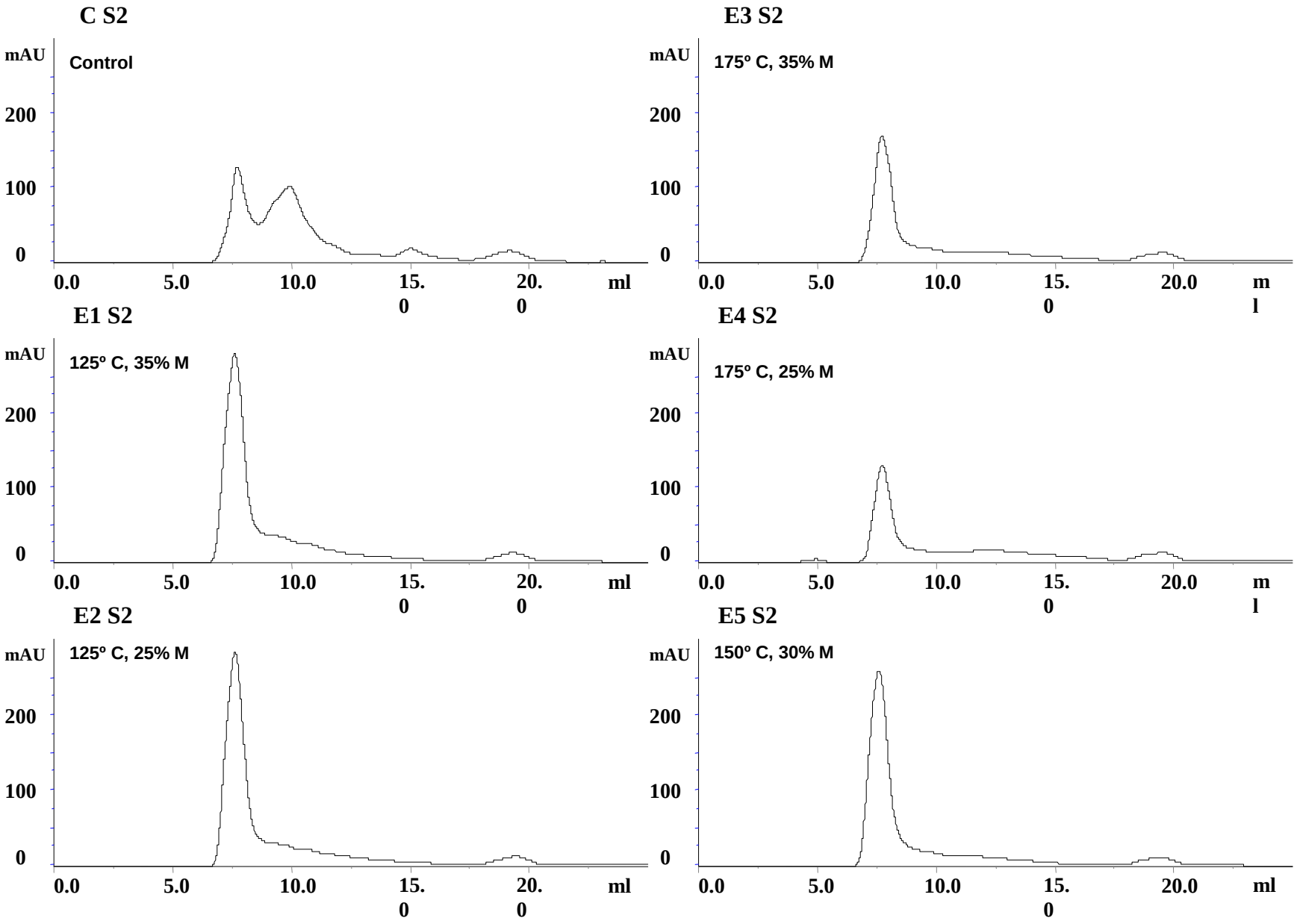


Figure 3.10: SDS-Page of buffer soluble extract of the peanut protein fractions from defatted peanut flour (control) and the five extruded samples (E1 to E5) – stage 2. The samples were extracted using 20 mM sodium phosphate buffer (buffer 2) (pH 7.9) containing 6 M Urea.

Figure 3.11: Molecular gel exclusion chromatographic analysis of unextruded (control) and extruded peanut flour samples of Stage 2.


C S2
Control
mAU
200
100
0
0.0
5.0
10.0
15.0
20.0
ml
E3 S2
175° C, 35% M
E1 S2
125° C, 35% M
E4 S2
175° C, 25% M
E2 S2
125° C, 25% M
E5 S2
150° C, 30% M

43-40, and 25 kDa ranges regardless of being subjected to the extrusion process. In the control, these bands might correspond to the components of large aggregates existing in peanut flour, which are normally insoluble in an aqueous non-denaturing buffer. Stage 2 extracts of extrudates contain most of the sample's protein and also represent phosphate buffer insoluble species containing mainly subunits in the 40-43, ~30 kDa, and in some cases 70 kDa ranges. Further, these results also pointed out that the composition of aqueous buffer insoluble protein aggregates in control and extruded protein samples of peanut flour are identical in the specific subunits present. It was also noticed that the relative amounts of the above said characteristic protein bands were high in low temperature extrusion (**E1 and E2**). However, a severe extrusion condition of 175 ºC and 25% moisture content resulted in a low yield of subunits in buffer 2 extraction (E4).

Molecular gel exclusion chromatographic analysis of S2 samples:

The protein samples of S2 extracted in buffer 2 were also analyzed using MGEC. The elution profiles of the samples were distinct in the control and extruded peanut flour extracts as seen in **Figure 3.11**. The control resolved into 4 distinct peaks upon gel exclusion chromatography. Peaks eluting at volumes of ~7 ml (arachin) and 10 ml (conarachin II) were more prominent than the other 2 peaks eluting at volumes of 15and 20 ml . Contrastingly, the S2 extracts from extruded samples were found to resolve into two peaks, the predominant one eluting at around 7 ml. The size of the major peak varied with extrusion conditions. Under the extrusion conditions of low temperature (**E1 S2** and **E2 S2**) this peak was comparatively higher than at high temperature extrusion (**E3 S2** and **E4 S2**). At intermediate conditions (**E5 S2**) an intermediate height of this peak was observed. This indicates a direct correlation between the amount of these aggregates and the temperature of extrusion. It was also noted that the peak of

high molecular weight protein (arachin) eluting at 7 ml was significantly less in the case of control compared to the amount of aggregate in the same size range in the extruded samples. It is speculated that, the proteins constituting the two major peaks are aggregating into high molecular weight species, upon extrusion. In order to understand the exact protein profile of these peaks SDS-Page was conducted as shown in **Figure 3.12**. In general, all the peaks, irrespective of extrusion, contained ~43, 42, 40 and 25 kDa protein bands. This shows that these subunits represent the major components of protein aggregates present in the peanut flour with or without extrusion. It was also observed that the prominent peak obtained in the case of extruded samples contained an increased proportion of low molecular weight protein components. Under severe conditions of extrusion (175 ºC and 25% moisture; **E4**) the yield of stage 2 soluble aggregates, as indicated by the height of the prominent peak, was significantly less. In summary, it is speculated that the extrusion conditions stimulate a high degree of aggregation of proteins. Further, aggregation yielding these species is enhanced when the temperature is low and moisture content is relatively high.

Ion exchange chromatographic analysis of S2 samples:

The ion exchange chromatographic data depicted in **Figure 3.13** suggests that there exists a charge distribution identity among various protein aggregates belonging to S2 samples of control and extrudates. Irrespective of any extrusion process a neutral/positively charged peak eluting out early in the gradient (5ml) could be detected in all the samples. In general, all the samples resolved into a broad peak eluting at a region of 20 to 45 ml representing an intermediately negatively charged protein aggregate peak. Samples that were extruded at high temperature conditions ranging from 150 to 175 ºC (**E3 S2** to **E5 S2**) were different compared to all other samples. In these samples this intermediately charged peak was having a significantly

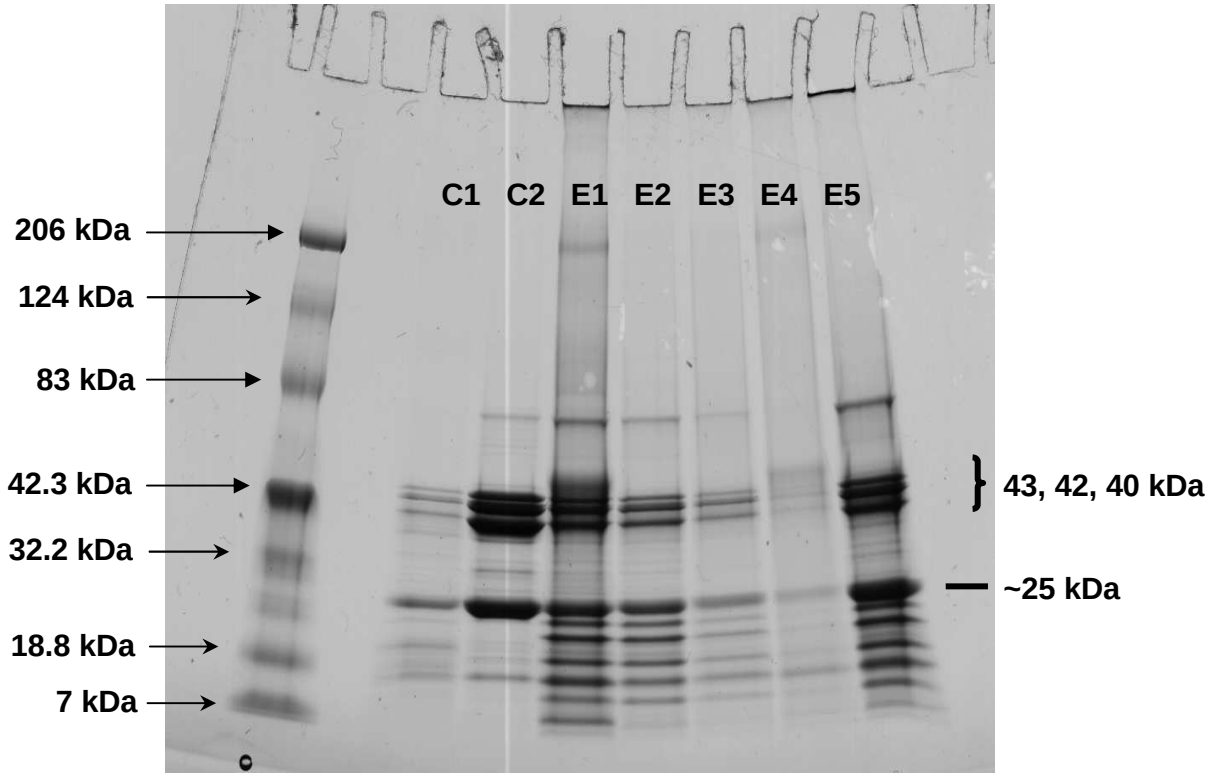
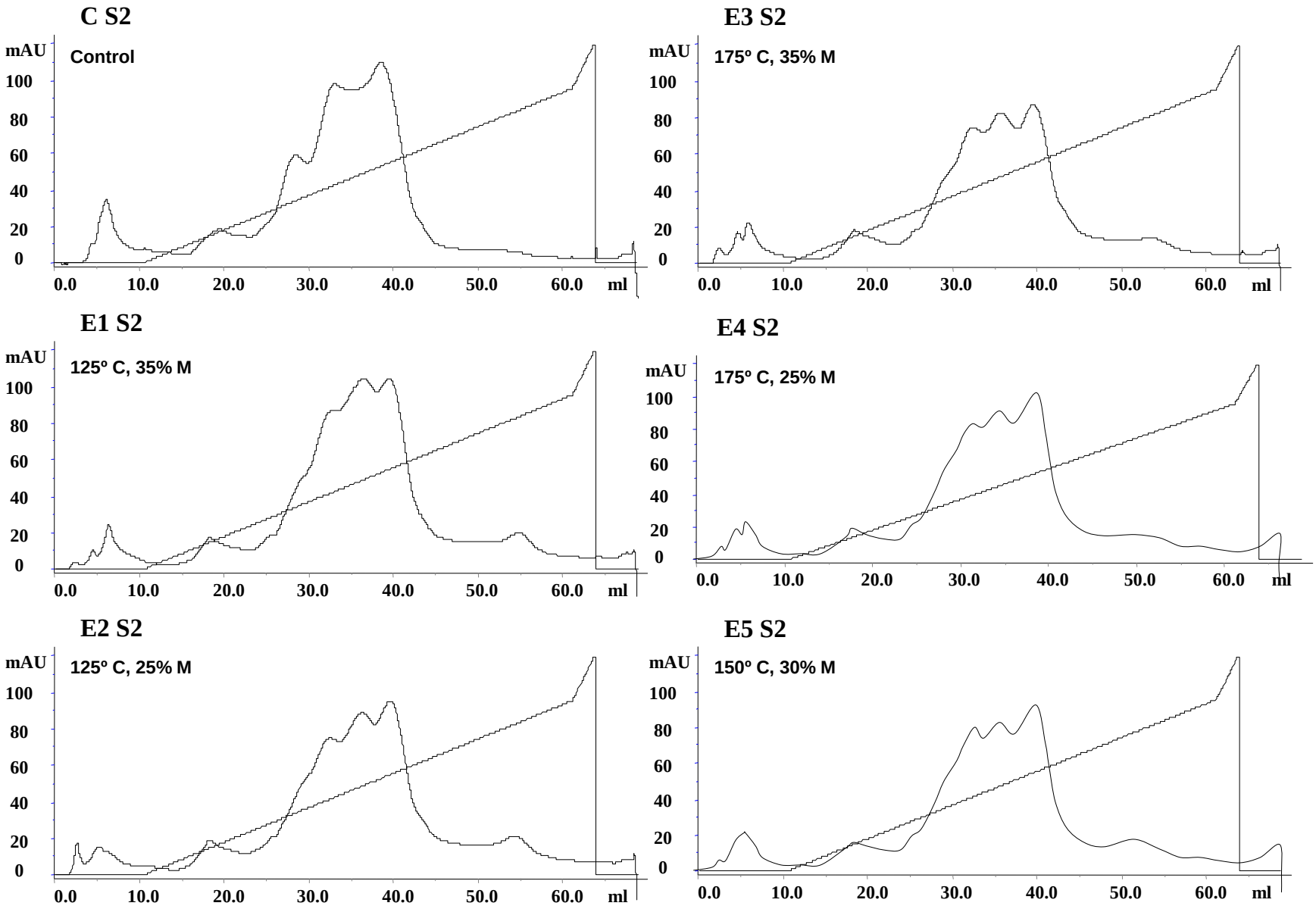


Figure 3.12: SDS-Page of proteins belonging to the prominent peaks obtained in molecular gel exclusion chromatographic analyses of control (2 peaks; C1 & C2) and extruded peanut protein samples of Stage 2.

Figure 3.13: Ion-exchange chromatographic analysis of control and extruded peanut flour protein samples of Stage 2.


C S2
Control
mAU
100
80
60
40
20
0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
ml
E3 S2
175° C, 35% M
E1 S2
125° C, 35% M
E4 S2
175° C, 25% M
E2 S2
125° C, 25% M
E5 S2
150° C, 30% M

broad profile. This shows that under these high temperature conditions of extrusion the protein aggregates undergo characteristic change in their folding pattern exposing out residues which result in a randomized distribution of charges. This hints that the extrusion conditions of very high temperature can definitely alter the aggregation pattern in the peanut flour proteins.

SDS-PAGE analysis of S3 samples:

Figure 3.14 shows the SDS-Page analysis of S3 buffer soluble extracts from the various samples. A good yield of buffer soluble proteins was obtained from extrudates upon using buffer 3 which indicates that addition of urea alone couldn't recover the bulk of protein aggregates into the supernatant. This means that DTT, by its direct interference on disulfide linkages and by reducing the free sulfhydril groups on the protein aggregates, facilitated more recovery of proteins into the solution. It was concluded that disulfide linkages were one among the major interactions stabilizing the protein aggregates in the various extrudates. The buffer 3 extraction of proteins from the extruded samples brought forwards a good yield of proteins in solution compared to that of control. Here it can be speculated that the protein aggregate formation stabilized by disulfide linkages was prevalent in extruded samples. In other words, the various extrusion conditions (E1, E2, E3 and E5) stimulate inter-molecular disulfide interactions. It was also observed that the subunit composition of aggregates, regardless of their extraction in buffer 2 or buffer 3, remained almost identical. The S3 samples also resolved into ~43, 42, 40, and 25 kDa characteristic protein bands similar to that observed in the case of S2 samples. The extreme conditions of extrusion such as 175 ºC and 25% moisture content resulted in a low yield of proteins while extracting with buffer 3. As observed in the case of S2 samples, the extrusion process resulted in an enhancement in the level of ~70 kDa subunit in aggregates compared to residual protein from the control. However, severe extrusion condition of high temperature and

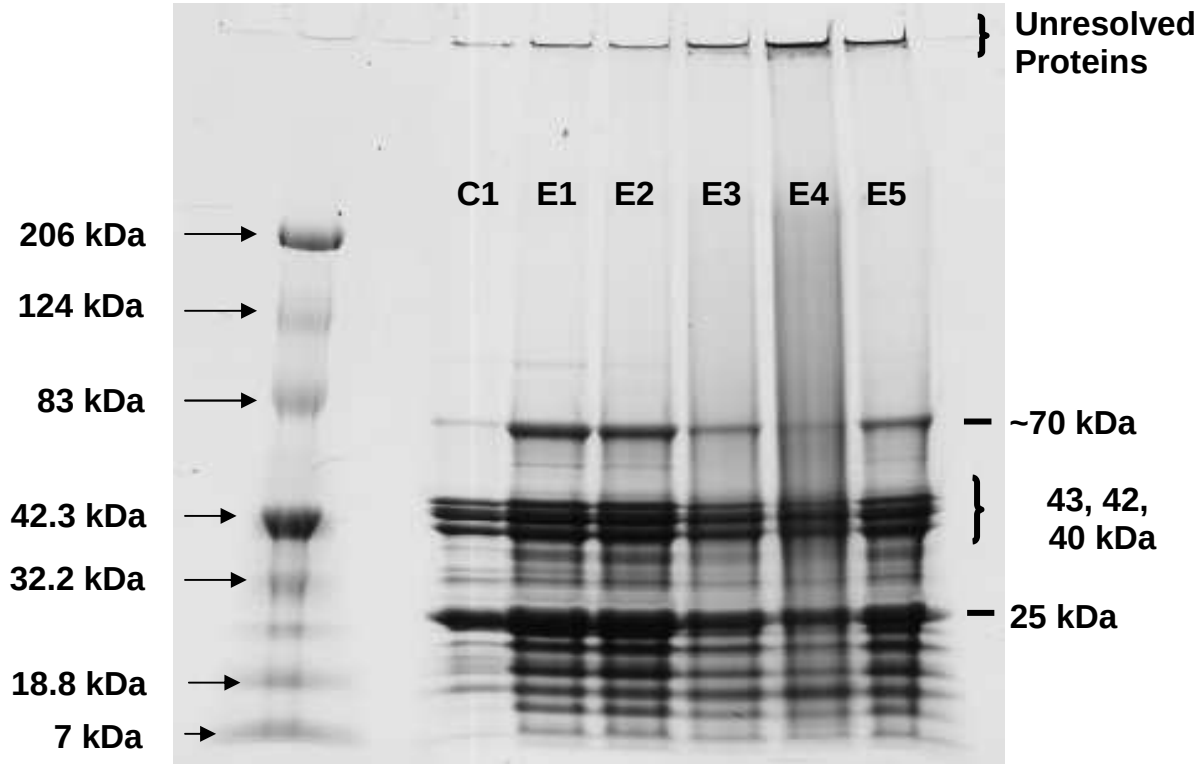


Figure 3.14: SDS-Page of buffer soluble extract of the peanut protein fractions from defatted peanut flour (control) and the five extruded samples (E1 to E5) – stage 3. The samples were extracted using 20 mM sodium phosphate buffer (buffer 3) (pH 7.9) containing 6 M Urea and 0.01 M DTT.

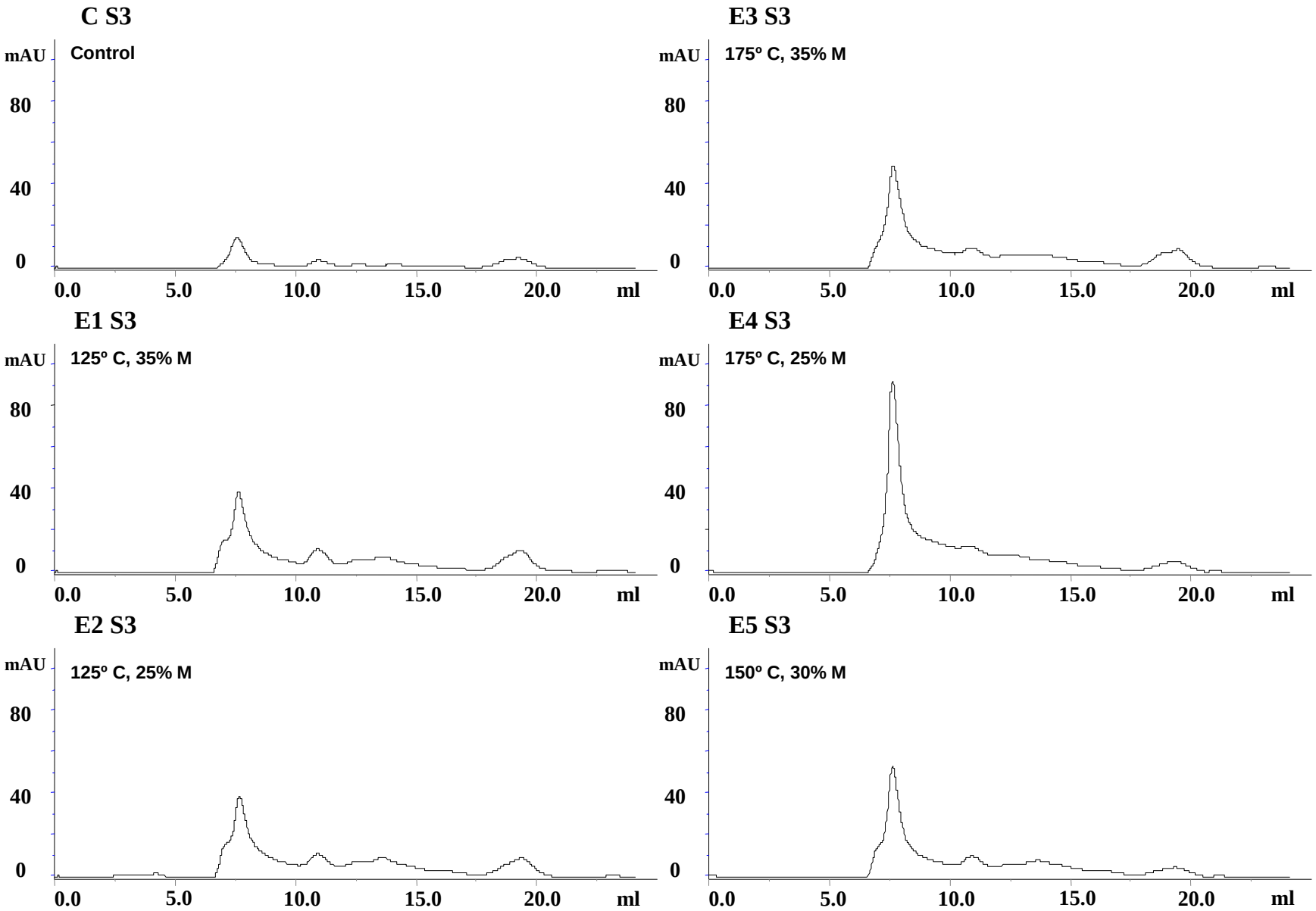
low moisture level exerted a drastic reduction in the level of this subunit showing its sensitiveness to high temperature environment. A substantial amount of proteins failed to enter the gel as shown in Figure 14. The highest amount of which corresponded to the sample E4. These unresolved proteins could be due to formation of stronger interactions, such as isopeptide bonds, amongst protein aggregates, which were not cleaved by SDS and mercaptoethanol, but were soluble in buffer 3 (urea and DTT).

Molecular gel exclusion chromatographic analysis of S3 samples:

The data representing the elution profile of S3 protein extracts from control as well as extruded samples are shown in **Figure 3.15**.

A distinct elution profile was observed in the case of control with prominent peaks eluting near the arachin and conarachin II regions. However, the protein extracts from the five different extruded samples using buffer 3 resolved into different sized peaks. Low temperature extrudates resolved into three peaks and a shoulder around 12-15 ml range where as higher temperature extrudates resolved into two peaks and a broad shoulder ranging from 10-15 ml range. As the temperature of extrusion increased, the higher molecular weight aggregate peak was also found to increase. One peculiar behavior of the elution pattern obtained in the case of most severe extrusion conditions was that the level of the peak representing the high molecular weight aggregate was found to be highest. This peak could be comprised of aggregates belonging to the unresolved proteins of the SDS-Page (**Figure 3.14**). Also, the possibility of the re-establishment of other important weak interactions among the disintegrated aggregates forming fresh aggregates cannot be ruled out.

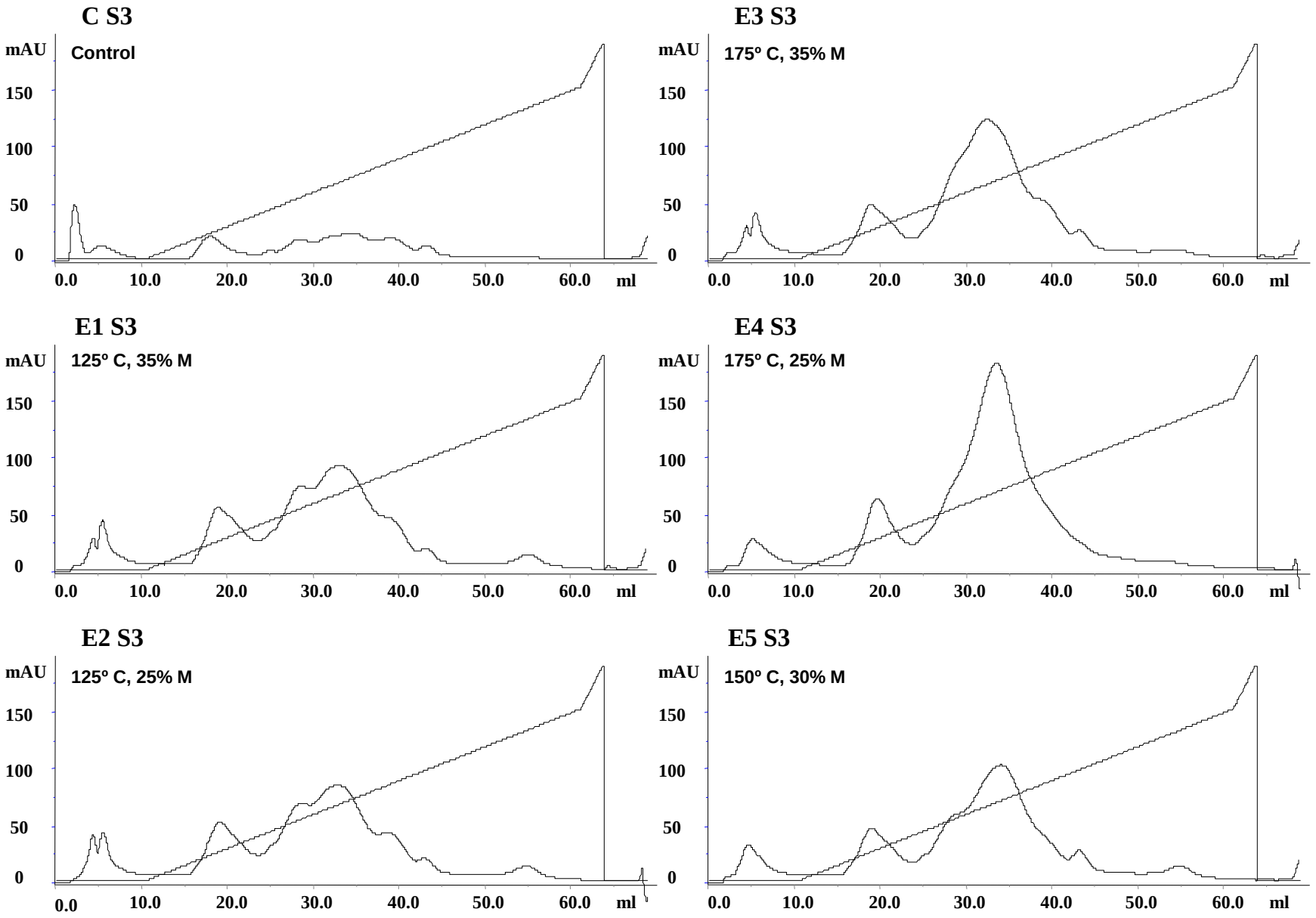
Figure 3.15: Molecular gel exclusion chromatographic analysis of unextruded (control) and extruded peanut flour samples of Stage 3.


C S3
Control
mAU
80
40
0
0.0
5.0
10.0
15.0
20.0
ml
E3 S3
175° C, 35% M
E1 S3
125° C, 35% M
E4 S3
175° C, 25% M
E2 S3
125° C, 25% M
E5 S3
150° C, 30% M

Ion exchange chromatographic analysis of S3 samples:

The retention of proteins on the column during gradient elution varied with extrusion conditions as seen in **Figure 3.16**. Earlier the SDS-Page and gel exclusion chromatography revealed the subunit composition and aggregate pattern of these protein components. Irrespective of any sort of processing a positive/neutrally charged protein peak eluting at very low concentration of NaCl in the elution buffer could be observed in all samples. The major variation in this gradient elution was seen in the case of a characteristic protein aggregate peak eluting at a concentration of approximately 50% NaCl (vol. 35ml). The level of this specific aggregate, which is intermediately negatively charged, was relatively high in the case of all extruded samples compared to that of control. Further, an extrusion condition of high temperature and low moisture level stimulated a maximum enhancement in the level of this specific aggregate.

Figure 3.16: Ion-exchange chromatographic analysis of control and extruded peanut flour protein samples of Stage 3.


C S3
Control
mAU
150
100
50
0
0.0
10.0
20.0
30.0
40.0
50.0
60.0
ml
E3 S3
175° C, 35% M
E1 S3
125° C, 35% M
E4 S3
175° C, 25% M
E2 S3
125° C, 25% M
E5 S3
150° C, 30% M

CONCLUSIONS

The present investigation is an attempt to understand the effect of extrusion, performed under varying conditions of temperature and moisture content, on the protein aggregate profile, the structural properties of protein aggregates/complexes belonging to peanuts.

The highest mean residence time was observed to be 88.8 s during extrusion performed under 175 °C temperature and 35% moisture (E3) due to decreased dough viscosity of melt under extrusion conditions of high temperature and high moisture. The highest SME (485.6 KJ/kg) was observed under low temperature and low moisture extrusion condition (E2) due to the fact that as the temperature and moisture decrease the melt viscosity increases and more energy is required to work the melt.

The three buffer systems employed to solubilize the proteins belonging to the control as well as the extrudates and the techniques incorporated to understand the protein chemistry of these samples offers a novel method for studying the effects of extrusions on proteins, in particular peanut proteins. With the high yield of buffer soluble proteins, majority of the protein aggregates existing in defatted peanut flour have been analyzed. These studies have been largely confined to protein molecules having a size greater than 8 kDa and all the analysis employed in this study have been on an equal protein basis. SDS-Page analysis showed that regardless of being subjected to extrusion process, all the samples contained characteristic proteins having molecular weights of ~70, 43, 42, 40, and 25 kDa. However, severe extrusion condition of 175 ºC and 25% moisture content resulted in a low yield of proteins. Further more, the abundant higher molecular weight protein (~70 kDa) in the control was greatly reduced by extrusion with the level being almost nil under most severe extrusion condition. Also, the extrudates showed to

contain more lower-molecular compounds. Thus, the process of extrusion stimulates disintegration of protein aggregates into low molecular weight components or species.

The extrusion process de-aggregates macro molecular protein aggregates/complexes forming low molecular weight products which are either neutral/positively charged or too strongly negatively charged. There also existed a charge distribution identity amongst the various samples analyzed. Substantial amounts of protein aggregates/complexes, having an intermediate negative charge distribution, were observed in the case of control and the various extruded products whose amount decreased upon extrusion.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The present investigation is an attempt to understand the effect of extrusion, performed under varying conditions of temperature and moisture content, on peanut protein profile and the properties of protein aggregates/complexes in partially defatted peanut flour-corn starch mixtures and defatted peanut flour. Extrusion is a unique process and generally involves the conversion of a “plasticized bio-polymer-based formulation” into a well and uniformly processed viscoelastic mass which undergoes forming and often puffing as it exits the die. It is characterized by the complex interplay of heat, mass, and momentum transfer in concert with the physical and chemical transformations the feed undergoes. It has been demonstrated by many researchers that extrusion causes protein denaturation leading to breaking non-covalent, disulfide, and sometimes peptide bonds which may again get reestablished in true or false manner upon extrusion. It was hypothesized that similar behavior occurs during extrusion of peanut proteins. We have concentrated our study to three major types of protein extracts isolated in three different buffer systems. The buffer 1 did not contain any of the known denaturizing additives, while buffer 2 and 3 comprised of denaturizing additives like urea and urea-DTT respectively.

The first part of the study (Chapter 2) explored the possibility of modification of structural and functional properties of peanut proteins-corn starch mixtures upon extrusion. Partially defatted peanut flour (~6% fat) was mixed with cornstarch (1:1) was used as the feed. Temperature of extrusion and moisture content of the feed were varied to obtain two different extrudates. The extrudates were a result of the following conditions: E1 - 135 ºC & 20% moisture (more severe); E2 - 115 ºC & 40% moisture (less severe). Moderately severe (in terms of mechanical energy input) screw configuration was employed at screw speed of 500 rpm. Sequential protein solubility profile in sodium phosphate buffer (pH 7.9) containing 0.5 M NaCl (buffer 1), sodium phosphate buffer (pH 7.9) containing 6 M urea (buffer 2), and sodium

phosphate buffer (pH 7.9) containing 6 M urea and 0.01 M DTT (buffer 3) were applied to study the effect of extrusion on peanut proteins in the control and the extrudates. Agarose gel electrophoresis, SDS-Page, Size exclusion chromatography, and Ion-exchange chromatography were employed to monitor the changes in the protein chemistry of peanut flour. It was concluded that extrusion caused the formation of not only disulfide bonds but also non-covalent bonds. Shear force, during extrusion, was one of the most important parameters governing formation of particular type of protein-protein interactions. The severity of extrusion was found to share an inverse relationship with formation of new aggregates which are either highly positively charged or moderately negatively charged.

The second part of the study (Chapter 3) concentrated on the possible changes taking place during the extrusion of native proteins belonging to peanuts. Defatted peanut flour (~1.5% fat) was extruded at 300 rpm using a very severe screw configuration. Temperature of extrusion (125 ºC, 150 ºC, and 175 ºC) and moisture content (25%, 30%, and 35%) of feed were varied to obtain five different extrudates (E1 – E5). The extrudates were analyzed using techniques similar to the previous study (Chapter 2). All buffer extractions were found to be efficient and brought about considerably large yield of buffer soluble proteins. We expect that with these three buffer systems we might have analyzed majority of the proteins and aggregates existing in defatted peanut flour. Dialysis was employed as a major tool for removing low molecular weight components from the various protein extracts. Thus, our studies were largely confined to protein molecules having a size greater than 8 kDa. All the analysis employed in this study was on an equal protein basis. This ensured an efficient comparison of protein level/structure and profile among the various samples. The major conclusions are as follows: Analysis of S1 samples - the peanut flour (control) showed an abundant level of a high molecular weight protein (~70 kDa).

The extrusion process brought about a reduction in the level of this protein. Severe extrusion condition of low moisture and high temperature resulted in a nil level of this protein. The relative level of high molecular weight protein aggregates were observed to be high in control compared to the samples subjected to extrusion. The process of extrusion stimulates disintegration of protein aggregates into low molecular weight components or products. The extrusion process de-aggregates macro molecular protein aggregates/complexes forming low molecular weight products which are either too weakly negatively charged or too strongly negatively charged. Also, a substantial amount of protein aggregates/complexes, having an intermediate negative charge distribution, were observed in the case of control and the various extruded products whose amount decreased upon extrusion. Analysis of stage 2 samples - the buffer 2 solubilization brought about a higher yield of proteins in extrudates than buffer 1 solubilization. SDS-Page analysis showed that regardless of being subjected to extrusion process, all the samples contained characteristic proteins having molecular weights of ~70, 43, 42, 40, and 25 kDa. Severe extrusion condition of 175 C and 25% moisture content resulted in a low yield of proteins. 43, 42, 40, and 25 kDa proteins represent the major components of protein aggregates in peanut flour regardless of being subjected to extrusion process. There is a direct co-relation between the amount of aggregates and the temperature of extrusion. There exists a charge distribution identity among various protein aggregates belonging to S2 samples of control and peanut flour. Analysis of stage 3 samples - when using Buffer 3, the component DTT by its direct interference on disulfide linkages and by reducing the free sulfhydril groups on the protein aggregates facilitate more recovery of protein in to the solution. Buffer 3 extraction of proteins from the extruded samples brought forward a good yield of protein in to solution compared to that of control indicating the prevalence of disulfide linkages amongst aggregates in the extruded

samples as compared to the control. Increased amount of higher molecular aggregates found in extruded samples compared to control. Also, a substantial amount of protein aggregates/complexes, having an intermediate negative charge distribution, were observed in the case of control and the various extruded products whose amount increased upon extrusion. RTD and SME - the highest mean time was observed to be 88.8 s during extrusion performed under 175 °C temperature and 35% moisture (E3) due to decreased dough viscosity of melt under extrusion conditions of high temperature and high moisture.The highest SME (485.6 KJ/kg) was observed under low temperature and low moisture extrusion condition (E2) due to the fact that as the temperature and moisture decrease the melt viscosity increases and more energy is required to work the melt.

Though the above mentioned techniques provide a good understanding of what, the proteins belonging to peanuts undergo upon a dynamic processing technique like extrusion, this knowledge still must be considered the tip of ice berg as far as proteomics of extruded peanut flour is considered. A further understanding of what really happens to peanut protein complexes, and protein-protein interactions upon extrusion can not only prove to be useful to utilize protein rich materials like peanuts, but also lead to the development of energy delivery food systems and novel food products.