THE EFFECTS OF MOIST HEAT ON THE NUTRITIVE VALUE OF SOY PROTEIN CONCENTRATE

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(ABSTRACT)

Soy protein concentrate (SPC) was autoclaved at 121° C and 15 p.s.i for 0, 10, 30 min., 2 and 4 hr. The solubility of the control, 10 and 30 min. autoclaved samples was quite low (about 12%) as compared with the 2 and 4 hr. samples (about 27%) in potassium phosphate buffer (pH 7.6, 0.5 ionic strength). When urea and beta-mercaptoethanol were added in increasing concentration to the buffer, the solubility of control, 10 and 30 min. samples increased dramatically up to 60%; while the solubility of 2 and 4 hr. autoclaved samples changed very little remaining at 25 to 40% throughout.

Digestibility of SPC samples was determined by three *in vitro* methods, all including treatment of SPC with selected proteases, followed by measurement of (1) TCA soluble N production (2) breakdown products via SDS-PAGE and (3) pH drop resulting from enzyme action. *In vivo* apparent digestibility was determined in a rat feeding study. The digestibility of SPC samples were found to be significantly affected by length of autoclaving. The digestibility of control was higher than autoclaved samples as determined by both *in vitro* and *in vivo*

assays. The digestibility of 10 and 30 min. autoclaved SPC samples was significantly higher than 2 and 4 hr. autoclaved samples in *in vitro* assays. While, apparent *in vivo* digestibility of 10, 30 min. and 2 hr. autoclaved samples was significantly higher than the 4 hr. sample. Correlation coefficients of *in vitro* digestibility as determined by the TCA-soluble N measurement, SDS-PAGE and pH drop method with *in vivo* apparent digestibility were 0.96, 0.92 and 0.95, respectively.

There was no actual destrution of amino acids except cysteine; 10, 30 min., 2 and 4 hr. SPC samples contained 6, 20, 27 and 39 % less cysteine respectively than the SPC control. The chemical score of SPC samples indicated that cysteine became the first limiting amino acid in the 2 hr. sample.

PERs of the 2 and 4 hr. autoclaved SPC samples were significantly less than control, 10 and 30 min. autoclaved samples. While C-PERs of control, 10 min. samples were higher than 30 min., 2 and 4 hr. samples. Decreased PER values of autoclaved SPC samples were likely due to i) decreased protein digestibility and rate of enzymatic hydrolysis, ii) destruction of essential amino acids, and iii) decreased food intake.

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1.0 INTRODUCTION

Soy protein is an important protein source to humans, because of its high nutritive value, low cost and excellent functional properties in food systems (Wolf, 1970, Erdman and Fordyce, 1989). Defatted soy flour, soy grits, soy protein concentrate (SPC) and soy protein isolate (SPI) are four commercially available protein products made from soybeans. There is increasing interest in the use of SPC by processors in a variety of food products because of their compatibility with other food ingredients.

Heat treatment is a necessary procedure in the processing of soybeans in order to enhance the functionality of soy protein in food systems. Apart from the functional properties, mild heat treatment also has beneficial effects on the nutritive value of soy protein, although excess heat treatment is detrimental.

Most investigators have evaluated the nutritive value of soybean protein on the basis of chemical analysis and/or experiments with laboratory animals. Although <u>in vivo</u> studies using laboratory animals and human subjects are regarded as reliable assay methods, they are expensive and time-consuming. On the other hand, amino acid analysis of protein is rapid, and relatively inexpensive. However, a major disadvantage of using only amino acid composition to predict the nutritive

value of protein, is that it overlooks protein digestibility which in some cases is a major determinant of protein bioavailability (Kies, 1981). Therefore, recent efforts have been directed towards the development of a simple, inexpensive, and rapid in vitro method for determining protein digestibility.

The purpose of this study was to investigate the effects of autoclaving for 0, 10, 30 min., 2 and 4 hr. on soy protein quality based on digestibility, amino acid composition and PER. Two existing in vitro assays (TCA soluble N measurement and pH drop), one new in vitro assay (SDS-PAGE) and one in vivo assay (apparent digestibility in rats) were employed in this study. In addition to these methods, the feasibility of using the simultaneous dialysis method for evaluation the use of SDS-PAGE as an in vitro digestion assay was studied. Nitrogen solubility of SPC samples was also determined to better understand the effects of moist heat on protein quality.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the techniques presently available for estimating the molecular weight of proteins. Recently, peptides with molecular weights less than 1,500 daltons were successfully resolved by SDS-PAGE (Anderson et al., 1983, Fling and Gregerson, 1986). This molecular weight cut-off is close to the size of an octa to decapeptide. Since most peptides larger than hexapeptides are inappropriate substrates

for enzymes in the brush-border membrane of the small intestine (Matthews and Adibi, 1976), hexapeptides and larger resolved on SDS-PAGE gels represent undigested proteolytic fragments remaining in the small intestine after 8 hour digestion. Percent in vitro protein digestibility can thus be estimated from densitometer scans of hydrolyzed protein samples resolved on SDS-PAGE.

The possibility of using Coomassie Blue R-250 stained SDS-PAGE gels for determinig the in vitro digestibility of proteins was explored in this study. The major difficulty in quantitation of proteins within a SDS-PAGE gel is variation in the amount of Coomassie Blue R-250 dye bound from one protein to another (Fishbein, 1972). This is one of the obstacles to quantitative analysis of in vitro protein digestibility using SDS-PAGE. The unique advantage of SDS-PAGE over other in vitro methods is that a sequential eight hour protein digestion process can be visualized using SDS-polyacryl amide gel patterns. Qualitative analysis using SDS-PAGE may provide greater understanding concerning the effects of heat on soy protein composition and digestibility.

2.0 REVIEW OF LITERATURE

Soybeans have for centuries been a staple food in oriental countries. Tofu, shoyu (soy source), miso, and tempeh are traditional soybean foods which are popular in oriental cultures (Wang et al., 1979). Soybeans have been extensively grown in the U.S. since the mid 1930's. Presently, most of the U.S. soybean crop is processed to produce soyoil and the remaining defatted soymeal is consumed primarily by animals as a high protein feed. However, the potential of the soybean as a high protein human food source has been gradually recognized. Recently, new technologies have been developed which produce more refined soy proteins in the form of flours, grits, isolates and concentrates. Food manufacturers are beginning to incoporate these various forms of soy protein into a variety of food products. The nutritonal, functional, chemical, and physical properties of these soy products have been reviewed by Wolf (1970), Mattil (1974), Kellor (1974), Kinsella (1979, 1984) and Kinsella et al. (1985).

2.1 Forms of Soybean Proteins

Defatted soy flour, soy protein concentrate (SPC) and soy protein isolate (SPI) are three commercially available protein

products made from soybeans (Waggle and Kolar, 1978). Typical composition of these soybean products is shown in Table 1. The defatted soy flour is used in the largest volume by food manufacturers and is the most economical source of soy protein available. SPC and SPI are more refined concentrated protein products. There is increasing interest in using SPC and SPI in a variety of food products because of their desirable functional properties and compatibility with other food ingredients. Successful incorporation of SPC in food systems also depends to a great extent on nutritional quality (Mattil, 1974, de Valle, 1981).

Soy protein concentrates are prepared from "low heat" undenatured, defatted soy flour. The protein content of defatted soybean flour is increased from ca. 50% to 70% in SPC by eluting soluble components such as carbohydrates, sugars, ash, peptides, and phytic acid using acid (pH = 4.5), aqueous alcohol (70%), or moist heat and hot water as leaching agents (see Fig. 1)

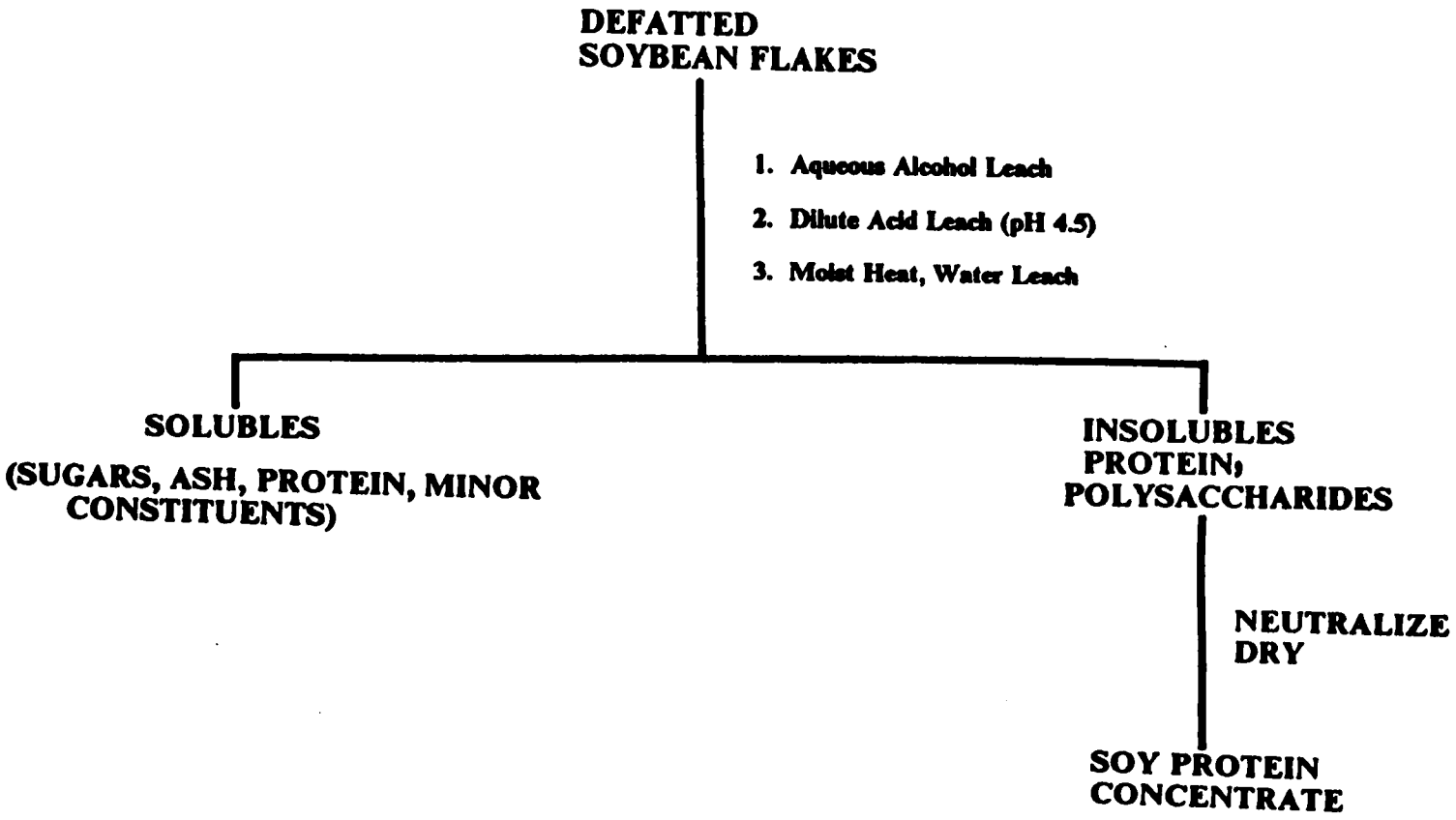
These three methods for producing SPC differ from each other primarily in the step used to insolubilize the major proteins. In the aqueous alcohol leach, the soluble constituents are leached out with aqueous alcohol leaving behind the proteins and polysaccarides, which are desolventized and dried. In the dilute acid leach, the major proteins are insolubilized by extracting with dilute acid,

Table 1. Typical Composition of Soybeans and Soybean Products[1]

	Protein (N x 6.25) (%)	Oil (%)	Total Carbohydrates[2] (%)	Ash (%)	Crude Fiber (%)
Whole Soybean	42	20	35	5.0	5.5
Defatted Soy Flour	54	1.0	38	6.0	3.5
Soy Protein Concentrate	70	1.0	24	5.0	3.5
Isolated Soy Protein	92	0.5	2.5	4.5	0.5

1. Moisture-free basis
2. Includes crude fiber

Reference: Waggle and Kolar, 1978



Reference: Waggle and Kolar, 1978

Figure 1. Flow Diagram of Process for Preparing Soy Protein Concentrates

near the isoelectric point of soy proteins (pH = 4.5), after which the insoluble protein-carbohydrate mixture is neutralized and dried. In the moist heat and hot water leach, the insolubilization of protein occurs due to the heat sensitivity of soy protein: the flakes or flours are heated with moisture to denature the proteins and insolubilize them. The soluble constituents are then leached out with hot water.

The overall amino acid composition of SPC prepared by the three processes is very similar, and their protein contents on a dry weight basis, fall within a relatively narrow range, 66 - 70% (see Table 2). There is also no appreciable difference in the amino acid composition of defatted soy flour and SPC (Kellor, 1974, Harper, 1989). Hence, the amino acid composition of soy proteins seems to be essentially unaltered during the preparation of SPC. Protein solubility, on the other hand, is very low for the alcohol and moist heat-treated products, and relatively high for the acid-extracted product (see Table 2). This suggests that the three dimensional structure or conformation of soy proteins is affected by the various methods employed during manufacture of SPC.

2.2 Physico-Chemical Behavior of Soy Protein During Thermal Denaturation

Approximately 90% of the protein in soybeans, mostly

Table 2. Typical Composition of Soy Protein Concentrates

Composition	Alcohol Leach	Acid Leach	Moist Heat Water Leach
Protein (N x 6.25)%	66	67	70
Moisture %	6.7	5.2	3.1
Fat %	0.3	0.3	1.2
Crude Fiber %	3.5	3.4	4.4
Ash %	5.6	4.8	3.7
Nitrogen Solubility Index %	5	69	3
pH (1:10 Water Dispersion)	6.9	6.6	6.9

Reference: Meyer, 1967

globulins, exist as dehydrated storage proteins located within cotyledon subcellular particles called "protein bodies". Ultracentrifugation analysis of the water soluble fraction of soybeans shows four types of components having sedimentation constants of approximately 2, 7, 11 and 15S at pH 7.6 - 7.8 and 0.5 ionic strength. Among them, the 11S globulin or glycinin (25-35%) and 7S globulin or conglycinin (30-35%) are major soy protein components, accounting for about 55-70% of the total protein in soybeans (Wolf and Cowan, 1971).

Many investigators have studied the physico-chemical properties of soy glycinin. It is now firmly established that soy glycinin is a globular protein containing 12 subunits with a molecular weight of about 350,000. Glycinin consists of three kinds of acidic (Mw 37,000-40,000) and three kinds of basic subunits (Mw 18,000-20,000) (Catsimpoolas et al., 1971b). The most stable form of the 11S globulin is the dimer form of two identical monomers made up of six subunits. Each acidic subunit is linked with a specific basic subunit through disulfide linkages. Glycinin has 18-20 disulfide bonds of both a inter and intramolecular nature that contribute to its compact structure (Draper and Catsimpoolas, 1978, Kella et al., 1986). From the amino acid composition of glycinin's polypeptides, it is known that about two-thirds of the S-S bonds of glycinin are contributed by the acidic polypeptides and the rest by basic polypeptides. Basic

polypeptides also have higher average hydrophobicity values than the acidic polypeptides (Kinsella et al., 1985). The 7S globulin, conglycinin, is composed of at least six combinations of three subunits, in which the subunits are associated via hydrophobic and perhaps hydrogen bonding. Three subunits denoted alpha, alpha' and beta, range in molecular weights from 42,000 to 57,000 (Brooks and Morr, 1985, Thanh and Shibasaki, 1976, 1977).

Screening of 108 soybean strains and varieties showed that none of the samples were free of trypsin inhibitor (T.I) activity (Kakade et al., 1972). Trypsin inhibitor is believed to account in part for the pancreatic hypertrophy and low weight gain of rats fed raw soybeans. However, soybean proteins without T.I. still caused pancreatic hypertrophy and growth inhibition. The residual pancreatic hypertrophy and growth inhibition of rats in the absence of T.I. were attributed to the resistance of native soybean proteins to proteolytic enzymes (KaKade et al., 1973). Heat treatment is necessary during the manufacture of processed soy protein products in order to inactivate T.I. and partially unfold the proteins present for the optimal nutritive value and physical properties.

The most obvious change that occurs in a heated solution of soy protein is the formation of aggregates. Many studies have investigated physical and chemical changes in soy protein

resulting from heat treatment. A study done by Mann and Briggs (1950) indicated that non-globulin soy proteins are sensitive to heat. When a soy protein solution was heated for 2 hr. at 75° C, several electrophoretic peaks of nonglobulin proteins were converted into a single aggregate peak. Ultracentrifugation analysis of soybean meal heated at 100° C showed 7S, 11S and 15S components aggregated with nonglobulin proteins.

The effects of dry or moist heat on the molecular structure of soy protein have been more clearly elucidated when heat treatments were carried out with isolated soy protein components (11S) than with whole soy protein. Glycinin appears to be stable upon heating to 50° C and very little change in conformation is observed between 50 and 70° C. When heated at temperatures above 70° C, glycinin solutions become increasingly turbid and the protein precipitates at 90° C (Catsimpoolas et al., 1969). Wolf and Tamura (1969) reported that a solution of isolated 11S protein (pH 7.6, 0.5 ionic strength) underwent profound structural changes when heated. Heating above 70° C disrupted the quaternary structure of soy 11S with subsequent separation of subunits into two distinct fractions. One fraction consisted of 3-4S soluble subunits, which were heat stable, and a second fraction consisted of a soluble aggregate.

The soluble aggregate was converted into an insoluble precipitate on continued heating. Insoluble aggregate formation was accelerated in presence of 0.01 and 0.5 M beta-mercaptoethanol without formation of soluble aggregate. The high molecular weight aggregate formation in presence of a sulfhydryl reagent was attributed to its ability to cleave disulfide cross-linkages. Newly exposed reactive sites in the protein are apparently then capable of forming an insoluble aggregate possibly via hydrophobic interactions. While, in the presence of 0.01 N N-ethylmaleimide, a sulfhydryl blocking agent, insoluble aggregate of 11S protein did not form upon continued heating. And soluble aggregate reached a maximum concentration in 5 to 10 min. followd by a decrease on continued heating. The relative amounts of free sulfhydryl groups and disulfide linkages also changed at various stages of heating. (Saio et al., 1971).

In another study, glycinin retained its immunological activity after heating for 30 min, at temperatures up to 65° C. However, rapid loss in antigenicity was observed in the temperature range between 70 and 90° C. It was observed that the amount of native glycinin that enters 7% polyacrylamide gels gradually decreases upon heating. This reduction in 11S protein content was accompanied by an increase in new fast moving bands on disc electrophoresis, which were regarded as released subunits. At 90° C, no undissociated glycinin could

be detected in the gels, while approximately 40% of the denatured protein appeared in the form of soluble subunits, or their soluble interaction products. The rest of the subunits converted into high molecular weight aggregates which did not enter the polyacrylamide gel. These results suggests that loss in antigenicity is strongly associated with conformational changes in the 11S protein (Catsimpoolas et al., 1971a).

Yamagishi et al. (1980) have explained the dissociation, rearrangement and aggregation of the heat denatured 11S globulin in terms of its acidic and basic subunits. They have demonstrated from gel electrophoresis experiments on heated 11S that soluble proteins, which correspond to 3-4S components in earlier studies, consist of acidic 11S subunits. SDS-PAGE analysis of the precipitate following gel filtration indicated that it consisted of monomers, oligomerized subunits and polymerized subunits. The electrophoretic mobility of the monomers were identical to those of basic subunits. Polymerized subunits and oligomers were shown to consist of various proportions of associated basic subunits and acidic subunits.

It has been suggested that hydrophobic bonds play an important role in the stabilization of the internal structure of glycinin (Fukushima, 1968, Catsimpoolas et al., 1969,). Since hydrophobic bond formation is favored by an increase in

temperature, it is conceivable that hydrophobic amino acid residues exposed by cleavage of disulfide bonds aggregate by hydrophobic interactions upon continued heating. Electrophoresis experiments suggest that the basic subunits of 11S are primarily responsible for protein aggregation (Yamagishi et al., 1980, Kella et al., 1986). Basic subunits, being hydrophobic, aggregate upon heating via hydrophobic interactions. Hydrophobic bonds interactions in turn enhance hydrogen bond formation. Polar groups interact more strongly when buried in hydrophobic regions (Nemethy et al., 1963). Thus, a combination of forces; hydrophobic, hydrogen and disulfide bond formation, may be responsible for thermal aggregation of soy glycinin.

Conformational changes in heated soy protein are also influenced by ionic strength (Wolf and Tamura, 1969, Catsimpoolas et al., 1970). When a water extract of defatted soy meal was heated at 80° C, glycinin and conglycinin were found to first dissociate into subunits followed by aggregate formation. Under these conditions, conglycinin disappeared almost completely whereas glycinin was stable. However when an acid-precipitated soy protein solution prepared from a water extract was heated at 80° C, glycinin was unstable and disappeared rapidly, whereas conglycinin was more stable (Hashizume et al., 1975). The difference in heat denaturation of soy protein was attributed

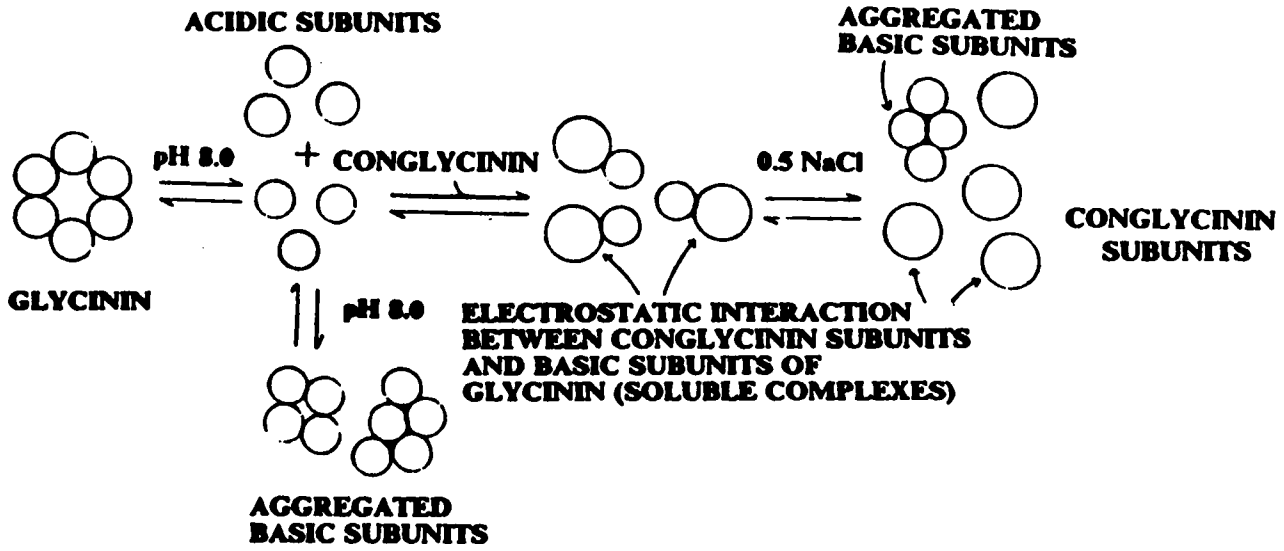
to the difference in ionic strength of two solutions.

In a follow up study, Hashizume and Watanabe (1979) studied the effects of ionic strength on heat denaturation of glycinin and conglycinin. When an acid precipitated soy protein solution prepared from a water extract was heated at low ionic strength (0.001), ultracentrifuge analysis showed the disappearance of 11S protein and concurrent apprearance of soluble aggregates and protein components of 2-4S at temperatures between 60-80° C. When the protein solution was heated at high ionic stregth (0.1), 11S protein did not change even at 70° C and still partly remained even at 85° C and completely disappeared at 90° C. The 7S globulin in acid precipitated soy protein solution reacts in a different manner upon heating. At low ionic strength, 7S protein remained almost unchanged after heating from 60 - 100° C. At high ionic strength, the amount of 7S protein began to decrease at 60° C. These studies suggest that glycinin becomes more stable as salt concentration increases, while the heat stability of conglycinin decreases with increasing ionic strength.

While the thermal properties of isolated 11S can be understood in terms of dissociation, and association of subunits through intermolecular and intramolecular disulfide and noncovalent bonds, 11S becomes more heat stable in presence of other globulins. Previously, Hashizume and

Watanabe (1979) reported that heating of low ionic strength, acid-precipitaed soy protein solution at 80° C did not result in precipitation even at 100° C, while glycinin precipitates when heated alone (Wolf and Tamura, 1969). The heat stability of 11S in a complex soy protein mixture is believed to be due to the formation of soluble complexes with conglycinin (7S) (Damodaran and Kinsella, 1982, German et al, 1982). Succinylation of conglycinin enhanced its inhibitory effect on glycinin aggregation, suggesting that succinylation of 7S increased its ability to interact with the basic subunits of glycinin via electrostatic interactions. However, the formation of a soluble complex was depressed when the ionic strength of solution was increased by adding NaCl (Damodaran and Kinsella, 1982). Hashizume and Watanabe (1979) also observed a precipitate formation in acid-treated soy protein solution at high ionic stength.

Based on these studies, Damodaran and Kinsella (1982) have suggested that various molecular changes occur in soy protein upon heating (see Figure 2). When soy glycinin (11S) is heated at 80° C, it undergoes thermal dissociation into acidic and the basic subunits, and the basic subunits formed insoluble aggregates via hydrophobic interaction. When glycinin undergoes thermal dissociation in the presence of conglycinin (7S), the subunits of conglycinin and the basic subunits of glycinin interact via electrostatic interactions.



Adapted from Damodaran, S. and Kinsella, J.E., 1982.

Figure 2. **Schematic Representation of Various Molecular Changes in Soy Protein Following Heating at 80° C in the Presence of 2-mercaptoethanol**

At high ionic strength, the electrostatic interaction between the basic subunits and conglycinin is suppressed and the basicsubunits aggregate via hydrophobic interactions.

Utsumi et al. (1984) carried out disc SDS-PAGE analysis to characterize the molecular size of various soluble protein complexes formed between conglycinin and glycinin following heating. However, he found that a significant amount of the soluble protein complex was unable to enter the 4% separating gel. The highly heterogeneous soluble macrocomplexes were believed to have a minimum molecular weight of about one million daltons. Two dimensional electrophoresis revealed that these macrocomplexes were composed primarily of the basic subunits of glycinin and the subunits of conglycinin. Most of the acidic subunits of glycinin were separated in the separating gel, indicating that these subunits apparently neither interact with the subunits of conglycinin nor form high molecular weight complexes via self-association

Saio et al.(1974) reported on high temperature expansion characteristics of soybean 7S and 11S proteins and the quantitative changes in proteins resulting from heating at 100 - 170° C (Saio et al., 1975). In the course of this investigation using SDS-disc polyacrylamide gel electrophoresis, no individual bands were observed on gels when protein samples were heated to over 150° C, even when large amounts of protein sample were loaded onto gels.

Further results from ultracentrifugation analysis and solubility measurements suggested that protein molecules were somehow degraded to lower molecular weight components.

In most applications of soy protein in food systems, a decrease in protein solubility is a prominent physical change which occurs when the food system is heated. It has been assumed that the insolubility of soy protein is accompanied by conformational changes in oligometric protein structure, with rearrangement of protein subunits through noncovalent and covalent interactions. Cumming et al. (1973) studied the effect of thermoplastic extrusion on water soluble soybean proteins using polyacrylaminde disc gel electrophoresis. They observed that as processing temperature increased, the main protein band in polyacrylamide disc gels disappeared and ones with faster migrating rates increased. This suggests that the water soluble soy protein dissociates into subunits and/or become insoluble.

Many studies have attempted to elucidate the mechanism(s) of molecular events which lead to soy protein insolubility when the protein is heated. Chiang and Sternberg (1974) reported that sulfhydryl-disulfide interchange is responsible for the decreased solubility of spun soy fibers during storage. Thiol reagents increased solubility of spun soy protein fibers, indicating that disulfide-based polymerization occurred during storage. Disc gel

electrophoresis measurement revealed a fast-moving peak which disappeared in aged fibers. This peak was restored after adding beta-mercaptoethanol, supporting the idea that the protein formed S-S linkage during storage. Fukushima et al. (1970) reported that the insolubilization of dried soybean milk was avoided in part by the addition of sulfhydryl reagents before drying. Hashizume et al.(1971) found that the formation of sponge like texture in frozen-tofu was due to protein denaturation caused by intermolecular sulfhydryl-disulfide exchange reactions. Hager (1984) employed two buffer systems with and without urea, sodium sulfite and acrylonitrile to dissolve extruded soy products. Solubility increased as the concentration of these reagents increased in buffer system, indicating that hydrogen, hydrophobic interactions and disulfide bond formations all play a role in stabilizing protein structure.

Sulfhydryl groups in glycinin are believed to be more active than those in conglycinin. Saio et al. (1971) changed the amount of sulfhydryl groups in soy proteins by the addition of N-ethylmaleimide, 2-mercaptoethanol and dithiothreitol and reported that the physical properties of tofu made from 11S protein were more significantly affected than tofu made from 7S protein. Differences in the texture of heat induced gels of 11S protein and 7S soy protein have also been attributed to a higher number of S-S cross linkages

present in heat induced gels of 11S protein (Saio et al., 1971).

2.3 Changes in the Nutritional Quality of Soy Protein Due to Heat treatment.

Inspection of the amino acid pattern of soybean protein reveals that methionine is the first limiting amino acid, and soybean has high potential nutritive value if supplemented with sulfur-containing amino acids. A high lysine content make soy proteins an ideal supplement for lysine-deficient cereal products (Kapoor and Gupta,1977). However, the physiological value of raw soy protein is inferior to predicted values based on amino acid composition. Raw beans do not support rat growth even when supplemented with methionine, but beans which have been autoclaved for 5 min. at 121° C and supplemented with methionine support normal growth (Kakade and Evans, 1965).

Soybeans contain several anti-nutritional factors. Soy trypsin inhibitors are known to decrease nutritive value by inhibiting the action of the pancreatic enzymes, trypsin and chymotrypsin, thus effectively impairing amino acid availability. Osborne and Mendel (1917) were the first to demonstrate that cooking soybeans increases their protein nutritive value. The increased nutritive value of heated

soybean meal is attributed primarily to inactivation of trypsin inhibitors. (Ham and Sandstedt, 1944, Ham et al., 1945, KaKade et al., 1973, Tuner and Liener, 1975, Liener, 1976). However, trypsin inhibitors are not the only factors involved in observed differences in the nutritive value of raw versus properly heated soybean meal (Gillette et al., 1978, Sitren et al., 1985). Liener (1981) has pointed out that heat treatment per se, apart from any heat inactivation of anti-nutritional factors, contributes to the improved nutritional quality of soy protein.

The availability, i.e., digestibility of soy protein depends on the extent of protein denaturation. The native soy protein molecules are quite compact and are not hydrolyzed by proteinase without disruption of the internal structure (Fukushima, 1968). Mild heat treatment modifies protein structure in such a way as to permit more complete digestibility and utilization of the growth-limiting sulfur amino aicds (Melnick et al., 1946, Guggenheim et al., 1960, Kakade, 1974). The beneficial effect of mild heat treatment on the soy protein quality has been extensively studied. On the other hand, severe heat treatment causes deteriorative effects in the nutritional quality of soy protein by destruction of essential amino acids and/or formation of iso-peptide bonds which are not susceptible to digestion enzymes (Mitchell et al., 1945, Friedman et al., 1981).

Amino acid analysis of soy proteins have shown that certain essential amino acids are destroyed by excessive heat treatment. When defatted soybean flour was autoclaved at 100° C for one, two and four hours, no discernible destruction of amino acids was observed. On the other hand, autoclaving at 126° C for 4 hr. significantly decreased cysteine, lysine and arginine content with smaller losses of tryptophan and serine (Taira et al., 1965). Stillings and Hackler (1965) studied the effects of deep fat frying on the amino acid content of tempeh and found that the content of most amino acids declined after deep fat frying for 7 min. Badenhop and Hackler (1971) employed PER and amino acid analysis to study the effect of the dry-roasting process on the bioavailability of soybean protein. Total amino acids and PER values of soybeans roasted to end point temperatures of 170, 180, and 185° C decreased with the degree of heating. PER values of roasted soybeans were higher than for raw beans, but decreased with prolonged heating. Average losses due to roasting of tryptophan, available and total lysine, cysteine, and histidine were 35, 31, 17, 15, and 6%, respectively. Cooking soymilk 1-6 hr. at 93° C had no adverse effect on protein efficiency ratio, growth, or available lysine. In contrast when soy milk was heated for 32 min., at 121° C, PER declined (Hackler et al., 1965) along with decreases in the content of available lysine.

Cysteine is one of limiting amino acids in soybean

protein. Cysteine is also particularly sensitive to heat. It may be destroyed by heat treatments employed to destroy growth inhibitors that are present in raw soybeans, thus becoming the first limiting amio acid (Rios Iriarte and Barnes, 1966). Evans et al. (1951b) reported that as much as one-half to one-third of the cysteine content of soybean protein may be lost by excessive heating.

However, the adverse effect of severe heating on the nutritive value of soy protein can not be completely explained by partial destruction of certain essential amino acids. Supplementation of overheated soy flour with these essential amino acids did not restore its nutritive value to that of properly heated flour (Rios Iriarte and Barnes, 1966, Taira et al., 1969).

Formation of molecular aggregates during severe heating may decrease the susceptibility of peptide bonds in proteins to the attack of proteolytic enzymes due to steric hindrance. The liberation of each of the essential amino acids in soy protein by pancreatic enzymes <u>in vitro</u> increased with proper heating but decreased upon prolonged heat treatment (Riesen et al., 1969). The availability of amino acids was assayed microbiologically after digesting the protein with the trypsin and erepsin enzymes and results were compared with <u>in vivo</u> digestibility in young chicks (Evans and McGinnis, 1947, Evans and McGinnis, 1948). Autoclaving raw soybean meal at 100 -

130 C for 30 min. increased both *in vitro* and *in vivo* digestibility; while autoclaving at 130° C for 60 min. decreased protein digestibility in both systems. Rios Iriarte and Barnes (1966) also studied the effect of excessive heat on commercially prepared soybean meal. In their study, excretory nitrogen significantly increased when overheated flour was used in rat diets, indicating that overheating caused decreases in the rates of protein hydrolysis and/or amino acid absorption. In 1979, Richardson and Catsimpoolas studied the digestibility of heat denatured glycinin using the pH-stat procedure. The initial rate of hydrolysis of the heat-denatured glycinin decreased by a factor of eight compared to the unheated glycinin.

The influence of autoclaving upon the availability of basic amino acids in soybean meal was extensively studied by Evans (1948, 1951, 1961). Among basic amino acids, the heat inactivation of lysine occurred to a greater extent than arginine and histidine. Evans and McGinnis (1949) determined the total amino acid contents of soybean oil meal after acid hydrolysis and following *in vitro* digestion with enzymes. Approximately 40% of the lysine was destroyed and 60% less lysine was liberated by enzymatic digestion *in vitro* from soybean oil meal autoclaved for four hours than from unautoclaved meal. In this study, 20% of the lysine in soybean meal was converted to a form which was freed by acid

but not by enzyme hydrolysis. Evans et al. (1951a, 1961) postulated that when soybean protein was autoclaved, the free carboxyl groups of aspartic and glutamic acid reacted with the free amino groups of lysine and arginine and with the imidazole group of histidine to form isopeptide-type linkages resistant to enzymatic attack, but cleaved by acid hydrolysis.

Lysine is also rendered unavailable by the Maillard or non enzymatic browning reaction, in which epsilon-amino groups react with the carbonyl groups of reducing sugars. In a study by Rhee and Rhee (1981), heating a mixture of soy protein isolate and glucose caused decreases in protein digestibility and PER. In the case of soybeans subjected to heat treatment, sucrose hydrolysis may give rise to appreciable quantities of reducing sugars which then interact with lysine. Lysine modified in this manner is no longer physiologically available, because the peptide bond containing the modified lysine is not susceptible to cleavage by trypsin; thus, the digestibility of proteins by pancreatic enzymes, whether in vitro or in vivo, is considerably reduced (Hansen and Millington, 1979).

Autoclaving also affects the availability of sulfur containing amino acids (Evans and McGinnis, 1948, Evans et al., 1951b). Taira et al.(1965), reported that the bioavailability of methionine and cysteine was affected by autocalving for four hr. at 15 lbs pressure. Autoclaving

resulted in a 32% decrease in the amount of methionine released by digestive enzymes. In the case of cysteine, between 31-36% of the cysteine was destroyed and only 13-18% of the cysteine present in soybean oil meal was available for enzyme hydrolysis.

2.4 The effects of pH, ionic stregth, and denaturing reagents on the enzymatic hydrolysis of glycinin.

In addition to heat treatment, any change in the tertiary and quaternary structure of soy protein affects the rate of enzymatic hydrolysis. The enzymatic hydrolysis of soy glycinin is influenced by environmental factors such as pH, ionic strength and protein denaturing agents. Higher rates of enzymatic hydrolysis are normally associated with the denaturation of protein molecules and the unfolding of the peptide chains. The unfolded protein is presumably subjected to rapid proteolytic cleavage of susceptible peptide bonds.

Low pH converts glycinin into a slowly sedimenting component apparently resulting from the dissociation of the protein into subunits. Low pH may cause dissociation of glycinin due to electrostatic repulsion of protein subunits (Wolf et al., 1958). Alkali treatment also causes dissociation of glycinin and subsequent unfolding of polypeptide subunits as a result of disulfide bond cleavage

(Catsimpoolas et al., 1971). Alkaline denatured glycinin was found to be more susceptible to enzymatic hydrolysis than acid denatured glycinin, indicating that protein unfolding occuring during or after acid dissociation was less than that following alkaline disulfide cleavage.

Unfolding of glycinin in 6 M urea, followed by cleavage of the disulfide bonds and blockage of the sulfhydryl groups, successively and dramatically reduced the time required for completion of tryptic hydrolysis of glycinin and its acidic subunits. However, it has been demonstrated that disulfide bond cleavage does not increase the rate of digestion of basic subunits (Lynch et al, 1977). Slower hydrolysis of basic subunits might be due to aggregation of released basic subunits via hydrophobic interactions (Kella et al., 1986).

The digestibility of urea denatured glycinin depends on the extent of protein renaturation. Kamata et al. (1979) studied the digestibility of glycinin denatured by 5 M urea and 8 M urea. At high ionic strength condition (0.5 M NaCl), the digestibility of 8 M urea-denatured glycinin was lower than 5 M urea-denatured glycinin. Gel filtration and electrophoresis analysis indicated that a stable digestion intermediate, found in native glycinin, was also present in the digestion products. These results suggest that unfolded glycinin renatures more readily following 8 M urea denaturation than after 5 M urea denaturation. Therefore,

the refolding may be the reason of the dereased digestibility of 8 M urea denaturation.

In earlier studies, Wolf et al. (1958) reported that both 7S and 11S soy globulins are sensitive to ionic strength in aqueous solutions. Both globulins underwent reversible dissociation-association reactions. At high ionic strength, the 7S globulin existed in a monomer form, while it dimerized at low ionic strength. Dissociation of 11S subunits on the other hand is suppressed at high ionic strength. The complexity of glycinin degradation by trypsin at high ionic stregth was investigated by Kamata and Shibasaki (1978a, 1978b). The time course of tryptic digestion (0 - 200 min.) was analyzed by SDS-urea polyacrylamide gel elctrophoresis. A total of seven tryptic fragments, ranging in size from 35,000 to 13,500 daltons were generated during protein hydrolysis. Larger fragments existed only for short time periods, while degradation of a so called glycinin-T component required more than 24 hours. This glycinin-T component may play an important part in the poor digestibility of the soybean storage protein. Two fragments (IST-3, IST-4) were isolated from glycinin-T and subjected to gel filtration and ion-exchange chromatography. Acidic-urea gel elctrophoresis indicated that IST-3 contained basic subunits (B1, B2, B3), whereas IST-4 contained B4 subunits.

2.5 In Vitro Methodologies for the Evaluation of Protein Digestibility

It is now well established that the amino acid composition of proteins does not necessarily indicate the extent to which each amino acid is released or made available upon enzymatic digestion. A major disadvantage of using amino acid composition data to predict the nutritive value of protein, is that it overlooks protein digestibility which is in some cases a major determinant of protein bioavailability (Kies, 1981). For the proper evaluation of the nutritive value of proteins, it is necessary to compare the results of chemical analysis with in vitro or in vivo studies which determine protein digestibility and/or the ability of the protein to maintain animals in nitrogen balance and sustain growth.

Protein digestibility is a critical factor affecting protein biovailability, i.e., the amount of protein in the form of amino acids ultimately absorbed into the blood stream. Most in vitro studies of protein digestibility have employed one-step or two-step digestion procedures and multi-enzyme systems. Several investigators have used a single enzyme such as pepsin (Scheffner et al., 1956), trypsin (Maga, 1973) or papain (Buchanan, 1969) in a one-step enzyme procedure. Sheffner et al. (1956) developed the "pepsin-digestive-residue

amino acid index (PDR)". This PDR amino acid index combined the pattern of essential amino acids released by in vitro pepsin digestion with the amino acid pattern in the remainder of the protein. The PDR index was closely correlated with the net protein utilization value of a variety of proteins.

Akeson and Stahmann (1964) used an autoanalyzer to make the amino acid analysis step simple and convenient. They also employed a two-step procedure, with pepsin followed by pancreatin hydrolysis yielding a pepsin pancreatin digest index. Excellent correlation (r = 0.99) was observed between the pepsin pancreatin index values of a wide range of protein samples and cited in vivo data. Buchanan and Byers (1969) reported that papain solubility was a better predictor for the in vitro digestibility of leaf protein than pepsin-pancreatin solubility. However, Saunders et al. (1973) found that although papain solubility was a better predictor of the in vitro digestibility of leaf protein; this was not true for other protein samples. They found an excellent correlation between values obtained from the enzyme system used by Akeson and Stahmann (1964) and in vivo data (r = 0.88). Saunders et al. (1973) developed a pepsin-trypsin digestion system. They reported a very good correlation between a pepsin-trypsin index and in vivo digestibility (r = 0.91). Although these methods are good predictors of in vivo digestibility, they all include a time-consuming nitrogen determination step or amino

acid analysis of the different digestion fractions.

In 1973, Maga et al. suggested using the initial rates of protein hydrolysis as a predictor of digestibility. The pH drop, which was caused by enzymatic cleavage of peptide bonds and freeing of amino acid carboxyl groups from the protein chain, was used as a measurement of initial proteolysis rates. They reported that recorded pH changes of pepsin hydrolyzed proteins were good indicators of their digestibilities. Hsu et al. (1977) modified this system by employing an automatic recording pH meter and a multi-enzyme system consisting of trypsin, chymotrypsin, and peptidase. A high correlation ($r = 0.90$) was found between pH at 10 min. and apparent *in vivo* digestibility for a wide range of food samples, although a direct relationship between the pH drop and extent of protein hydrolysis has been questioned (Mozersky and Panetieri, 1983, Barbeau and Kinsella, 1985).

In vitro digestion procedures mentioned so far are carried out in closed systems. *In vivo*, amino acids released during protein digestion are absorbed by the intestine into the blood stream. In closed *in vitro* systems, accumulated digestion products could cause a feedback inhibition slowing the rate of enzyme hydolysis. Recently, Gauthier et al. (1982) and Savoie and Gauthier (1986) employed a dialysis bag with a 1,000 molecular weight cut-off in a two step *in vitro* digestion procedure. In this procedure, protein is hydrolyzed

with pepsin at pH 1.9 for 30 min. in a closed system. The hydrolyzate is transferred to a dialysis tubing and hydrolyzed with pancreatin at pH 8 for an additional 24 hr. To eliminate dialysates (digestion products), the buffer solution is continuously replaced. Percent *in vitro* digestibility of casein, soybean, and rapeseed proteins was markedly improved by increasing the frequency of buffer replacement.

In an effort to predict PER based on *in vitro* digestibility and amino acid composition, Satterlee et al. (1979, 1982) constructed an equation for computed-PER (C-PER). In this C-PER model, the essential amino acid profile of each sample is expressed as a percentage of a FAO/WHO reference essential amino acid profile (1973) after correction for protein digestibility obtained from the modified procedure of Hsu et al. (1977). This C-PER model was evaluated using protein samples having rat PERs ranging 0.67 to 3.22. Although the C-PER values obtained from the model tend to overestimate the PER value of low quality proteins, and underestimate high quality proteins, the combination of amino acid analysis and *in vitro* digestibility has one important advantage over rat-PER assays. This method can yield a predicted PER in 72 hr. or less, and is applicable to a wide range of food ingredients and processed foods.

2.6 Development of SDS-PAGE as an Analytical Tool

Since Shapiro et al (1967) introduced the use of the anionic detergent sodium dodecyl sulfate (SDS) in polyacrylamide gel electrophoresis (PAGE) systems, SDS-PAGE has been proved to be a useful tool for the estimation of molecular weights of polypeptides and peptides and analysis of complex mixtures of proteins. SDS denatures polypeptides and forms SDS-polypeptides complexes. As a result of the anion complex formation with SDS, the native charge differences of proteins are minimized and electrophoretic mobility of proteins is closely correlated with their molecular weights. The usefulness of SDS-PAGE introduced by Shapiro et al. (1967) for the molecular weight determination of proteins was demonstrated by Weber and Osborn (1969) who found SDS-PAGE to be empirically valid for at least 40 different proteins with a wide range of molecular weights from 10,000 to 70,000.

In spite of the excellent reliability of SDS-PAGE for molecular weight determination of proteins, this system has failed to accurately predict the molecular weights of polypeptides and peptides less than 15,000 daltons. The plot drawn for log molecular weights versus relative mobility shows scatter from a smooth curve for peptides with molecular weights less than 15,000 (Shapiro et al, 1967, Weber and Osborn, 1969, Neville, 1971). This deviation from linearity is believed to be somehow related to the size of the micelle

complexes formed between SDS and short-chain polypeptides (Dunker and Rueckert, 1969), peptides less than a certain length all end up in complexes of protein and detergent of the same size. Also, it has been suggested that intrinsic charge and conformation, which does not greatly influence SDS-binding capacity of large molecular weight proteins, can modify SDS-binding capacity for short-chain peptides (Dunker and Rueckert, 1969).

The resolving power of SDS-PAGE in neutral continuous buffer systems was enhanced by the development of disc electrophoresis in a discontinuous buffer system (Davis, 1964). The advantage of a discontinuous buffer system is based on different mobilities of electrolytes in such a system. Proteins in a discontinuous system become trapped at moving boundaries between buffer ions as an ultra-thin band. This stacking process significantly enhances the subsequent actual separation of protein, since original protein samples shrinked to ultra-thin bands and start the electrophoretic run at the same time.

Since the discontinuous buffer system was first introduced by Orstein and Davis (1964), many buffer systems have been studied with respect to SDS-PAGE. The most popular buffer systems are those developed by Laemmli (1970), Neville (1971) and Wyckoff et al. (1977). Laemmli (1970) used a tris-glycine discontinuous buffer system for the resolution of

Bacteriophage T4 structural proteins, and reported 28 bands including proteins with molecular weights less than about 15,000. Neville (1971) discovered that a borate-sulfate SDS-PAGE buffer system resolved plasma membrane proteins into 40 discrete bands over a wide molecular weight range from 2,300 to 320,000 daltons. The amount of SDS in the upper buffer chamber of both of these systems was reduced from 0.1% to 0.03% by Wyckoff et al. (1977). In Wyckoff system, ammediol is used as a base and glycine and chloride as trailing and leading ions. Wyckoff found that broadening of stacked protein sample was proportional to the amount of SDS, existing in the sample and upper buffer. Impaired resolution due to a wide stack was improved using 0.03% SDS in upper buffer, no SDS in the gel, and 150 ug or less SDS in the sample. In 1981, Bury compared the three buffer systems of Laemmli (1970), Neville (1971), and Wyckoff et al. (1977) using 12.5% polyacrylamide concentration. He reported that the system of Wyckoff et al. (1977) gave the best resolution especially for polypeptides below 10,000 daltons, when the original 0.03% SDS concentration was increased to 0.1% SDS in the upper buffer. It seemed that 0.1% SDS, but not 0.03% SDS, is sufficient to saturate all the polypeptides with SDS forming SDS-polypeptides complexes.

Although SDS-PAGE systems mentioned so far work well for large proteins and polypeptides with molecular weights from

approximately 15,000 to 300,000, the resolution of low molecular weight polypeptides of less than 15,000 daltons is not accomplished. This prevents the estimation of the molecular weight of small protein subunits or fragments following chemical or enzymatic digested of proteins. However, various attempts have been made to provide sharp banding, high resolution, and reliable detection of these smaller peptides.

The resolving power of an SDS-PAGE gel is in part due to its gel sieving properties. Generally speaking, increasing the concentrations of acrylamide and the crosslinking agent, and/or including urea in SDS-PAGE gels reduces the effective pore size of gels. Swank and Munkres (1971) proposed increasing both acrylamide and cross-linker concentrations, and including 8 M urea in gels to decrease gel porosity. They found that 12.5% gels with a crosslinker to acrylamide of 1:15 ratio gave the best resolution. They succeeded in the separation of oligopeptides in the molecular weight range of 1,200 to 10,000 daltons. Discrete electrophoretic mobilities of low molecular weight polypeptides were observed which had comigrated in previous studies (Shapiro et al., 1967, Weber and Osborn, 1969, Laemmli, 1970, Neville, 1971). They attributed better resolution of small peptides to the decreased size of SDS micelles caused by addition of 8 M urea. However, Swank and Munkres's system requires a relatively long

running time and in spite of improved separation of low molecular peptides, diffused protein bands were also observed, which could be prevented by employing a discontinous, or multiphasic PAGE system.

Consequently, subsequent methods have focused on discontinuous buffer systems. Recently, Merle and Kadenbach (1980) used a Laemmli discontinuous buffer system together with 16.5% separating gel acrylamide and inclusion of 8 M urea in the sample buffer to separate rat liver cytochrome C oxidase into 12 different polypeptide chains. The molecular weight of the smallest subunit was identified as 3,900. However this study was criticized because a systematic survey of standard protein was not conducted.

Among the problems associated with the determination of low molecular weight peptides using SDS-PAGE are inflection points in calibration curves at intermediate molecular weight ranges of 10,000 - 20,000 (Swank and Munkers, 1971), and the uncertainty in assigning molecular weights for small peptide components (Merle and Kadenbach, 1980). These problems can be partially overcome by controlling the SDS-PAGE buffer composition and concentration. In 1983, Anderson et al. employed a discontinuous SDS-PAGE slab gel electrophoresis system to separate and resolve low molecular weight polypeptides as a function of molecular weight. Their system utilizes a relatively low-mobility acetate ion in the stacking

gel and high-mobility strong anion, sulfate and chloride, as leading and trailing ions in the separating gel without pH discontinuity (pH = 7.8). The separating gel in their system contains 8 M urea. In this system, a sulfate-protein-acetate stack is temporarily formed in the stacking gel. As electrophoresis proceeds, the chloride ion overtakes the sulfate-protein-acetate stack, and the acetate and proteins become successively unstacked. Once they reach the lower resolving gel, proteins separate on the basis of both molecular sieving and the electrophoretic mobolity differences. A calibration curve of log molecular weight vs electrophoretic mobility obtained was linear over a molecular weight range from 2,500 to 90,000, regardless of acrylamide concentration. The success of this system indicates that the resolving power and molecular weight separation achieved by SDS-PAGE is entirely a function of buffer composition and is not critically dependent on acrylamide concentration.

Other SDS-PAGE methods for estimating the molecular weights of proteins, protein subunits, and other macromolecules, depend on gradient gel systems. Gradient SDS-PAGE has proven valuable for enhancing the resolution and size estimations of proteins. When protein mixtures are applied to gradient SDS-PAGE gels, protein bands are compressed and sharpened at the part of the gel where pore size matches the hydrodynamic volume of the protein (i.e. size of SDS-protein

complex). Campbell et al. (1983) prepared gradient gels in which the acrylamide concentration rose from 3 to 40% and the degree of cross-linking increased from 4 to 12.5%. These gels were reported as suitable for fractionating proteins with molecular weights from 10,000 to several million daltons.

Although the above SDS-PAGE systems are powerful tools for separating small molecular weight polypeptides, they all include a high molarity of urea, high concentration of acrylamide or cross-linker. Recent research has focused on the development of discontinous SDS-PAGE system without high concentrations of urea, acrylamide or cross-linker. In 1985, Bothe et al. used a urea-free SDS-PAGE system to successfully resolve proteins and polypeptides with molecular weights from 1,500 to 100,000. This system combines a linear acrylamide gradient (10.2 to 30.2%) in the separating gel and the discontinuous ammediol/glycine buffer system suggested by Bury (1981). Fling and Gregerson (1986) found that a simple doubling of Tris concentration in the Laemmli buffer system yielded superior banding, and resolution for both large proteins and small peptides. Their system was suitable for the separation and resolution of the peptides as small as 1,300 molecular weight.

The resolution of polypeptides and peptides on Fling and Gregerson's discontinuous SDS-PAGE system (1986) is based on pH differences in sample, stacking gel, resolving gel and

running buffers. The pH of the sample buffer is the same as that of the stacking gel buffer (pH = 6.8), which is two pH units lower than running and resolving gel buffers. SDS-polypeptide complexes carry a net negative charge and have high electrophoretic mobility. In this SDS-PAGE system, tris is used as a basic ion and the pH is adjusted with HCl. Glycine (pKa1 = 2.34, pKa2 =9.6) in the running buffer (pH 8.8) exists as glycinate ion with a charge of minus one. When glycine enters the stacking gel with a pH of 6.8, it becomes immobilized as a zwitterion. Under constant current conditions there is a deficiency of mobile ions between the trailing ion glycine and leading chloride ion producing a large voltage gradient within the stacking gel. The pore size of the stacking gel is larger than that of separating gel reducing the resistance to electrophoretic mobility of the protein samples. In this strong local electric field, the anionic proteins move very fast until they reach the area containing chloride ions where they drastically slow down. The protein molecules are then stacked in a tight disc between the glycinate and chloride ions. As the protein disc encounters the running gel, its migration slows down even further within the small pores of the gel. This permits the small glycinate ions to catch up with the proteins. When glycinate ions reach the resolving gel, they become fully charged and there is no longer an ion deficiency. Therefore,

there is a constant field strength througout the gel, and protein molecules are separated according to their molecular size (Cooper, 1977).

3.0 OBJECTIVES

The objectives of this study were:

1. To determine the effects of moist heat on the nitrogen solubility, amino acid composition, PER and C-PER of soy protein concentrate.
2. To determine the effects of moist heat on *in vitro* digestibility as determined by three assays (TCA soluble N measurement, SDS-PAGE and pH drop method) and *in vivo* apparent digestibility of soy protein concentrate.
3. To evaluate the use of a modified SDS-PAGE procedure of Fling and Gregerson (1986) as an *in vitro* digestion assay for soy protein concentrate.
4. To study the feasibility of using the simultaneous dialysis method for evaluating the use of SDS-PAGE as an *in vitro* digestion assay.

4.0 Materials and Methods

4.1 Materials

Central Soya company (Fort Wayne, Ind.) was contacted in writing and asked to donate soy protein concentrate (SPC) for this study along with copies of any pertinent product specifications and performance brochures. According to information in their brochure, SPC (Promosoy (R) Plus), is prepared via the "dilute acid" method and in a wet state is heated to above pasteurization temperatures for several minutes during processing. In final form it is a spray dried powder. Trypsin inhibitor is in the range of 5 - 8 mg/g of product.

Heated SPC samples were prepared from the same lot of SPC. The SPC was mixed with distilled water (1:6 weight/volume ratio) to form a paste. This SPC paste was spread in a stainless steel pan at a thickness of about 1.0 cm and autoclaved at 15 lb pressure (121 °C) for 10, 30 min., 2, and 4 hr. After freeze drying, samples were stored at 5° C. Unautoclaved SPC served as a control.

4.2 Study Design

A schematic diagram of the experimental design is shown

in Figure 3. This study includes seven in vitro assays (N solubility measurement, trichloroacetic acid (TCA) soluble N measurement, SDS-PAGE, simultaneous dialysis, pH drop, amino acid analysis, and C-PER) and two in vivo assays (apparent digestibility and PER). Digestibility of control and autoclaved SPC samples was evaluated by three existing in vitro methods (TCA soluble N measurement, pH drop and simultaneous dialysis method), one new in vitro method (SDS-PAGE), and apparent in vivo digestibility.

The moisture and total N content of samples were determined by AOAC method (1984). The total protein content was calculated by multiplying N values by the appropriate conversion factors (6.38 for the casein and 6.25 for soy protein). All analyses for this study were conducted in triplicate.

Analysis of variance (ANOVA) procedure and Duncan's multiple range test were used to test significant differences in digestibility of control and autoclaved SPC samples at a p value = 0.05. Since digestibility data is expressed as percentage, the arcsine root transformed data was used for the statistical analysis. Correlation coefficients were also determined between in vivo and in vitro digestibility.

4.3 Determination of Nitrogen Solubility

Nitrogen solubility of SPC samples in potassium phosphate

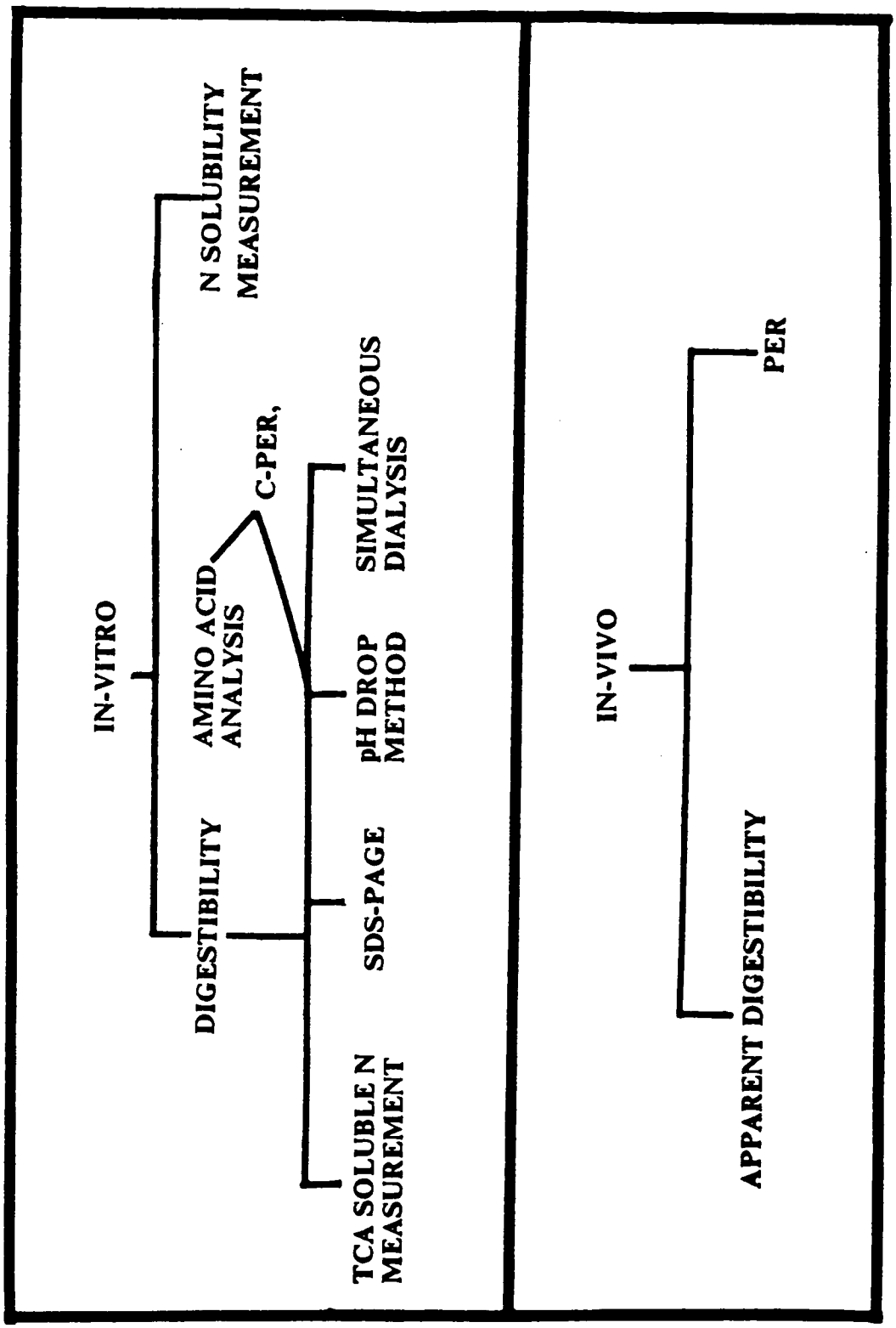


Figure 3. Experimental Design

buffer solution (pH 7.6, 0.5 ionic strength) containing 1.5 - 8 M urea and 0.1 M beta-mercaptoethanol was determined according to the modified procedure of Chiang and Sternberg (1974). The potassium phosphate buffer was prepared by mixing 0.0325 M K_2HPO_4 with 0.0026 M KH_2PO_4 to pH 7.6, then adding 0.4 M NaCl and 0.02% (w/v) sodium azide.

Aliquots (40 ml) of buffer containing 1.5 - 8 M urea and 1.5 M beta-mercaptoethanol were added to 400 mg of the SPC samples. The mixture was shaken in a water bath at 23° C for 1 hr. and then centrifuged at 5,000 rpm for 30 min. (IEC CRU-5000 Centrifuge, Needham HTS, Mass). After centrifugation, the supernatant was filtered with #44 Whatman filter paper. Samples containing urea were dialyzed for 48 hr. at 4° C using a Spectro Por 1 dialysis tubing (Molecular weight cut-off; 6,000 - 8,000 daltons) to remove urea. The amount of solubilized N was determined by the Kjeldahl method. Solubility was expressed in terms of the percentage of soluble N to total N in the original sample.

4.4 Determination of <u>In Vitro</u> Digestibility by TCA-Soluble Nitrogen Measurement.

4.4.1 Sample preparation

All protein samples used for <u>in vitro</u> digestion studies were ground to a fine powder that passed through an 80 mesh

screen. These samples were subjected to a 2 hr. pepsin digestion followed by a 6 hr. pancreatin digestion. Porcine pepsin(EC 3.4.23.2, Cat. No. P-6887) and a mixture of pancreatic enzymes; Pancreatin (Cat. No. P-1750, grade VI) were purchased from Sigma Chemical Co., St. Louis, MO.

One hundred mg of each SPC sample was suspended in 100 ml of 0.1 N HCl in a elermeyer flask and stirred for 5 min. For the pepsin digestion, the pH was adjusted to 1.9 with granulated NaOH. The samples were incubated at 37° C in a water bath for 30 min., and digestion was initiated by the addition of 10 mg crystalline pepsin to each sample resulting in a 1:100 enzyme to SPC ratio. After exactly 2 hr., the pH of each of the samples was rapidly adjusted to pH 7.0 with granulated NaOH and then carefully raised to pH 8.0 using a carbonate-bicarbonate buffer (55 mg carbonate mixed with 45 mg bicarbonate). A sample aliquot of 20 ml was removed prior to pancreatic digestion and throughly mixed with 20 ml of 20% (w/v) TCA solution.

After the samples were re-equilibrated in a 37° C water bath for 30 min., 25 mg of crystalline pancreatin was added to each digest. Aliquots of 20 ml were removed at time intervals; 1, 2 and 6 hr. during the 6 hr. pancreatin hydrolysis. Each of the sample aliquots was diluted in 20 ml of 20% (w/v) TCA solution.

4.4.2 TCA Soluble Nitrogen Measurement

Diluted samples were allowed to stand for 1 hr. before centrifugation at 5,000 rpm for 30 min. After centrifugation, the supernatant was filtered through #44 Whatman filter paper. A 30 ml aliquot was removed from each supernatant and its nitrogen content was determined by the Kjeldahl method.

The total N in 15 ml of a 1% unhydrolyzed protein solution was determined by Kjeldahl method to obtain total N. The percent *in vitro* digestibility of each sample was expressed in terms of the percentage of TCA soluble N divided by total N.

4.5 Determination of *In Vitro* Digestibility by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

4.5.1 Sample Preparation.

Protein samples (250 mg) were dispersed in 25 ml of 0.1 N HCl. The pepsin and pancreatin digestion prodedures followed were exactly as described in the TCA soluble N measurement (see Section 4.4.1) except 2.5 mg of enzymes were used. At time intervals (0, 2, 3, 4 and 8 hr.), an aliquot of 0.5 ml was removed from each sample and diluted in 2.5 ml SDS-PAGE sample buffer (5.5 mM tris-HCl, pH 6.8, 8M urea, 2% SDS and 0.025% pyronin y) to give 1 mg SPC in 1 ml of SDS-PAGE sample buffer. Diluted samples were kept overnight at room temperature to complete SDS-protein binding, and then stored

at 5° C until loaded onto an SDS-PAGE gel. Aliquots were also removed from each SPC prior to initiating digestion to obtain an unhydrolyzed sample.

Molecular weight marker proteins were purchased from Sigma Chemical Co., St. Louis, Mo. The marker proteins/peptides used for SDS-PAGE are listed in Table 3, with their respective molecular weight, relative mobility (Rm) and retention time (Rt). The marker proteins represent a range of molecular weights from 205,400 to 1,046 daltons. Markers were diluted in SDS sample buffer to a concentration of 1 mg/ml. A small portion of marker proteins (200 microliter) were stored at 4° C until loaded onto SDS-PAGE gel, and rest of the marker proteins were stored at - 20° C to prevent deterioration.

4.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The SDS-PAGE discontinuous buffer system of Fling and Gregerson (1986) was modified for this study. Modifications were made in fixing procedures. An additional 1 hr. fixing step was employed using a fixing solution containg 1.4% picricacid (W/V), 1.25% glutaraldehyde (V/V) and 20% acetic acid (V/V). In preliminary studies, this additional fixing step was found necessary in order to fix low molecular weight peptides, angiotensin I and bacitracin.

Table 3. Standard Protein Markers and Their Relative Mobility (Rm) and Retention Time (Rt) on SDS-PAGE Gels

Std Protein Markers	Mw	Rm	Rt
Myosin,Rabbit Muscle	205,000	0.09	0.94
Galactosidase	116,000	0.17	2.09
Phosphorylase B	97,400	0.20	2.55
Bovine Serum Albumin	66,000	0.25	3.29
Ovalbumin	45,000	0.33	4.59
Glyceraldehyde 3-phosphate Dehydrogenase	36,000	0.38	5.34
Carbonic Anhydrase	29,000	0.45	6.45
Soy Trypsin Inhibitor	20,100	0.57	8.01
Myoglobin(Polypeptide backbone)	16,950	0.61	8.72
Myoglobin(fragment I + II)	14,400	0.66	9.34
Myoglobin(fragment I)	8,160	0.71	10.29
Myoglobin(fragment II)	6,210	0.73	10.50
Myoglobin(fragment III)	2,510	0.80	11.56
Bacitracin	1,500	0.82	11.79
Angiotensin I	1,296	0.87	12.50
Angiotensin II	1,046	-	-

Protein resolution on SDS-PAGE systems depends to a considerable degree on purity of electrophoresis reagents that are used. All reagents for electrophoresis were purchased from Bio-Rad, Richmond, Cal. All solution formulations used in SDS-PAGE analysis are given in Appendix A. Stock solutions were freshly prepared every 4 weeks, and running buffer was limited to two uses. The time of polymerization is also affected by tetramethyl ethylene diamine (TEMED) and ammonium persulfate. A longer time for polymerization or incomplete polymerization results when TEMED is oxidized. TEMED was stable for 8 months and the oxidized TEMED has a yellow color. TEMED was kept in the refrigerator, and ammonium persulfate was prepared daily.

Air bubble formation in gels was avoided since air bubbles produce electrical resistance, thus changing migration distances of proteins in gels. The problem was avoided by soaking glass plates at least 1 hr. in regular detergent before cleaning and by air drying. Precaution should be taken in selecting detergent. Regular detergent was used not to remove the precoat on glass plates. Degasing of gel solution is also important to prevent air bubble formation.

The ratio of protein sample to the sample buffer also affected the resolution. Best results were obtained with a concentration of 1 mg protein/ml SDS-PAGE buffer solution.

Diffusion of the bands during the electrophoresis run was prevented by running the gel at a lower current (30

milliamps), with continuous cooling of the running buffer to 12° C during electrophoresis.

One pair of glass plates (width 18 cm and height 16 cm) were throughly cleaned and air-dried, and then set into a casting stand to form a vertical gel sandwich. Two resolving gel solutions (acrylamide concentration of 8% and 25%) were prepared and degased for 5 min. each. Ammonium persulfate and TEMED were added prior to pouring gel solutions into a Hoefer gradient former. The 8% and 25% solutions were pumped by a peristaltic pump (Isco Wiz Pump/Diluter) through a needle (gauge number of 18) into a level vertical gel sandwich (SE 600 Series Vertical Slab Gel Unit, Hoefer Scientific Instruments, San Francisco, CA) at a flow rate of approximately 2.5 ml per min. The needle was removed from the plates when the gradient solution reached 3.0 cm from the top edge of the plates. The top layer of the gel was covered with a layer of 1% SDS to give a flat surface. The gel was allowed to polymerize overnight at room temperature.

A 5% acrylamide stacking gel was prepared the following day. The 1% SDS layer was poured off of the resolving gel and the top of the plates were carefully dried with a paper towel. Ammonium persulfate and TEMED were added to the stacking gel solution prior to loading the stacking gel solution on top of the polymerized resolving gel with a pasteur pipet. A 15 lane comb was inserted to form 1.5 cm depth wells for sample injection. The stacking gel was alowed to polymerize for 2

hr. prior to removal of the comb. After the stacking gel had polymerized, the entire gel plate unit was carefully removed from the stand and readjusted for connection to the buffer chamber apparatus. Running buffer chilled to approximately 15° C was poured into the top buffer chamber and the system was checked for leaks. The unit was placed into the lower buffer reservoir and filled with running buffer. The electrophoresis unit (St. Petersburg, Fl) was connected to a circulating water bath (Brinkman MGU LAUDA RM6 Refrigerating Circulator, Sybon Corp., Westbury, NY) which had been equilibrated to 12° C.

Aliquots (30 ul) of five digest samples plus aliquots (5 ul) of four molecular weight markers were loaded carefully into the sample wells with a Hamilton syringe. Electrophoresis was initiated at 15 milliamps constant current while the samples migrated through the stacking gel. After 2 hr. the samples formed a thin layer (stack) before entering the resolving gel. When the samples entered the resolving gel the current was increased to 30 milliamps for the duration of the run. The electrophoresis run was terminated when the dye front (pyronin y) reached the bottom of the plate. An average electrophoresis run was finished in 9 hr.

After removal from the plates, SDS-PAGE gels were first placed in 250 ml of the peptide fixing solution (20% glacial acetic acid, 1.4% picric acid, 1.25% glutaraldehyde) for 1 hr. Then the gels were transferred to 250 ml of a second fixing

solution (10% glacial acetic acid, 40% methanol) and shaken during overnight fixing. The gels were stained for 4 hr. in 500 ml Coomassie Brilliant Blue R-250 staining solution (10% glacial acetic acid, 10% methanol, 0.025% Coomassie Brilliant Blue R-250), and shaken to prevent pooling of the stain. The staining solutions were used twice, and were filtered prior to each use. The gels were destained for 12 hr. (10% glacial acetic acid, 10% methanol) in a destaining chamber (4 l). The destaining solution was filtered through charcoal and circulated to enhance destaining. After 12 hr. the gel was transferred to a container with fresh destaining solution and shaken for another 24 hr., to complete the destaining procedure. Destained gels were stored in a 10% glacial acetic acid solution.

The SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 were scanned using an optical densitometry system consisting of an Isco Gel Scanner (Model 1312, Isco Model 228 Absorbance Detector, Lincoln, NE) connected to a Hewlett Packard 3390-A Electronic Reporting Integrator (Hewelett Packad, Palo Alto, CA). The scanning conditions for densitometry were as follows: attenuation - 9, chart speed - 1.5, peak width - 0.6, threshold - 7, scan speed - 1 cm/min and automatic color staining setting. The integrated area for each band was used to calculate the percent digestibility of each sample digest.

The percent <u>in vitro</u> digestibility of each sample was

determined using the following formula:

% *in vitro* digestibility =

$$\frac{\text{total peak areas of undigested sample} - \text{total peak areas of digested sample}}{\text{total peak areas of the undigested sample}} \times 100$$

The scans obtained from unhydrolyzed and digest samples were overlaid using a Shimadazo C-R4A Chromatopac computer program in order to visualize the digestion process.

4.6 Determination of *In Vitro* Digestibility by Simultaneous Dialysis Method

An *in vitro* digestion procedure based on a modified simultaneous dialysis of Gauthier et al. (1982) was also used to determine the *in vitro* digestibility of the SPC samples. This procedure was used as an alternative method to evaluate the SDS-PAGE system for quantitative estimation of *in vitro* protein digestibility.

4.6.1 Sample Preparation

Soy protein concentrate samples of 250 mg were dispersed in 25 ml of 0.1 N HCl solution. The pepsin digestion procedure followed was exactly as described in the SDS-PAGE method in section 4.5.1. After the pH of each of the samples

was adjusted to 8 for the pancreatin digestion, the pepsin digests were transferred into a dialysis bag with a molecular weight cutoff of 1,000 daltons (Spectra/Por 6 Wet Cellulose Dialysis Tubing, Spectrum Medical Industries Inc., Los Angeles, CA). The dialysis bag was suspended in contour glass bottles filled with 170 ml of pre-warmed (37° C), pH 8.0, 0.1 M sodium phosphate buffer. Aliquots of 1 ml of pancreatin solution (2.5 mg/ml) was added to each pepsin digest prior to sealing the dialysis bag.

Enzyme blanks was prepared by incubation under the described conditions without the protein source to be digested and with 1 gm of each of enzyme to make N measurement easier.

4.6.2 Dialysis

The pancreatin digestion was allowed to continue for a total of 24 hr. in the 37° C continuous shaking water bath. The dialysate was replaced with 170 ml of fresh pre-warmed (37° C) 0.1 M phosphate buffer every 2 hr. At the end of 8 hr. digestion the content of the dialysis bag was removed.

The percent <u>in vitro</u> digestibility of five SPC samples was determined in triplicate by determining the N content within the dialysis bag following the 8 hr. digestion process. The percent <u>in vitro</u> protein digestibility of the SPC samples was calculated using the following formula:

$$\%\text{Digestibility} = \frac{\text{N in sample} - (\text{N in dialysis bag} - \text{N in blank})}{\text{N in sample}} \times 100$$

4.7 Determination of *In Vitro* Digestibility by the pH Drop Method.

A drop in pH occurs when proteins are hydrolyzed by enzymes and protons are released from freed carboxyl groups. This drop in pH during protein hydrolysis forms the basis for an *in vitro* digestibility technique of Satterlee et al. (1979, 1982) known as the pH drop method.

4.7.1 Enzymes Used

All enzymes were purchased from Sigma (St. Louis, Mo).

1. Trypsin (1 g); Cat. No. T-0134, crystallized, dialyzed and lyophilized from porcine pancreas.
2. alpha-Chymotrypsin (1 g); Cat. No. C-4129, from bovine pancreas.
3. protease (1 g); Cat. No. P-5147, bacterial, purified. A nonspecific protease from *streptomyces griseus*.
4. Peptidase (1g); Cat. No. p-7500, from porcine intestinal mucosa.
5. Pepsin (1 g) (used to clean pH electrode); Cat. No. P-6887, crystallized and lyophilized from porcine stomach

mucosa.

4.7.2 Sample Preparation

Based on the calculations listed in Appendix B, appropriate amounts of protein samples including the sodium caseinate control were weighed into glass vials (O.D. x H. of 28 mm x 90 mm). Enzymes, including a multi-enzyme (mixture of chymotrypsin, trypsin and peptidase) and protease, were weighed into two other vials. The enzymes were kept at -20° C in the freezer. All samples were analyzed within 24 hr. to prevent loss of enzyme activity. Two vials containing 0.730 g sodium caseinate were used as an internal standard. One sodium caseinate sample is assayed before and one after other samples to verify that the enzymes are functioning properly for the entire assay period. The final pH drop of the sodium caseinate controls after 20 min. should be between 6.37 and 6.47.

Protein samples were reconstituted by adding 10 ml of distilled deionized water to each vial. The samples were vortexed for at least 2 min. and stored at 5° C for at least 1 hr. to allow for complete hydration. The samples were then removed from the refrigerator and place in a 37° C water bath for 10 min. Using a pasteur pipet, the pH of the samples was adjusted to pH 8.0 + 0.03 with acid (0.001 and 0.01 N HCl) and alkali solutions (0.05 and 0.1 N NaOH) while samples were

stirred in 37° C water bath. This pH adjustment was performed within 15 min. The pH was measured by Accumet 925 pH meter (Fisher Scientific, Pittsburg, PA) calibrated with standard pH 7.0 and pH 4.0 buffers at 37° C. The enzymes were removed from the freezer and reconstituted by adding 10 ml deionized water. After gentle stirring, the pH of the enzyme solutions was quickly adjusted to pH 8.00 + 0.03 at 37° C, using the same acid and alkali solution and stored in an ice bath during the entire assay. A pH adjustment was made within 2 min. for both enzymes.

4.7.3 pH Drop Determination

After the pH of all the samples was equilibrated to pH 8.00 ± 0.03, the digestion process was initiated. An aliquot (1 ml) of the multi-enzyme solution was added to the casein control sample, and to the remaining samples at 2 min. intervals. An aliquot (1 ml) of the protease was added exactly 10 min. after addition of the multi-enzyme solution. After addition of the protease enzyme, samples were transferred from the 37° C water bath to a 55° C water bath. Nineteen min. after the addition of multi-enzyme, the samples were returned to a 37° C circulating water bath and the pH of the solution was read and recorded exactly 20 min. after the digestion process was initiated. After each assay, the pH meter was soaked in 0.1% pepsin/0.1 N HCl for at least 2 hr.

to prevent protein coating of the electrode and placed afterwards in a pH 4 buffer.

The percent in vitro digestibility of proteins in the samples was calculated from the pH drop using the following equation (Saterlee et al, 1979, 1982):
% in vitro digestibility = 234.84 - 22.56 (X), where (X) is the pH recorded at 20 min.

4.8 Amino Acid Anlysis

4.8.1 Sample Preparation

Forty mg of sample was weighed into a hydrolysis flask. one ml (2.0 mg) of the norleucine internal standard made up in 6N HCl was added into a hydrolysis flask. Approximately 12 to 15 ml of 6N HCl was added into the flask and hydrolysis was perfomed under a nitrogen flushing for 22 hours. After hydrolysis, the sample was filtered through a GFA microfilter into a 50 ml round bottom flask. Three drops of octanol was added into the flask, and the filtered hydrolysate was evaporated to dryness on the rotary evaporator at 40° C. When dry, 2 ml of deionized water was added to the flask, swirled and evaporated to dryness again. When dry, the flask was rinsed with methanol and evaporated to dryness again. The methanol rinse and evaporation was done once more. Sample dilution buffer of 25 ml was added to the flsk, swirled to

dissolve all residues. The residues were transferred to a microcentrifuge tube and centrifuged to two min.

4.8.2 Amino Acid Analysis

The sample was injected on the Carlo Erba 3A29 Amino Acid Analyzer. For each set of samples run on the analyzer, a calibration standard was run. This standard contains known amounts of each amino acid, and a known amount of the internal standard, norleucine. The relative responses of each amino acid to norleucine were determined from a calibrated standard.

4.8.3 Sample Preparation for Protected Cystine

An aliquot (1 ml) of standard cystine was pipetted into a standard taper 24/40 joint 125 ml erlenmeyer flask. Into this same flask, 2.0 ml of the 200 ug/ml norleucine solution was pipetted into the same flask. The solution was evaporated to dryness on a rotary evaporator at 40° C. One mg of norleucine internal standard solution made up in 0.1 N HCl was pipetted into a standard taper 24/40 joint 125 ml erlenmeyer flsak. The solution was evaporated to dryness. Thirty mg of sample was weighed into the erlenmeyer flask. Ten ml of the 0° C performic acid was added to standard cystine and sample in each flask, swirled in an ice bath to wet sample. While swirling in an ice bath, 2.0 ml of 48% HBr was added to

standard and samples under a hood. The solution was evaporated to dryness on a rotary evaporator at 40° C. 6N HCl was added to each flask and refluxed for 22 to 24 hours. After hydrolysis, the sample preparation procedure and calculations were done as same in the amino acid analysis.

4.9 Determination of Apparent In Vivo Digestibility and PER.

4.9.1 Sample Preparation

Diet formulations for the rat bioassays including PER and apparent digestibility were based on a modification of the AOAC method (1984). Diet formulations are shown in Table 4. All diets used in the in vivo studies were formulated to contain 10% protein. Diets were stored in double wrapped polyethylene bags at 4° C. ANRC casein was used as the protein source inthe control diet.

4.9.2 Rat Bioassay

Sprague-Dawley male weaning rats (21 - 22 days old) were placed into individual cages with suspended water bottles. The rats were housed in a room maintained at 22.2° C, 50 - 100% RH, with alternating 12 hr. periods of light and dark. Rats were first placed on 4 day adaptation diet of standard

Table 4. Composition of Experimental Diets for Rat Bioassay

	Percent of Diet Components gms/100 gms	
	Casein	SPC
Cornstarch	73.13	67.87
Casein[1]	10.87	-
SPC[2]	-	15.39
Corn Oil[3]	10.0	10.0
Vitamin Mix[4]	2.0	2.0
Mineral Mix[5]	4.0	4.0

1. ANRC Vitamin-Free Casin (Protein = 92%), Nutritional Biochemicals Corp., Cleveland, Ohio
2. Soy Protein Concentrate (Protein = 65%), Central Soya Company, Fort Wayne, Indiana
3. Mazola Corn Oil, Best Foods, CPC International, Inc., Englwood Cliffs, New Jersey
4. AIN Vitamin Mixture 76. Nutritional Biochemicals Corp., Cleveland, Ohio
5. AIN Mineral Mixture 76. Nutritional Biochemicals Corp., Cleveland, Ohio

rat chow. Rats were weighed at the end of the adaptation period. A total of 60 rats were randomly assigned to 6 experimental groups (10 rats per group). Each group was fed an experimental diet for 28 days. Food and water were made available to the animals ad libitium. Food consumption was determined throughout the study and feces were collected for a period of 8 days during test days (18 - 26 days). Separate fecal collections were dried in an air oven and analyzed for nitrogen by the Kjeldahl method. Apparent *in vivo* digestibility was calculated as follows:

Apparent Digestibility =

$$\frac{\text{N in diet consumed(g)} - \text{N in feces(g)}}{\text{N in diet consumed(g)}} \times 100$$

To calculate PER, rats were weighed bi-weekly, and weight gain and food consumption were recorded. The PER of each diet was calculated using the following equation:

$$\text{PER} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

4.10 C-PER

C-PER was calculated based on *in vitro* digestibility obtained by the pH drop method in conjunction with amino acid analysis. The calculation C-PER's for each soy protein sample was done using the appropriate formula (Satterlee et al., 1982).

5.0 RESULTS and DISCUSSION

5.1 Protein and Moisture Content of SPC Samples.

The protein content of SPC samples on a dry weight basis was 65.25%. The moisture contents of SPC and all autoclaved SPC samples after freeze drying were 3.5% and 4.35%, respectively.

5.2 The Effect of Autoclaving on Protein Solubility

Heat treatment is one of the most important procedures employed in commercial processing of soy protein. The most common physical change in heat denatured protein is decreased solubility. Solubility not only influences the functional properties of soy protein but also affects the biological value of protein. In the studies done with ruminants, high correlation exists between the solubility of protein in low ionic strength salt solution and the extent of protein degradation in the rumen (Mitchell et al., 1967, Dingley et al., 1975, Wohlt et al., 1976, Schingoethe and Ahrar, 1979). Therefore, the determination of protein solubility is important in evaluating the quality of heated soy protein.

Many studies have attempted to elucidate the mechanism(s) of molecular events which lead to soy protein insolubility

when the protein is heated. Glycinin contains approximately 48 half-cystine residues which could form a substantial number of disulfide linkages in the soy protein. Heating cleaves disulfide linkages and sulfhydryl-disulfide interchange is believed to be responsible for the decreased solubility of heated soy protein (Fukushima et al., 1970, Hashizume et al., 1971, Cumming et al., 1973). In addition to intermolecular disulfide bonding, hydrogen and hydrophobic bond formation of proteins have been proposed as a likely explanation for the decreased solubility of heat denatured soy protein (Chiang and Sternberg, 1974, Hager, 1984).

In this study, solubility measurements of SPC samples were performed in potassium phosphate buffer (pH 7.6, 0.5 ionic strength) containing varying concentrations of urea and beta-mercaptoethanol. Urea was selected as a solute in order to solubilize protein molecules held together by hydrogen or hydrophobic bonding. And beta-mercaptoethanol was selected as solute in order to solubilize protein molecules held together by inter-molecular disulfide linkages. The protein solubility of SPC samples in different solvent composition are shown in Table 5.

The protein solubility of SPC samples in potassium phosphate buffer ranged from 11.2% for the control sample to 30.9% for 4 hr. autoclaved SPC. Soy proteins have a compact globular structure and are insoluble in water near their isoelectric point (pH = 4.5). Protein solubility increases

Table 5. The Effect of Autoclaving on Solubility of Soy Protein Concentrate[1]

	Autoclaving Time, min				
Solvents	0	10	30	120	240
Potassium Phosphate Buffer	11.2	11.0	13.3	25.3	30.9
Buffer + 1.5M urea	15.9	12.6	14.3	22.3	28.6
Buffer + 3.0M urea	25.5	14.6	17.5	25.7	30.8
Buffer + 8.0M urea	48.2	23.0	23.5	27.6	31.5
Buffer + 0.1M ME	23.2	24.0	22.7	25.4	29.0
Buffer + 1.5M urea + 0.1M ME	32.6	26.4	24.4	27.7	32.2
Buffer + 3.0M urea + 0.1M ME	45.1	30.8	35.0	37.8	38.5
Buffer + 8.0M urea + 0.1M ME	62.8	61.3	62.5	39.3	42.5

1. Percentage of soluble protein in each sample.

when salt is added to water at the isoelectric point and further increases in solubility occur as the pH is shifted away from isoelectric point. Based on this phenomenon, the protein solubility of SPC samples in potassium phosphate buffer (pH 7.6, 0.5 ionic strength) is attributed to partial unfolding of soy protein due to electrostatic repulsion between negatively charged soy protein subunits. The higher protein solubility of 2 and 4 hr. autoclaved samples of potassium phosphate buffer implies that other factors besides conformational unfolding of soy protein contribute to changes in solubility. Heat treatment is known to denature protein by altering both noncovalent and covalent (sulfhydryl-disulfide interchange) bonds between protein molecules. Therefore, the increase in the solubility of the 2 and 4 hr. autoclaved samples is likely due to major changes in three dimensional protein structure.

The solubility of control SPC increased dramatically as the urea concentration of the buffer increased. The initial solubility in buffer increased four fold when 8 M urea was added. The solubilities of 10 and 30 min. autoclaved samples in buffer containing different concentrations of urea also increased but to a smaller extent than that observed with the control sample. Increased protein solubility in presence of urea is probably due to the disruption and solubilization of small aggregates of protein molecules previously held together by hydrogen or hydrophobic bonding (Hager, 1984). Therefore,

the observed differences in protein solubility of the control, 10 and 30 min. autoclaved samples might indicate that soy protein molecules interact by noncovalent forces such as hydrophobic interaction and hydrogen bonding, which although present in the control were weaker than those in autoclaved samples.

There were no differences observed in protein solubility of control, 10 and 30 min. autoclaved samples in buffer containing 0.1 M beta-mercaptoethanol. The solubility of control, 10 and 30 min. autoclaved samples in buffer containing both urea and beta-mercaptoethanol is higher than in buffer containing either urea or beta-mercaptoethanol. Even though overall solubility of 10 and 30 min. autoclaved samples were lower than control, the solubility of 10 and 30 min. in buffer containing 8 M urea and beta-mercaptoethanol was similar to that of control. The increased protein solubility in buffer containing beta-mercaptoethanol is likely due to the solubilization of aggregates formed by intermolecular disulfide bonds. Therefore, these results suggest that the aggregates formed after 10 and 30 min. autoclaving are held together by intermolecular disulfide bonds in such a way that urea was less effective in disrupting hydrophobic and hydrogen bonds and solubilizing the protein.

The protein solubility of 2 and 4 hr. autoclaved samples in buffer did not change appreciably by adding even 8 M urea. An increase in solubility in buffer containing 3 M urea and

beta-mercaptoethanol was also observed with 2 and 4 hr. autoclaved samples. The solubility of 2 and 4 hr. autoclaved samples in buffer containing 8 M urea and 0.1 M beta-mercaptoethanol was much less than control, 10 and 30 min. autoclaved samples, indicating that protein molecules were more extensively cross-linked in 2 and 4 hr. autoclaved samples.

5.3 *In Vitro* Digestibility Determined by the Measurement of TCA Soluble Nitrogen.

The effects of autoclaving on soy protein digestibility was determined based on the N content of supernatant remaining after enzymatic hydrolyzates of soy protein were treated with a 20% trichloro acetic acid (TCA) solution. The average molecular weight of the TCA soluble fraction ranges from 330 to 380 daltons (Greenberg and Shipe, 1979). The molecular weight of TCA soluble components corresponds to peptides containing three or four amino acid residues, which is in the range of peptides which would be further hydrolyzed by mucosal and cellular peptidases and absorbed into the bloodstream. This method was developed by Anson (1938) to measure proteolysis from increases in 280 nm absorbance of the supernatant remaining after TCA precipitation of undigested proteins. However, spectrophotometric and colorimetric measurements of optical density for the assay of proteolysis

can be misleading. Changes in conformation could expose different amino acids and/or chromophoric groups. Increase in optical density of a solution can be detected, even when there is no actual degradation of large proteins (Ory and Sekul, 1977). Since conformational changes in soy protein after autoclaving were expected in this study, the total nitrogen content of the supernatant was measured by Kjeldahl analysis.

The mean percent in vitro digestibility after 8 hr. pepsin and pancreatin hydrolysis observed with control, 10, 30 min., 2 and 4 hr. autoclaved SPC samples was 87.53, 80.06, 82.08, 72.68 and 67.08, respectively (Table 6). The mean percent in vitro digestibility of the control was significantly higher than those of autoclaved samples. Among autoclaved samples, the mean percent in vitro digestibility observed with 10 and 30 min. autoclaved samples was significantly higher than those of 2 and 4 hr. autoclaved samples. The mean percent in vitro digestibility of 2 hr. autoclaved sample was significantly higher than that of 4 hr. sample.

The values for the unhydrolyzed samples indicated that low molecular weight N containing materials already existed in samples. Unhydrolyzed 2 and 4 hr. autoclaved samples had a higher TCA soluble N content than control, 10 and 30 min. autoclaved samples. Excessive heating disrupts protein structure and degrades protein subunits into low molecular

Table 6. The Effect of Autoclaving on In-Vitro Digestibility of Soy Protein Concentrate as Determined by TCA Soluble Nitrogen[1]

	Autoclaving Time, min				
Hours Digs.	0	10	30	120	240
0	3.54 ± 0.22	3.16 ± 0.21	3.21 ± 0.33	8.34 ± 0.59	15.6 ± 0.46
2	65.42 ± 3.84	60.06 ± 1.92	61.09 ± 0.53	53.34 ± 2.48	45.53 ± 1.15
3	76.20 ± 1.36	72.70 ± 0.91	75.01 ± 0.36	67.11 ± 1.85	59.56 ± 1.30
4	78.98 ± 4.35	76.43 ± 0.62	78.76 ± 0.56	70.84 ± 2.51	63.17 ± 1.15
8	87.53^{a} ± 1.63	80.06^{b} ± 2.02	82.08^{b} ± 1.71	72.68^{c} ± 3.55	67.08^{d} ± 2.21

1. Mean percent in-vitro digestibility with standard deviation of three trial runs. Data excludes TCA soluble N in unhydrolyzed SPC sample.

abcd. Means with the same superscript are not significantly different ($p < 0.05$).

weight componentrs (Saio et al., 1975). Therefore, the higher TCA solulble N content in 2 and 4 hr. autoclaved samples might be attributed to degraded low molecular wieght components present after 2 and 4 hr. autoclaving. In the TCA soluble N measurement study, the digestibility was calculated based on enzymatic hydrolysates determined by TCA soluble N originating only from pepsin-pancreatin hydrolysis. The content of TCA soluble N in unhydrolyzed sample was substracted from the content of TCA soluble N in the hydrolyzed sample to obtain TCA soluble N originating only from pepsin-pancreatin hydrolysis.

Enzymatic hydrolysis rate is an important factor in determining protein quality. Slow absorption of amino acids due to a decrease in the rate of enzymatic hydrolysis further decreases the efficiency of amino acid utilization (Melnick et al., 1946). The effect of autoclaving on enzymatic hydrolysis rate was estimated by measuring the mean values for percent <u>in vitro</u> digestibility after 2 hr. pepsin and subsequent 3, 4 and 8 hr. pancreatin hydrolysis (see Table 6). The rate of pepsin hydrolysis was faster than pancreatin hydrolysis for all the samples. More than 50% of protein was hydrolyzed after 2 hr. pepsin hydrolysis except in the case of the 4 hr. autoclaved sample.

Lynch et al. (1977) reported that pepsin digestion of glycinin was at least tenfold faster than the corresponding trypsin digestion. The fast hydrolysis of glycinin by pepsin

at pH 2.0 is believed to be due to the acidic dissociation of the subunits with concurrent unfolding of the polypeptide chain (Wolf et al., 1958, Catsimpoolas et al., 1969). Evidence for such conformation changes in glycinin subunits at pH 2.0 has been previously confirmed by changes in optical rotary measurements, hydrogen ion titration and UV difference spectra (Wolf et al., 1958, Catsimpoolas et al., 1971). The effects of pH on the 11S protein suggests that dissociation is due to electrostatic repulsion forces between protein subunits. The synergistic effect of protein denaturation at acidic pH coupled with the optimum pH of pepsin activity enhances the rate of protein hydrolysis (Lynch et al., 1977).

The percentage of soy protein hydrolyzed by pepsin in the control, 10, 30 min., 2 and 4 hr. autoclaved samples was 65, 60, 61, 53 and 46%, respectively (see Table 6). It has been reported that pepsin hydrolyzed casein faster than soy protein, even though both have equal amounts of aromatic amino acids (Gauthier et al., 1982). This implies that differences in conformation of casein and soy protein can influence the rate of enzyme hydrolysis in addition to the percentage of peptide bonds in each protein for which pepsin had specificity. The decreased pepsin hydrolysis rate of autoclaved SPC samples further implies that conformational changes in soy protein occurred which limited the rate of pepsin hydrolysis. The noticeable decrease observed with 2 and 4 hr. autoclaved samples also suggests that conformational

changes occurred to a greater extent in these samples which coincides with the relatively low protein solubility of the 2 and 4 hr. autoclaved samples in potassium phosphate buffer containing urea and beta-mercaptoethanol (see Table 5).

The percent of protein hydrolysis due to pancreatin was about 20% for each of the samples. This indicates that conformational changes in soy protein due to autoclaving affected the initial rate of pepsin hydrolysis, but did not affect subsequent pancreatin hydrolysis. Most protein digestion occurred during the first 4 hr. After 4 hr., the pancreatin hydrolysis proceeded more slowly up to 8 hr. The time course of pancreatin hydrolysis showed that the extent of pancreatin hydrolysis for 1 hr., after pepsin hydrolysis, was faster than the rest of the time intervals. This trend suggests that conformational changes under acidic conditions of pepsin digestion cause the unfolding of glycinin, thus enhancing the susceptibility of soy protein to pancreatin hydrolysis. Following pepsin and pancreatin hydrolysis of accessible peptide bonds, intermediate digestion products are formed. Further degradation of the protein by pancreatin may require additional conformational changes in order to expose the remaining susceptible peptide bonds to enzymes, thus slowing down the subsequent rate of pancreatin hydrolysis.

5.4 Evaluation of SDS-PAGE as an *In Vitro* Digestion Assay

SDS-PAGE has been proven to be a useful tool for the estimation of molecular weights of proteins and peptides. In order to estimate the molecular weights of resolved polypeptide and peptide present in the hydrolyzates and unhydrolyzed SPC samples, marker proteins were loaded onto the same gel. A plot of log molecular weight versus relatve mobility and a plot of log molecular weight versus retention time for marker proteins are depicted in Figure 4 and Figure 5, respectively. Weber and Osborn (1969) found SDS-PAGE to be empirically valid for at least 40 different proteins with polypeptides, covering a wide range of molecular weights from 10,000 to 70,000 daltons. In spite of the excellent reliability of SDS-PAGE for molecular weight determination of proteins, there are problems associated with the resolution and molecular weight estimation of low molecular weight peptides. A plot of log molecular weight versus relative electrophoretic mobility is nonlinear, with a break in the curve occuring in the molecular weight range from 10,000 to 20,000 daltons (Weber and Osborn, 1969, Laemmli, 1970, Swank and Munkers, 1971, Fling and Gregerson, 1986). The deviations from linearity observed with low molecular weight peptides have been attributed to the relative importance of intrinsic charge and conformation in the binding of SDS to small molecular weight peptides. Since the size and charge of the SDS-peptide complex depends on the relative content of various amino acids in the peptide, relative variations should

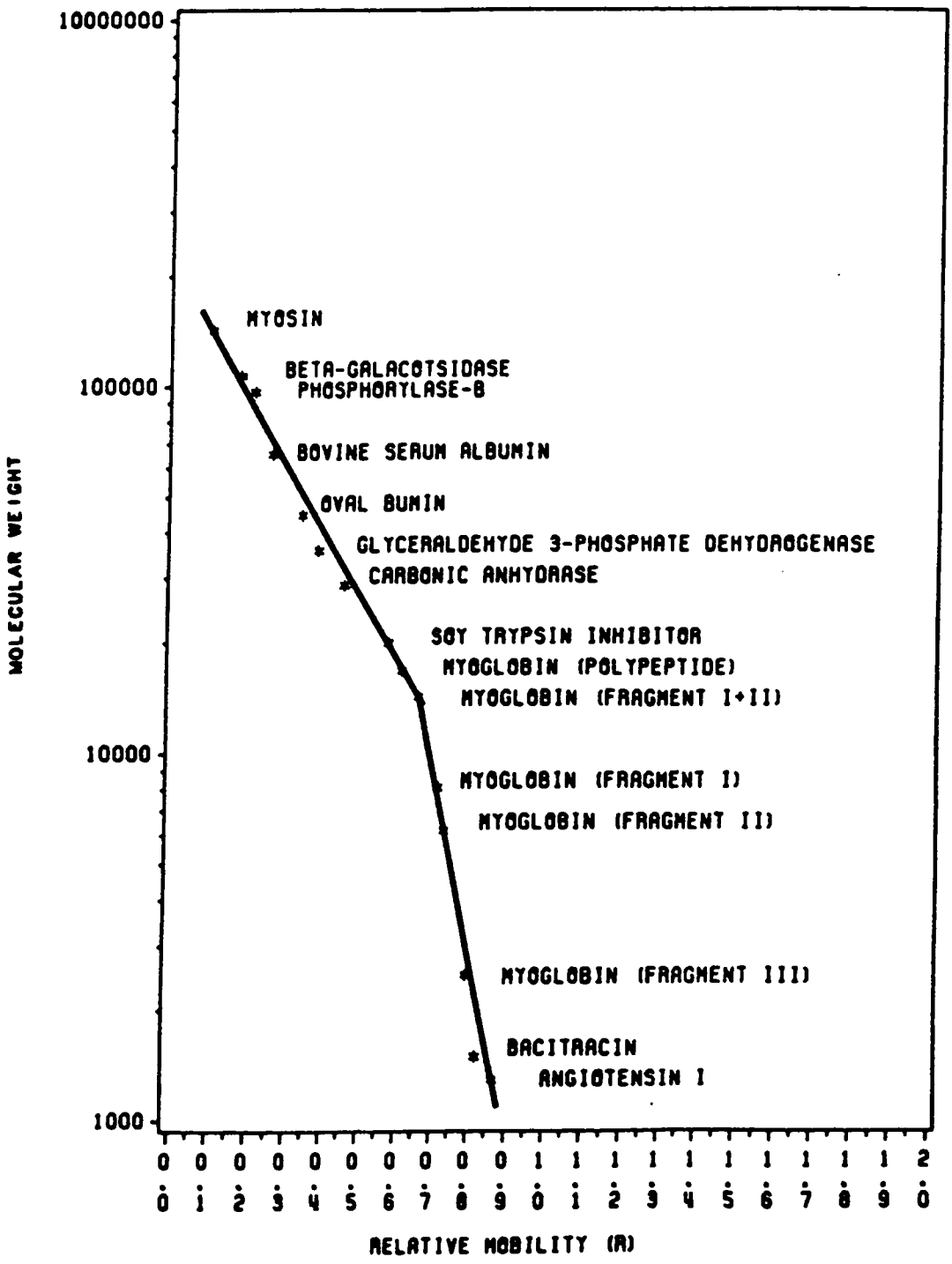


Figure 4. **SDS-PAGE Molecular Weight Calibration Curve (Log Mw versus Relative Mobility)**

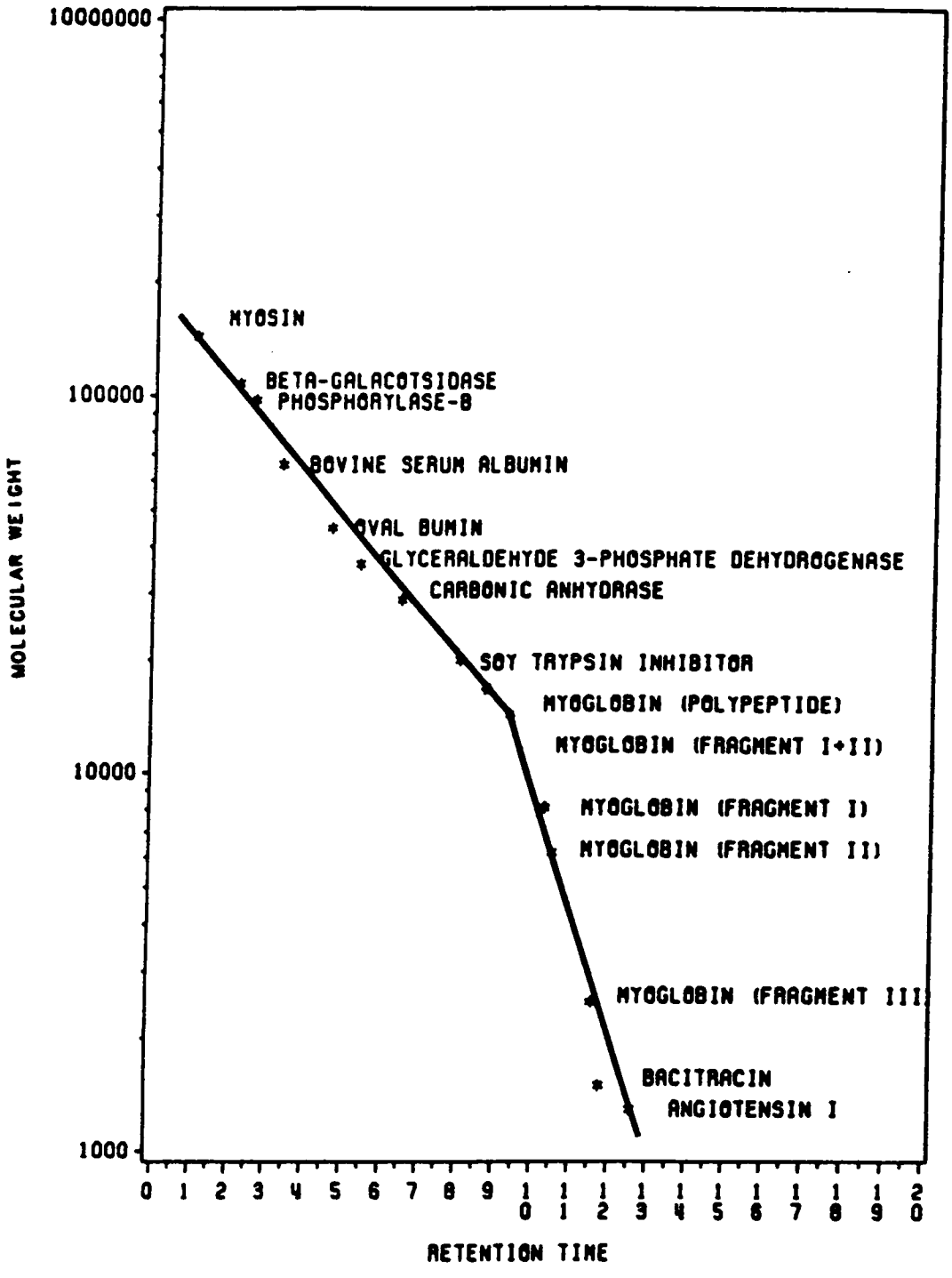


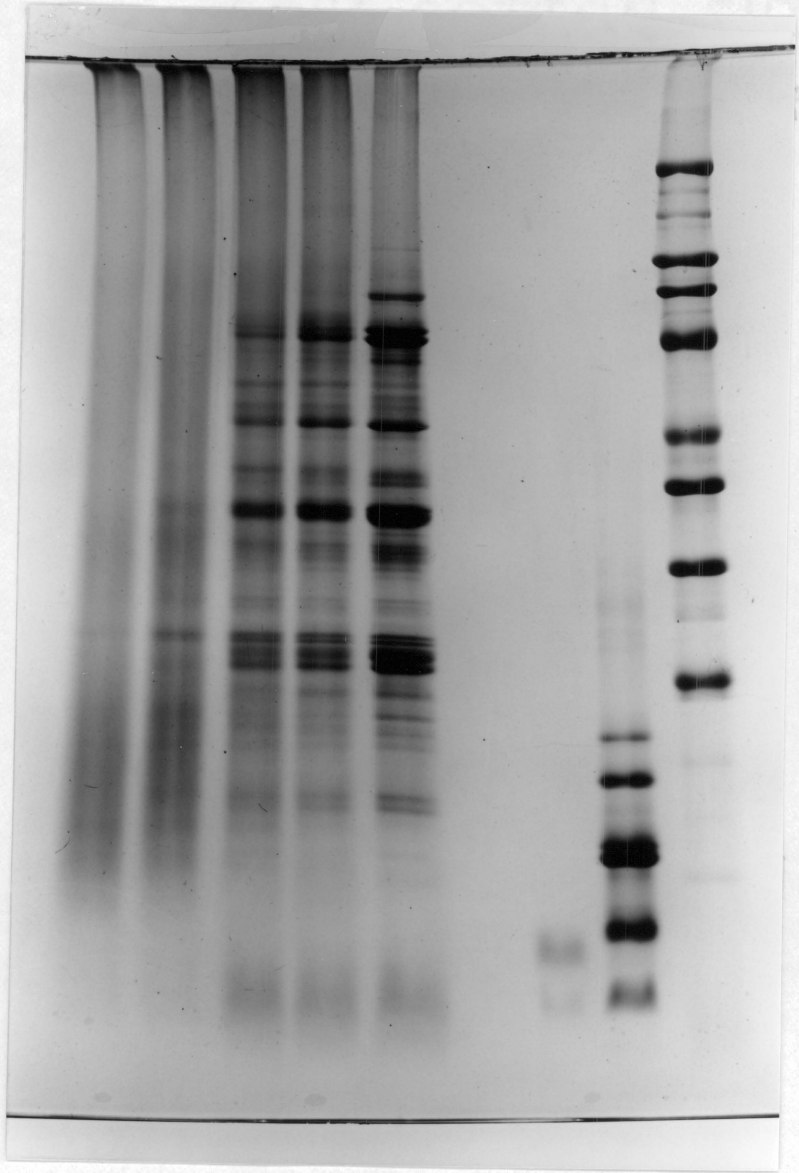
Figure 5. SDS-PAGE Molecular Weight Calibration Curve (Log Mw versus Retention Time)

be greater in small peptides than in proteins.

Consistent with the results of other workers, we also observed an inflection point near 14,400 daltons in Figures 4 and 5.

A photo of the SDS-PAGE gel pattern for the five unhydrolyzed samples are shown in Figure 6. The five lanes represent unhydrolyzed control, 10, 30 min., 2 and 4 hr. autoclaved SPC samples. Ten major bands and approximately seventeen minor bands were resolved in the SPC control. The molecular weight of each band in the unhydrolyzed SPC control sample was estimated using the calibration curve drawn in Figure 4. The relative mobility of each band and its corresponding molecular weight are shown in Table 7.

For convenience, the electrophoretical pattern of each SPC sample was divided into five parts. The estimated molecular weight of a minor band located just above a fifth major band (see Part 3) was 47,000. According to the literature (Moreira et al., 1970, Kitamura et al., 1976), the molecular weight of acidic 11S subunit A4 is 45,000 and the molecular weights of acidic subunits A1 - A3 are 37,000 - 42,000 daltons. The molecular weigh of the fifth and sixth major bands (see Part 3) are 41,000 and 40,000, respectively. The minor band and two major bands are thought to correspond to acidic A4 and acidic A1-A3 subunits. The molecular weights of major bands seven to ten (see Part 4) are in the range of 23,500 - 20,500, which correspond to literature values for the



A:SPC-4 hour Autoclaved
B:SPC-2 hour Autoclaved
C:SPC-30 min. Autoclaved
D:SPC-10 min. Autoclaved
E:SPC-Control
F:Reference Markers (Mw range:1,500 - 1,296)
G:Reference Markers (Mw range:16,950 - 2,510)
H:Reference Markers (Mw range:205,000 - 20,100)

Figure 6. SDS-PAGE Gel of Control and Autoclaved SPC Samples.

Table 7. Relative Mobility of Bands in SDS-PAGE Electrophoresis Pattern of Control SPC and Their Corresponding Molecular Weights

Parts	Number of Major Bands	Rm	Mw	Number of Minor Bands	Rm	Mw
1	1	0.22	115,000			
	2	0.25	90,000			
	3	0.26	88,000			
2				1	0.28	79,000
				2	0.30	72,000
				3	0.31	68,000
				4	0.32	66,000
	4	0.34	60,000			
3				5	0.38	50,000
				6	0.39	47,000
	5	0.42	41,000			
	6	0.43	40,000			
4				7	0.44	38,000
				8	0.46	34,000
				9	0.47	33,000
				10	0.51	27,000
				11	0.52	26,000
	7	0.54	23,500			
	8	0.55	22,500			
	9	0.56	21,500			
	10	0.57	20,500			
5				12	0.6	18,000
				13	0.62	16,000
				14	0.63	15,500
				15	0.64	15,000
				16	0.70	9,000
				17	0.71	8,200

basic 11S subunits (Kitamura et al., 1976).

Densitometric scanning of gels and quantification of the area under peaks corresponding to the acidic A1 - A3 subunits and basic subunits indicated that they account for 26.3 and 22.4%, respectively of the total protein. Thanh and Shibasaki (1976, 1977) have purified beta-conglycinin to near homogeneity and shown that it is made up of three subunits alpha, alpha' and beta. Beachy et al (1981) reported a molecular weight of 83,000 for alpha', 76,000 for alpha and 53,000 for beta subunits. Two minor bands under a third major band (see Part 2) have estimated molecular weights of 79,000 and 72,000 and the estimated molecular weight of a minor band just under a fourth major band (see Part 3) is 50,000. These three bands are believed to be the three subunits of beta conglycinin (7S). The estimated molecular weights for the acidic subunits of soy glycinin are somewhat higher and molecular weights of subunits for beta-conglycinin are somewhat lower than those reported in the literature. Discrepancies in molecular weight estimation of protein subunits presumably resulted from differences in electrophoretic conditions and molecular weight standards that were used.

Three major bands with apparent molecular weights of 115,000, 90,000 and 88,000 daltons (see Part 1) are thought to be dimers and/or oligomers of protein subunits. These high molecular weight bands were unstable upon autoclaving.

The first band was not detected in the 10 and 30 min. autoclaved samples and other high molecular weight bands also disappeared faster than the acidic and basic 11S subunits in the 10 and 30 min. autoclaved samples. It is possible that they are dimerized or oligomerized subunits held together by S-S bonds, which were cleaved by heat treatment.

The effect of autoclaving on soy protein components is also demonstrated by the SDS-PAGE patterns of the five unhydrolyzed SPC samples in figure 6. Gel patterns shown in Figure 6 were very similar for control, 10 and 30 min. autoclaved samples, except for some differences in minor bands and the intensity of some major bands. The presence of faint bands of low molecuar weight on the bottom of the gel observed with control, 10 and 30 min. autoclaved samples, may be attributed to low molecular weight components produced by enzymatic proteolysis of SPC samples during storage or may be low molecular weight components solubilized by 8 M urea in the sample buffer. Each of these bands gradually becomes less intense and more obscure in the 10 and 30 min. autoclaved samples. The original 27 bands could not be detected in 2 and 4 hr. autoclaved samples, while the intensity of the low molecular weight components increased. Poor resolution and smearing of bands on SDS-PAGE gels has been previously reported when extensively heated protein samples were loaded on a gel system (Cumming et al., 1973, Marshall et al., 1988). These electrophoretical patterns suggest that soy protein

subunits were unchanged in the control but started to dissociate after 10 and 30 min. autoclaving, and were degraded to low molecular weight components after 2 and 4 hr. of autoclaving.

The dissociation of subunits which accompanies heat denaturation of soy protein has been extrensively studied. According to Fukushima's study (1968), the oligomeric stucture of soy glycinin (11S) begins to dissociate around 80° C. One 11S fraction stays as an undissociated soluble component and the other fraction is converted into a soluble aggregate. On continued heating, the soluble aggregate increases in size and forms a precipitate (Wolf and Tamura, 1969). Saio et al. (1975) reported that a SDS-PAGE pattern similar to that of unheated soybean proteins was obtained when soy protein was heated up to 130° C. However no bands were detected after heating to 150° C due to degradation of protein subunits to low molecular weight components. In 1980, Yamagishi et al. reported that a precipitate forms after heating soy glycinin (11S) at 100° C consisting of various proportions of associated basic and acidic subunits. In the presence of conglycinin (7S), glycinin does not form aggregates when heated at 100° C. Instead, the basic subunits of glycinin form a soluble complex with the subunits of conglycinin via electrostatic interactions. The molecular weight of this soluble complex is reported as around one million daltons and does not enter into separating gels (Utsumi et al., 1984).

Based on these previous studies and electrophoretical patterns of autoclaved SPC samples (see Fig. 6), it was believed that 11S and 7S subunits dissociated and formed aggregates or macromolecular complexes during autoclaving. When SPC samples were loaded on gels, it was found that some of the soy protein molecules did not enter into the resolving gel. These aggregates are visible in Figure 6 as high molecular weight bands on the top of the resolving gel. The control, which was not autoclaved, apparently has smaller amounts of protein aggregates than other autoclaved samples.

The unique advantage of SDS-PAGE over other in vitro methods to determine protein digestibility is that a sequential eight hour protein digestion process can be visualized as SDS-PAGE gel pattern, and the moleclar weight of digestive products can also be estimated (Wasserman, 1986). SDS-PAGE gel patterns of hydrolyzed SPC samples are shown in black and white photographs presented in Figures 7, 9, 11, 13 and 15. Each gel contained eight lanes. The eight lanes included the unhydrolyzed SPC sample and 2, 3, 4 and 8 hr. hydrolyzed samples and three lanes consisting of molecular weight markers. The electrophoretic pattern of SPC control shows that after 2 hr. hydrolysis the bands corresponding to acidic 11S and 7S subunits disappeared, but the bands of the basic 11S subunits were still visible, although very faint. After 3 hr. hydrolysis, the basic subunits were hardly detectable. Electrophoretic patterns indicated that the basic

11S subunits were more resistant to enzyme hydrolysis than acidic 11S subunits. The resistance of 11S basic subunits to enzymatic hydrolysis has been reported by Lynch et al.(1977) and Kamata and Shibasaski (1978a, 1978b). The basic subunits, being more hydrophobic than the acidic subunits, associate by hydrophobic interactions in such a way as to expose only a limited number of peptide bonds to proteolytic enzymes (Kella et al., 1986).

The digestion pattern is more cleary visualized when the densitograms of the 2 hr. pepsin digest and subsequent 3, 4 and 8 hr. pancreatin digests of an SPC sample are over-layed. The densitograms depicting the enzymatic hydrolysis of the SPC samples are shown in Figures 8, 10, 12, 14 and 16. The densitograms of SPC samples indicate that high molecular weight polypeptides were rapidly hydrolyzed by pepsin to lower molecular weight peptides. Also the peak areas of the densitogram are hardly discernible after the 8 hr. digestion period.

Problems were acounted with estimating the molecular weights of SPC hydrolyzate components based on their relative mobility (Rm) on SDS-PAGE gels because of difficulties with discerning discrete bands on each gel. The distribution of molecular weights of SPC hydrolysates was determined from a calibration curve of log molecular weight of marker proteins versus retention times on densitogram (see Fig. 5). Estimated molecular weights of polypeptides and peptides remaining in

A:SPC-Control: 2 hour pepsin digest/6 hour pancreatin digest
B:SPC-Control: 2 hour pepsin digest/2 hour pancreatin digest
C:SPC-Control: 2 hour pepsin digest/1 hour pancreatin digest
D:SPC-Control: 2 hour pepsin digest
E:SPC-Control: Unhydrolyzed
F:Refernce Markers (Mw range:1,500-1,296)
G:Reference Markers (Mw range:16,950-2,510)
H:Reference Markers (Mw range:205,000-20,100)

Figure 7. SDS-PAGE Gel of the Unhydrolyzed and Hydrolyzed SPC Control

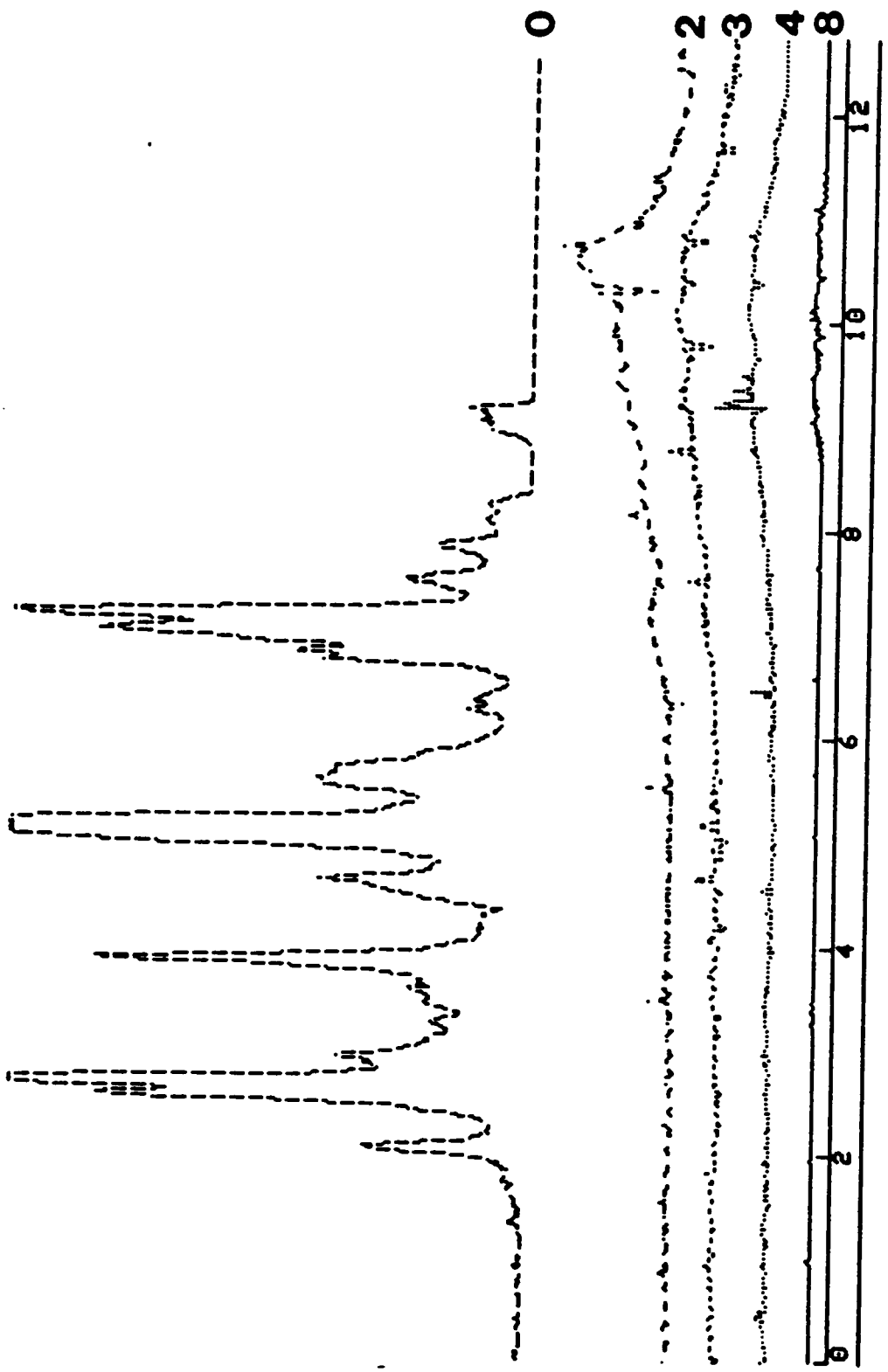
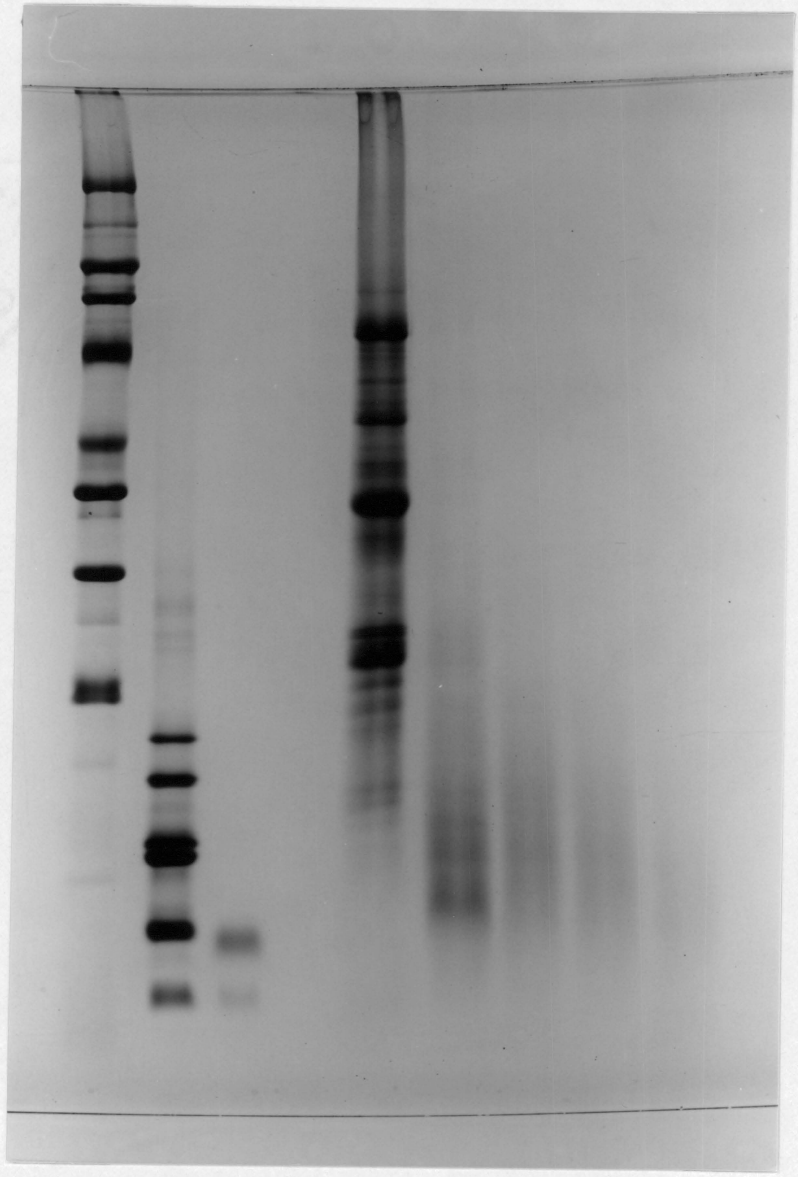


Figure 8. Densitometer Scans of Unhydrolyzed and Hydrolyzed SPC Control



A:Reference Markers (Mw range:205,000 - 20,100)
B:Reference Markers (Mw range:16,950 - 2,510)
C:Reference Markers (Mw range:1,500 - 1,296)
D:SPC-10 min. Autoclaved:unhydrolyzed
E:SPC-10 min. Autoclaved:2 hour pepsin digest
F:SPC-10 min. Autoclaved:2 hour pepsin/1 hour pancreatin digest
G:SPC-10 min. Autoclaved:2 hour pepsin digest/2 hour pancreaticn digest
H:SPC-10 min. Autoclaved:2 hour pepsin digest/6 hour pancreatin digest

Figure 9. SDS-PAGE Gel of the Unhydrolyzed and Hydrolyzed 10 Min. Autoclaved SPC.

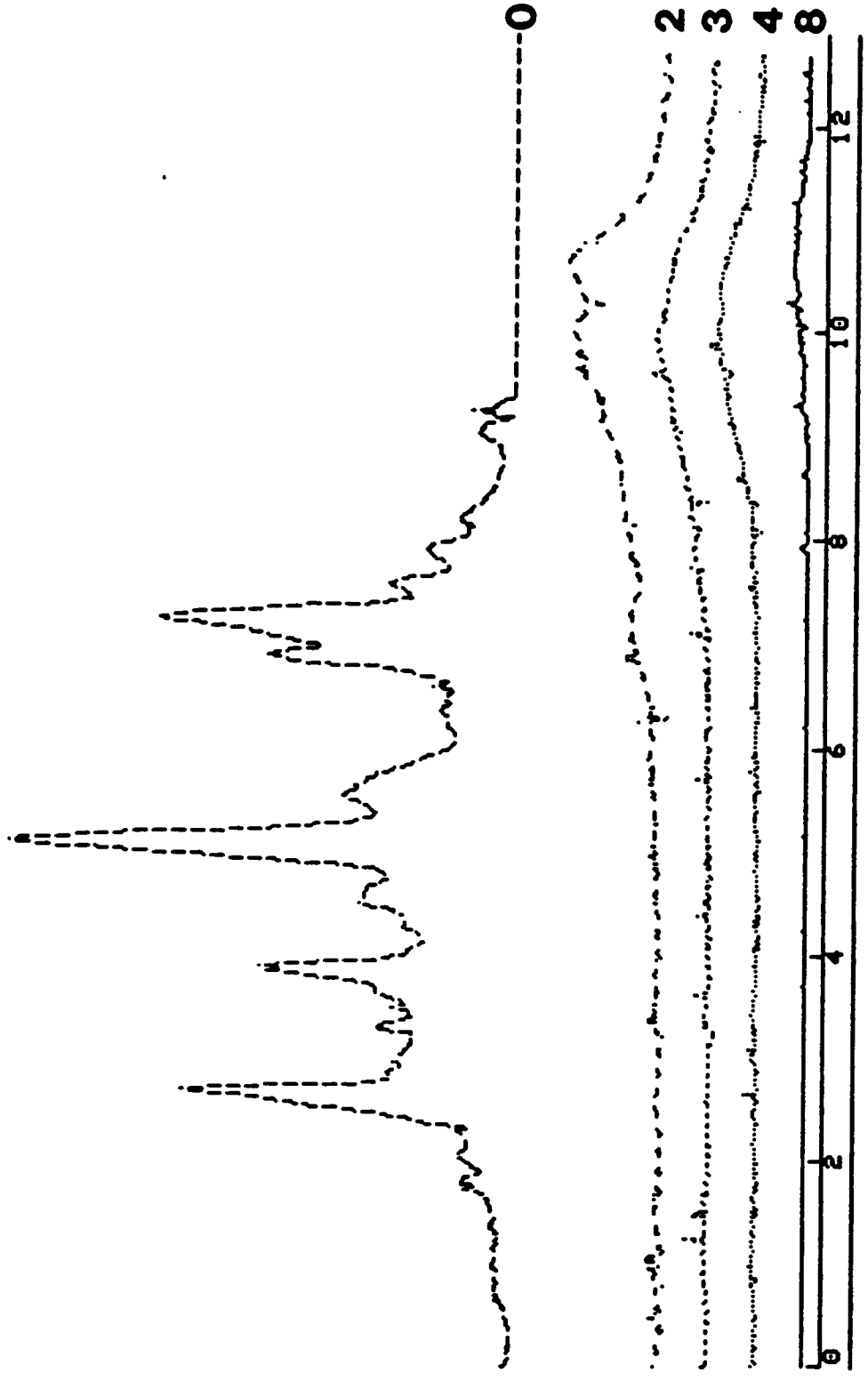
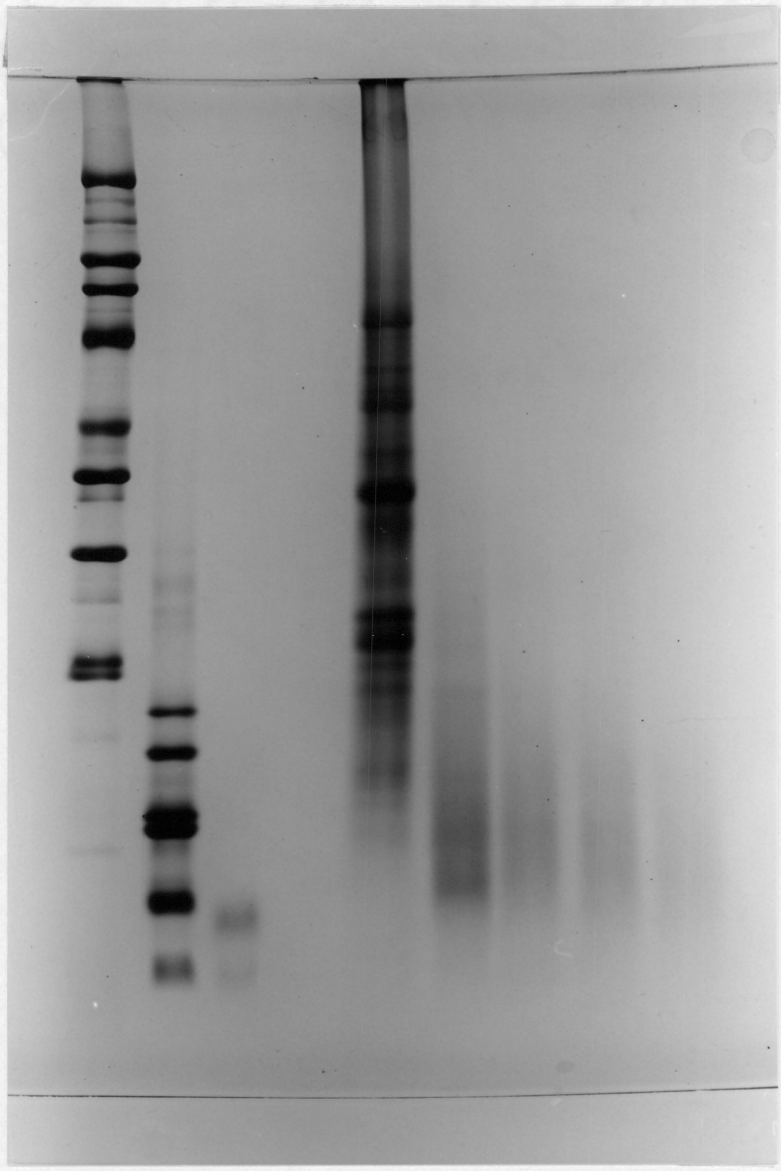


Figure 10. Densitometer Scans of Unhydrolyzed and Hydrolyzed 10 Min. Autoclaved SPC.



A:Reference Markers (Mw range:205,000 - 20,100)
B:Reference Markers (Mw range:16,950 - 2,510)
C:Reference Markers (Mw range:1,500 - 1,296)
D:SPC-30 min. Autoclaved:unhydrolyzed
E:SPC-30 min. Autoclaved:2 hour pepsin digest
F:SPC-30 min. Autoclaved:2 hour pepsin/1 hour pancreatin digest
G:SPC-30 min. Autoclaved:2 hour pepsin digest/2 hour pancreaticn digest
H:SPC-30 min. Autoclaved:2 hour pepsin digest/6 hour pancreatin digest

Figure 11. SDS-PAGE Gel of the Unhydrolyzed and Hydrolyzed 30 Min. Autoclaved SPC.

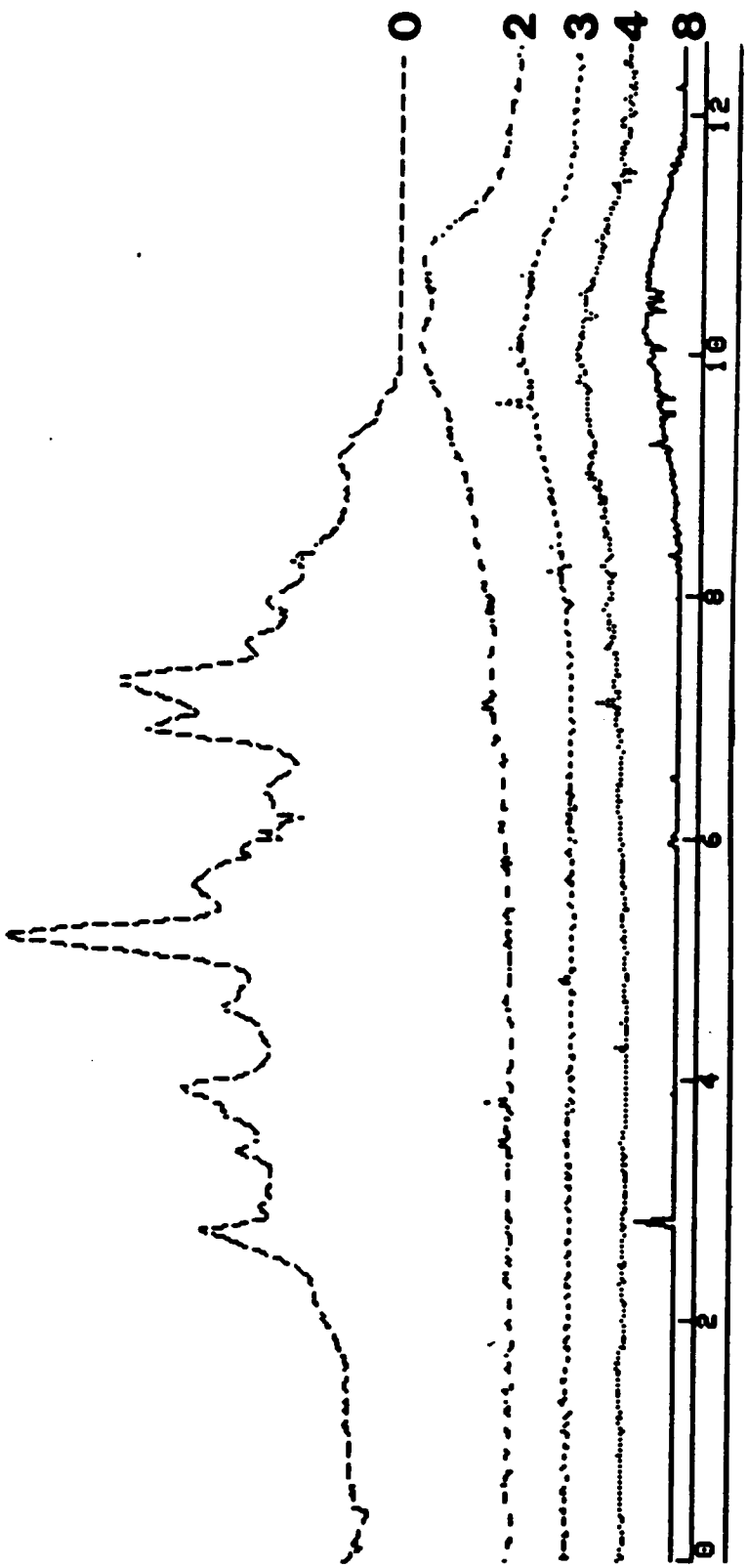
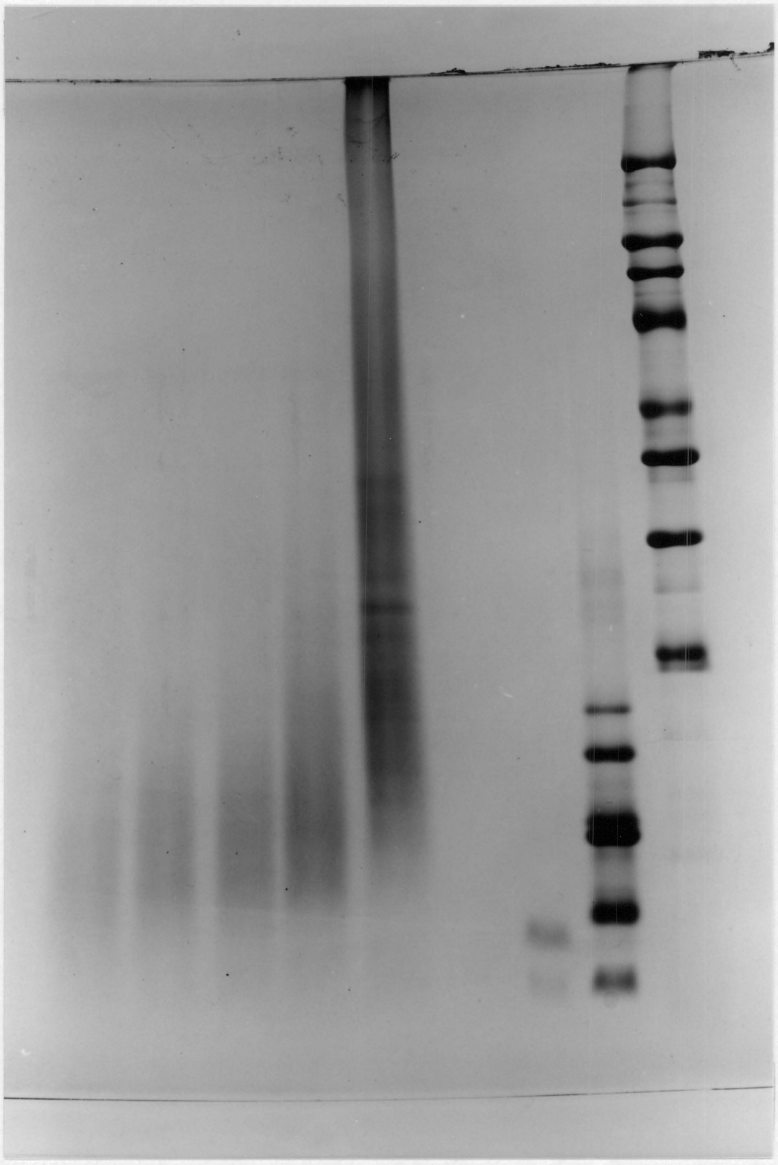


Figure 12. Densitometer Scans of Unhydrolyzed and Hydrolyzed 30 Min. Autoclaved SPC.



A:SPC-2 hour Autoclaved:2 hour pepsin digest/6 hour pancreatin digest
B:SPC-2 hour Autoclaved:2 hour pepsin digest/2 hour pancreaticn digest
C:SPC-2 hour Autoclaved:2 hour pepsin/1 hour pancreatin digest
D:SPC-2 hour Autoclaved:2 hour pepsin digest
E:SPC-2 hour Autoclaved:Unhydrolyzed
F:Reference Markers (Mw range:205,000 - 20,100)
G:Reference Markers (Mw range:16,950 - 2,510)
H:Reference Markers (Mw range:1,500 - 1,296)

Figure 13. **SDS-PAGE Gel of the Unhydrolyzed and Hydrolyzed 2 Hour Autoclaved SPC.**

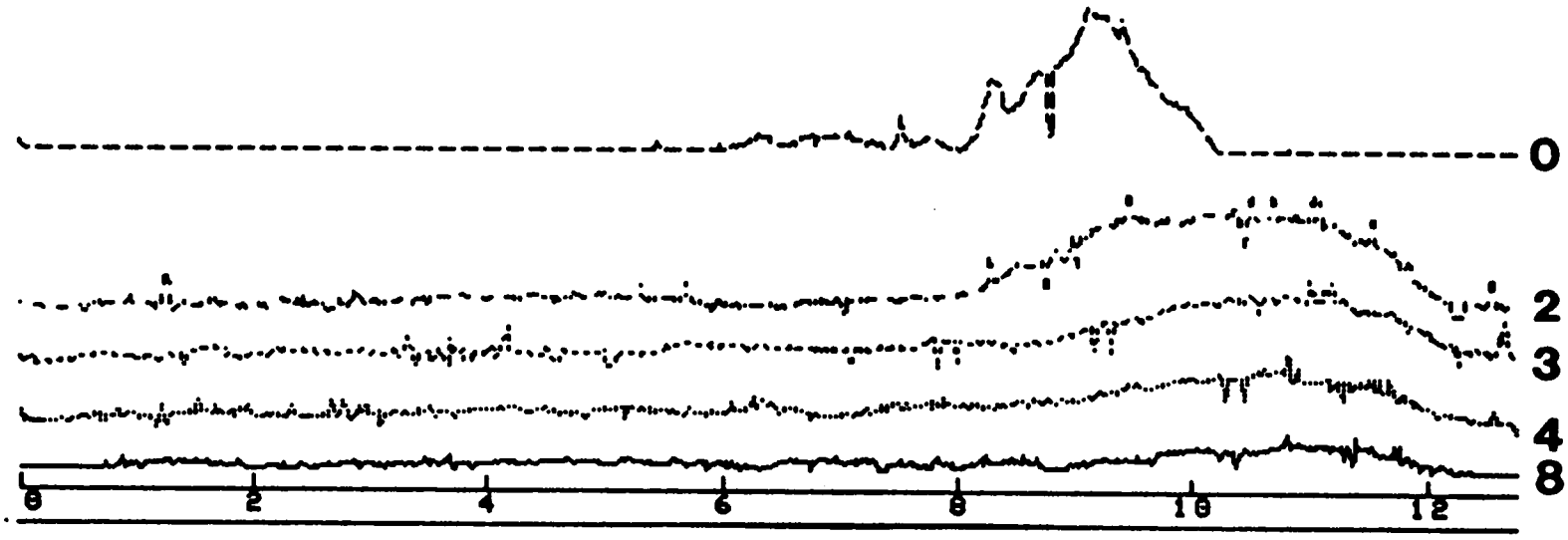
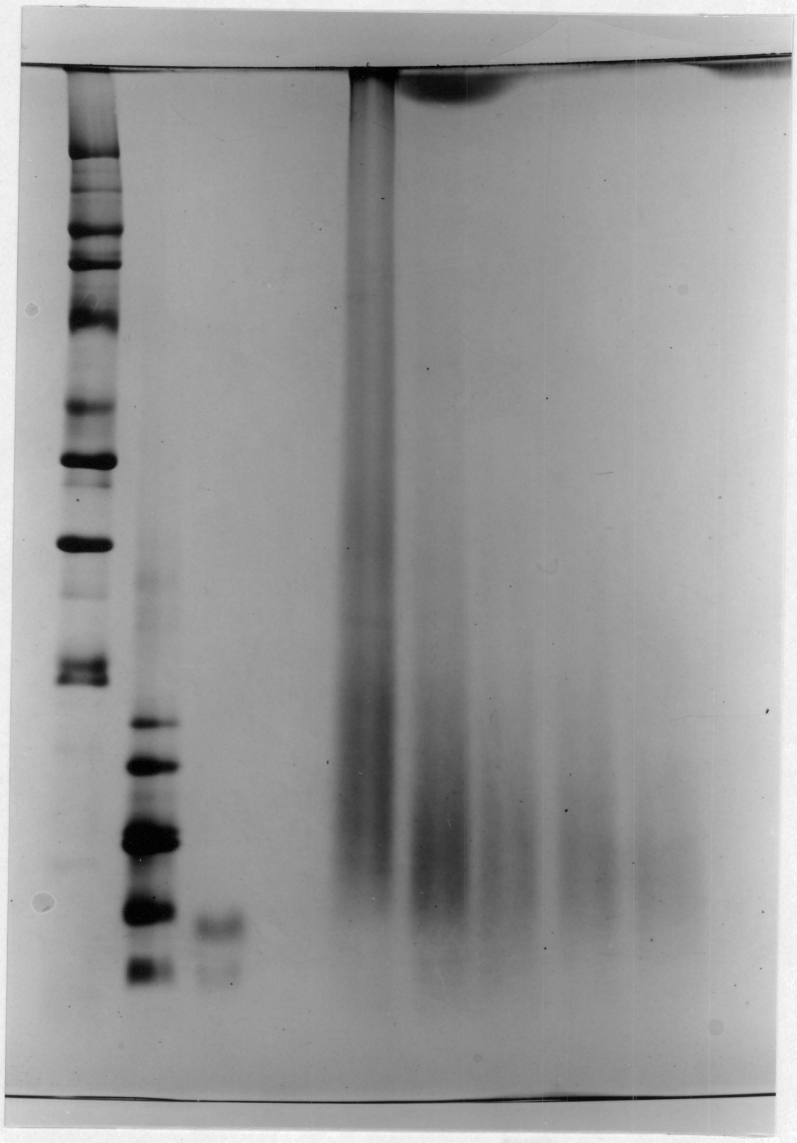


Figure 14. **Densitometer Scans of Unhydrolyzed and Hydrolyzed 2 Hour Autoclaved SPC.**



A B C D E F G H

A:Reference Markers (Mw range:205,000 - 20,100)
B:Reference Markers (Mw range:16,950 - 2,510)
C:Reference Markers (Mw range:1,500 - 1,296)
D:SPC-4 hour Autoclaved:unhydrolyzed
E:SPC-4 hour Autoclaved:2 hour pepsin digest
F:SPC-4 hour Autoclaved:2 hour pepsin/1 hour pancreatin digest
G:SPC-4 hour Autoclaved:2 hour pepsin digest/2 hour pancreaticn digest
H:SPC-4 hour Autoclaved:2 hour pepsin digest/6 hour pancreatin digest

Figure 15. SDS-PAGE Gel of the Unhydrolyzed and Hydrolyzed 4 Hour Autoclaved SPC.

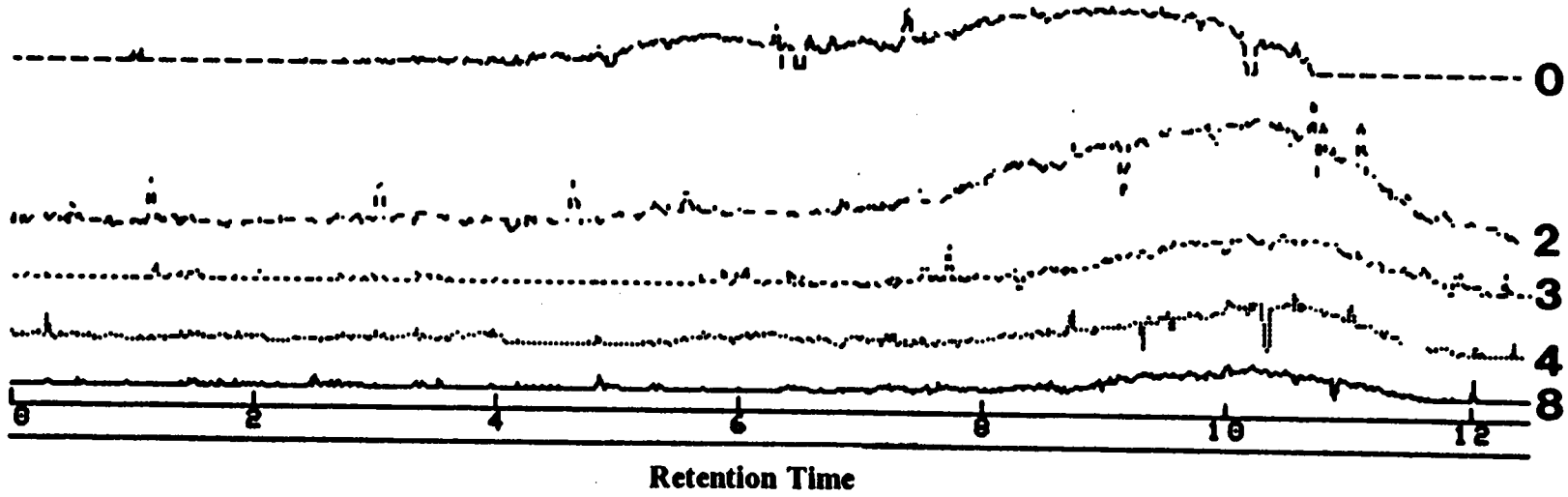


Figure 16. **Densitometer Scans of Unhydrolyzed and Hydrolyzed 4 Hour Autoclaved SPC.**

SPC hydrolyzates are given in Table 8. The molecular weights of control, 10 and 30 min. autoclaved samples before enzyme hydrolysis ranged from 115,000 - 8,200 daltons. The molecular weights of intact 2 and 4 hr. autoclaved samples were difficult to determine because of smeared bands. The apparent molecular weights of SPC hydrolysates after 2 hr. pepsin digestion were less than 28,000, 28,000 and 19,000 for the control, 10 and 30 min. autoclaved samples, respectively. The molecular size of polypeptides/peptides in these samples continued to decrease during enzymatic hydrolysis. The molecular weights of polypeptides/peptides remaining after 8 hr. hydrolysis in control, 10 and 30 min. autoclaved samples were less than 8,400, 14,00 and 16,000 daltons, respectively. The molecular weights of polypeptides remaining in 2 and 4 hr. autoclaved samples following pepsin digestion were less than 49,000 and 35,000 daltons respectively; and continued to decrease during digestion resulting after 8 hr. in remaining polypeptides with estimated molecular weights less than 17,000 and 13,000 daltons.

The minimum molecular weight of 8 hr. SPC hydrolysates was estimated to be approximately 1,300 daltons, based on the assumption that enzyme hydrolysates larger than Angiotensin 1 (Mw = 1,296) were resolved on gel. In this study, angiotenisn II (Mw = 1,046) was not resolved on gels. The molecular weight distribution of polypeptide/peptide fragments remaining after 9 hr. tryptic hydrolysis of glycinin were

Table 8. Estimated Molecular Weight of Polypeptides Remaining in Enzyme Hydrolysates of SPC as a Function of Time

	Autoclaving Time,min				
Hours Digs	0	10	30	120	240
2	> 28,000	> 28,000	> 19,000	> 49,000	> 35,000
3	> 22,000	> 23,000	> 19,000	> 37,000	> 33,000
4	> 15,000	> 16,000	> 15,000	> 20,000	> 16,000
8	> 8,400	> 14,000	> 16,000	> 17,000	> 13,000

reported to be between 30,000 and 7,000 and below 5,000daltons (Lynch et al., 1977). Landmann et al. (1985) has also separated large peptides from the in vitro digestion of wheat gluten. The molecular weights of peptides in undigested gluten ranged from 700 to 2,000 daltons.

One of the objectives of this study is quantitative analysis of in vitro protein digestibility using a modified SDS-PAGE system of Fling and Gregerson (1982) based on densitometric analysis of protein stained with Coomassie Brilliant Blue R-250 dye. The major sources of errors in quantitative densitometry are variable backgrounds and the staining property of the dye.

In the determination of digestibility of SPC samples by the SDS-PAGE method, the optical density of Coomassie Brilliant Blue R-250 stained gel bands was integrated by constant comparison to the background value as zero. Some difficulties were encountered in setting the backgound zero value due to smeared protein bands throughout a lane, which were observed with 2 and 4 hr. autoclaved samples (see Figure 13, 15). There is also variation in the amount of Coomassie Brilliant Blue R-250 dye-bound from one protein to another (Fishbein, 1972). Since any change in molecular structure after hydrolysis by enzymes and/or severe heat treatment can influence the dye binding capacity to protein, differences in dye binding capacity can in turn affect quantification of peak areas.

Mean percentage *in vitro* digestibility of the SPC samples as determined by SDS-PAGE are given in Table 9. There were significant differences in the mean digestibility values of the five samples as determined by Duncan's Multiple Range Test ($p < 0.05$). The highest digestibility was observed with control followed by 10 and 30 min. autoclaved samples. Lower digestibilities were observed with 2 and 4 hr. autoclaved samples. One way to evaluate this SDS-PAGE method as an *in vitro* digestion assay is to compare the digestibility values based on densitometric scans of SDS-PAGE with *in vivo* apparent digestibility. This comparison is shown in Table 10, along with predicted values of apparent digestibility based on a linear regression model. Digestibility values obtained with SDS-PAGE were higher for control, 10 and 30 min. autoclaved samples and lower for 2 and 4 hr. autoclaved samples than *in vivo* digestibility. These results indicate that *in vitro* digestibility determined by SDS-PAGE is not consistent with *in vivo* digestibility results for all SPC samples. But the predicted value of *in vitro* digestibility based on the linear regression model shown in Table 10 is impressively close to the *in vivo* digestibility.

5.5 *In Vitro* Digestibility Determined by the Simultaneous Dialysis Method.

All the other *in vitro* methods for estimating protein

Table 9. The Effect of Autoclaving on In-Vitro Digestibility of Soy Protein Concentrate as Determined by SDS-PAGE

Autoclaving Time (min)	Digestibility (%)
0	$98.39^{a} \pm 0.26$
10	$93.58^{b} \pm 1.18$
30	$87.42^{c} \pm 0.40$
120	$63.91^{d} \pm 3.04$
240	$59.72^{d} \pm 8.89$

1. Mean percent in-vitro digestibility with standard deviation of three trial runs.
abcd. Means with the same superscript are not significantly different($p < 0.05$)

Table 10. Comparison of Actual and Predicted In-Vitro Digestibility Determined by SDS-PAGE with Apparent In-Vivo Digestibility

actual value	predicted value[1]	apparent digestibility
98.32	85.82	86.46
93.58	85.26	84.82
87.42	84.54	84.0
63.91	81.77	83.03
59.72	81.28	80.36

1. Predicted value based on linear regression model of $y = 0.12x + 74.26$

digestibility employed in this study were carried out in a closed system which may result in the inhibition of enzymatic hydrolysis due to the accumulation of digested products over time. In 1982, Gauthier et al. introduced simultaneous dialysis method for determining in vitro digestibility of proteins. Using this in vitro method protein digestibility is calculated from the amount of nitrogen containing compounds which dialyze through a membrane during the course of protein hydrolysis. A dialysis membrane with a molecular weight cut-off of 1,000 was selected to resemble in vivo conditions in the human gastrointestinal tract where the end products of digestion range from free amino acids to hexa-peptides.

One of the objectives of this study was to study the feasibility of using the SDS-PAGE system of Fling and Gregerson (1986) for estimating in vitro protein digestibility. Quantitative analysis of in vitro protein digestibility using SDS-PAGE is based on densitometric analysis of proteins stained with Coomassie Brilliant Blue R-250 dye. However, a major difficulty in quantitation of proteins with a SDS-PAGE gel is variation in the amount of dye bound from one protein to another. The molecular weight cut-off of the dialysis bag (1,000 dalton) used in Gauthier's system is similar to the minimum molecular weight which can be resolved on the SDS-PAGE system of Fling and Gregerson,i.e., 1,300. Thus, the quantitation of protein hydrolyzates based on densitometric scans of SDS-PAGE was

evaluated by comparison to estimates of *in vitro* protein hydrolysis based on the simultaneous dialysis method of Gauthier et al. (1982).

The mean percentage *in vitro* protein digestibility of SPC samples at 8 hr. determined by simultaneous dialysis method are shown in Table 11. Mean digestibility values ranged from 43.69 to 48.93%, with no significant differences among SPC samples. Digestibility values determined by this method were quite low compared to results obtained by other methods employed in this study.

One reason for lower *in vitro* digestibilities determined by the simultaneous dialysis method than the TCA soluble N measurement and SDS-PAGE method, where there was no removal of digestive products, could be attributed to differences in experimental conditions of the dialysis method modified in the present study from those of Gauthier et al. One of the major differences in dialysis conditions employed in this study and that used by Gauthier et al. is the frequency of buffer replacement during protein digestion. In this study, protein digestion was terminated after 8 hr. in order to be consistent with conditions of the SDS-PAGE system, and dialysates were replaced every 2 hr. Gauthier's group recommends a 24 hr. period for protein hydrolysis with continuous buffer replacements using a peristalic pump. The importance of continuously replacing dialysates with phosphate buffer was emphasized in their study. They found that when

Table 11. The Effect of Autoclaving on In-Vitro Digestibility of Soy Protein Concentrate as Determined by Simultaneous Dialysis[1]

Autoclaving Time (min)	Digestibility (%)
0	$48.93^{a} \pm 6.16$
10	$46.17^{a} \pm 5.26$
30	$49.29^{a} \pm 5.47$
120	$45.15^{a} \pm 6.11$
240	$43.69^{a} \pm 5.93$

1. Mean percent in-vitro digestibility with standard deviation of three trial runs.
a. Means with the same superscript are not significantly different($p < 0.05$)

dialyzates were infrequently replaced, the digestion of soyprotein never exceeded 50%, but increased to 70% after changing the buffer continuously. In a closed *in vitro* system, accumulated digestion products could cause a feed back inhibition which slows down the rate of enzyme hydrolysis. While *in vivo*, digested products are continuously absorbed through intestine. Therefore, increasing the frequency of buffer replacement will produce an *in vivo* like situation increasing the enzymatic hydrolysis rate.

The low digestibility of SPC samples obtained with simultaneous dialysis in this study may be in part also due to the short digestion period. As digestion proceeds, the solute concentration inside the dialysis bag will increase due to the accumulation of digested protein products. The higher solute concentration inside the dialysis bag will result in higher osmotic pressure, thus decreasing the rate of dialysis through the bag. Therefore, it was considered that 24 hr. assay period carried out by Gauthier et al. may be necessary.

Considering the differences in digestibilities due to conditions employed in each *in vitro* study, it seems difficult to compare the digestibility obtained from simultaneous dialysis method to the digestibility obtained with SDS-PAGE system, even though the molecular weight cut-off of dialysis bag is similar to minimum molecular weight of peptides resolved by the SDS-PAGE.

5.6 *In Vitro* Digestibility Determined by the pH Drop Method

The multi-enzyme technique for determining *in vitro* protein digestibility was developed by Hsu et al. (1977), and modified by Satterlee et al. (1979). This method is based on the pH drop during digestion of an aqueous protein suspension at 37° C, pH 8, subjected to a sequence of multienzymes (a combination of trypsin, alpha-chymotrypsin and peptidase) and protease. As the enzymes hydrolyze peptide bonds within the protein's primary structure, the free carboxyl groups that are formed immediately ionize and release protons, which in turn lowers the pH of the protein suspension (Satterlee et al., 1979, 1982). The pH drop of control and autoclaved SPC samples was recorded following a 20 min. digestion period using the multienzyme system.

In vitro digestibilities of SPC samples calculated based on pH drop are shown in Table 12. The mean percent *in vitro* digestibility of the five SPC samples ranged from 84% to 89%. The SPC control sample had the highest mean percent *in vitro* digestibility followed by 10, 30 min., 2 and 4 hr autoclaved samples. There were significant differences in the mean values of the five samples as determined by Duncan's Multiple Range Test ($p < 0.05$). The mean percent *in vitro* digestibility value of the SPC control was significantly higher than those of autoclaved samples. And the digestibilities of autoclaved samples were significantly

Table12. The Effect of Autoclaving on In-Vitro Digestibility of Soy Protein Concentrate as Determined by pH Drop[1]

Autoclaving Time (min)	Digestibility (%)
0	$89.36^{a} \pm 0.21$
10	$88.15^{b} \pm 0.20$
30	$85.86^{c} \pm 0.06$
120	$85.03^{d} \pm 0.08$
240	$83.79^{e} \pm 0.37$

1. Mean percent in-vitro digestibility with standard deviation of three trial runs.
abcde. Means with the same superscript are not significantly different ($p < 0.05$).

different from each other.

The basic concept of the pH drop method is that the change in pH upon protein hydrolysis depends directly on the release of free carboxyl groups after enzymatic hydrolysis of peptide bonds. However, Mozersky and Panettieri (1983) reported that the extent of protein hydrolysis does not directly correlate with digestibility obtained using pH drop method. The extent to which some protein substrates were hydrolyzed was found to be much lower than calculated digestibility based on pH drop. The difference in pH drop and extent of protein hydrolysis was attributed to other factors such as the number and pKa values of amino groups released by proteolysis and exposed buffering side chains initially buried in the unhydrolyzed protein. Even though, Hsu et al. (1977) reported a high correlation between in vivo studies and the pH drop method, Barbeau and Kinsella (1985) questioned whether digestibility determined by pH drop could be lower than actual in vivo digestibility. Since the amino groups released by proteolysis buffer in the pH range 7-8, as more proteolysis take place, the pH change is less than expected.

In this study, the in vitro digestibilities of the SPC samples determined by pH drop method were slightly higher than in vivo apparent digestibility. The digestibilities of 2 and 4 hr. autoclaved samples were also higher than values obtained by other in vitro methods (TCA soluble N measurement and SDS-PAGE method). The reason for these high digestibility values

may be due to the solubility of 2 and 4 hr. autoclaved samples. The solubility of 2 and 4 hr. autoclaved samples in potassium phosphate buffer was higher than control, 10 and 30 min. autoclaved samples (see Table 5). Since the pH drop measures proteolysis for only 20 min., enzymes may at least more rapidly attack soluble protein in 2 and 4 hr. autoclaved samples. The other possible reason for higher digestibilities of 2 and 4 hr. autoclaved SPC samples could be attributed to different enzyme systems. Since the pH drop method included peptidase and bacterial protease in addition to pancreatic enzymes, the small molecular weight peptides existing in 2 and 4 hr. autoclaved samples could be more hydrolyzed than in other *in vitro* systems.

5.7 Apparent *In Vivo* Digestibility Determined by Rat Bioassay

Digestibility is often used as an indicator of protein bioavailability. The apparent digestibilities of rats fed either autoclaved SPC samples or ANRC casein were determined by measuring total fecal N and total dietary N, for a period of 8 days during test days 18 - 26. This study did not include a group of animals fed a protein free diet. Therefore, the amounts of endogeneous N, contributed by protein turnover (sloughed mucosal cells and digestive enzymes from the gastrointestinal tract) were not accounted for. In our case, endogeneous N was measured as part of the fecal

nitrogen. Thus, the true digestibility of SPC samples and ANRC casein which takes into account endogeneous N will be higher than apparent digestibility.

The apparent in vivo digestibilities of ANRC casein and the SPC samples are given in Table 13. The apparent digestibility of the SPC control is 86.46% which is close to a literature value of 88.85% for soy protein (Keith and Bell, 1988) and 86.2% (rios Iriarte and Barnes, 1966). The reported true digestibility of soy protein is 95.8% (Keith and Bell, 1988).

Digestibility of soy protein depends in part on the extent of protein denaturation. The three dimensional structure of native soy protein is quite compact and not hydrolyzed by proteinase without disruption of the internal structure (Fukushima, 1968). Mild heat treatment modifies protein structure in such a way as to make peptide bonds more susceptible to cleavage by digestive enzymes (Kakade, 1974) and increases the liberation of sulfur-containing amino acids (Evans and McGinnis, 1946). The digestibility of SPC control significantly decreased after autoclaving. For soy proteins, a mild heat treatment is also necessary to inactivate trypsin inhibitors. Since the SPC control previously received a mild heat treatment at the Central Soya company (Harper, 1989), lower digestibility after autoclaving indicates that further heat treatment caused changes in the protein conformation of the SPC control, thus decreasing the rate of protein

Table 13. The Effect of Autoclaving on Apparent In-Vivo Digestibility of Soy Protein Concentrate as Determined by Rat Bioassay

Treatment	Protein Digestibility (%)
Casein	$90.78^{a} \pm 1.38$
Autoclaving Time (min)	
0	$86.46^{b} \pm 1.27$
10	$84.82^{c} \pm 0.93$
30	$84.00^{c} \pm 1.51$
120	$83.03^{c} \pm 2.47$
240	$80.36^{d} \pm 2.31$

1. Data are reported as mean ± std. dev. of values for ten animals.
abcd. means with the same superscript are not significantly different ($p < 0.05$).

hydrolysis.

Although the digestibility of soy protein after heat treatment depends on the soy protein sample, temperature, time of heating and moisture content, etc., previous studies showed decreases in digestibility after severe heat treatment (Rios Iriarte and Barnes, 1946, Evans and McGinnis, 1948, Riesen et al., 1969, Taira et al., 1969). However, in this study, there were no differences in the in vivo apparent digestibilities of the 10 min., 30 min., and 2 hr. autoclaved samples, but the apparent digetibility of 4 hr. autoclaved sample was significantly less than the other SPC samples. This result indicates that cross-linkage formation and/or isopeptide formation may occur in SPC during 4 hr. autoclaving.

Evans and Butts (1949) and Evans et al. (1951a) reported inactivation of basic amino acids, aspartic acid and glutamic acid via formation of isopeptide bonds in soy protein autoclaved for 4 hr. During severe heat treatment at neutral pH, a beta-elimination reaction starts with cysteine forming dehydroalanine (DHA), DHA then condenses with lysine through cross-linkage formation (Cheftel, 1977, Dworschak, 1980). Since the pH of a water dispersion of the SPC sample was 7.0, lysinoalanine formation is possible during 4 hr. of autoclaving. Isopeptide formation eg. glutamyl-lysine and aspartyl-lysine and/or lysinoalanine formation can attribute either to a reduction in protein digestibility or to decreases in the availability of lysine. Since essential amino acids

such as lysine and cysteine are involved in these reactions, decrease in availability of these essential amino acids will further decrease the efficiency of protein synthesis.

One of the objectives of this study was to evaluate the effect of autoclaving on the *in vitro* digestibility of SPC samples as determined by three different digestion methods of assay. The three *in vitro* digestion assays were TCA soluble N measurement, SDS-PAGE and the pH drop method. In order to compare results from each of these *in vitro* assays with apparent *in vivo* digestibility, relative differences between *in vitro* digestibility and mean apparent digestibility were calculated as follows:

$$\text{Relative difference} = \frac{(\textit{In vitro}\ \text{result}) - (\textit{In vivo}\ \text{result})}{\textit{In vivo}\ \text{result}}$$

Results of these calculations are shown in Table 14 - 18. A value with plus sign indicates that the digestibility obtained by *in vitro* assay is higher than that obtained by *in vivo* assay. The reverse is true for any value with a minus sign. In these tables, any *in vitro* digestiblity with a relative difference of ± 0.05 was consider ed to be close to apparent *in vivo* digestibility.

There were large relative differences in the *in vitro* and *in vivo* digestibilities of the 2 and 4 hr. autoclaved SPC samples as determined by TCA soluble N measurement. However, the digestibilities of control, 10 and 30 min. autoclaved SPC

Table 14. Relative Difference between Mean In-Vitro Digestibility and Mean Apparent In-Vivo Digestibility at 0 Min. Autoclaving

	TCA soluble N measurement[1]	SDS-PAGE	pH drop	Rat
Mean	87.53	98.32	89.36	86.46
Relative[2] Difference	+ 0.012	+ 0.14	+ 0.034	

1. Data excludes TCA soluble N in unhydrolyzed SPC sample.
2. Where Relative Differences (RD) = $\frac{(In-VitroResult)-(In-VivoResult)}{In-VivoResult}$

Table 15. Relative Difference between Mean In-Vitro Digestibility and Mean Apparent In-Vivo Digestibility at 10 Min. Autoclaving

	TCA soluble N measurement[1]	SDS-PAGE	pH drop	Rat
Mean	80.06	93.58	88.15	84.82
Relative[2] Difference	- 0.056	+ 0.10	+ 0.039	

1. Data excludes TCA soluble N in unhydrolyzed SPC sample.
2. Where Relative Differences (RD) $= \frac{(In-VitroResult)-(In-VivoResult)}{In-VivoResult}$

Table 16. Relative Difference between Mean In-Vitro Digestibility and Mean Apparent In-Vivo Digestibility at 30 Min. Autoclaving

	TCA soluble N measurement[1]	SDS-PAGE	pH drop	Rat
Mean	82.08	87.42	85.86	84.00
Relative[2] Difference	- 0.023	+ 0.041	+ 0.022	

1. Data excludes TCA soluble N in unhydrolyzed SPC sample.
2. Where Relative Differences (RD) = $\frac{(In-VitroResult)-(In-VivoResult)}{In-VivoResult}$

Table 17. Relative Difference between Mean In-Vitro Digestibility and Mean Apparent In-Vivo Digestibility at 120 Min. Autoclaving

	TCA soluble N measurement[1]	SDS-PAGE	pH drop	Rat
Mean	72.81	63.91	85.03	83.03
Relative[2] Difference	- 0.123	- 0.230	+ 0.024	

1. Data excludes TCA soluble N in unhydrolyzed SPC sample.
2. Where Relative Differences (RD) = $\frac{(In-VitroResult)-(In-VivoResult)}{In-VivoResult}$

Table 18. Relative Difference between Mean In-Vitro Digestibility and Mean Apparent In-Vivo Digestibility at 240 Min. Autoclaving

	TCA soluble N measurement[1]	SDS-PAGE	pH drop	Rat
Mean	67.08	59.72	83.79	80.36
Relative[2] Difference	- 0.165	- 0.257	+ 0.043	

1. Data excludes TCA soluble N in unhydrolyzed SPC sample.
2. Where Relative Differences (RD) = $\frac{(In-VitroResult)-(In-VivoResult)}{In-VivoResult}$

Table 19. Linear Regression and Correlation Coefficients for Mean In-Vitro Digestibilities and Mean Apparent In-Vivo Digestibility

In-Vitro Method	Slope	Intercept	r
TCA soluble N measurement	0.27	67.76	0.96
SDS-PAGE	0.12	74.26	0.92
pH drop	0.94	2.53	0.95

samples were closer to the corresponding *in vivo* digestibility. Determination of relative differences showed a high discrepancy between digestibility determined by SDS-PAGE and apparent *in vivo* digestibility. The *in vitro* digestibility values obtained by pH drop method were slightly higher than apparent *in vivo* digestibilities. Relative differences in digestibility determined by pH drop and apparent *in vivo* digestibility shown in Table 14 -18 are less than 0.05.

The correlation coefficients of *in vitro* digestibility with apparent *in vivo* digestibility are shown in Table 19. All three *in vitro* methods correlated well with apparent *in vivo* determined in rats. Among the *in vitro* methods employed in this study, results of the pH drop method were not only the closest to apparent *in vivo* digestibility but also highly correlated with apparent *in vivo* digestibility of SPC samples, $r = 0.95$.

5.8 Amino Acid Composition

Simple theories concerning protein quality are based on the assumption that the efficiency of protein utilization depends primarily on the relative concentration of limiting essential amino acids. Therefore, amino acid analysis is a basic step in evaluating the nutritive value of a protein.

The amino acid contents of control and autoclaved SPC

samples were determined and the results are shown in Table 20. A value reported by Central Soya (Harper, 1989) was used for the tryptophan content of SPC samples, since tryptophan is destroyed during acid hydrolysis. Lysine has been reported to be much more sensitive to heat destruction than tryptophan (Evans and Butts, 1951). Since there was no apparent decrease in lysine after 2 and 4 hr. autoclaving, it was assumed that there would be no heat destruction of tryptophan in this study. Thus, the same trytophan value was used for control and autoclaved samples.

Essential amino acid values obtained from amino acid analysis are sometimes inaccurate, since destruction of certain amino acids may occur during acid hydrolysis. Generally, the amino acid contents of the 30 min., 2 and 4 hr. autoclaved samples were higher than control and 10 min. autoclaved samples. However, the overall % difference in amino acids from the values reported by Central Soya (Table 21) were less than 10% with the exception of valine and cysteine in the control, 30 min., 2 and 4 hr. autoclaved samples. The cysteine content in the control was 22% less than the value provided by company. Even though the cysteine content of SPC samples was analyzed by a special acid hydrolysis procedure following performic acid oxidation (Moore, 1958), the lower cysteine value indicated that there still may have been destruction of cysteine during acid hydrolysis. The higher % deviation in valine content was

Table 20. Amino Acid Pattern of Control and Autoclaved Soy Protein Concentrates[1]

Amino Acid [1]	Treatment Autoclaving Time, min.					Lit. Value[a]
	0	10	30	120	240	
Aspartic	10.54	9.84	12.43	12.97	12.81	
Glutamic	18.74	18.44	21.46	22.19	22.38	
Serine	4.86	4.81	5.61	5.59	5.59	
Glycine	4.05	4.26	4.32	4.52	4.55	
Histidine	2.67	1.99	2.56	2.65	2.71	2.7
Arginine	5.99	5.99	7.36	7.59	7.0	
Proline	5.21	6.13	4.46	5.12	5.30	
Alanine	4.38	4.48	4.54	4.67	4.64	
Threonine	3.72	3.59	4.23	4.25	4.20	4.1
Tyrosine	3.63	3.66	3.80	3.94	3.77	3.9
Valine	4.93	4.31	4.52	5.00	4.83	5.6
Methionine	1.35	1.24	1.33	1.44	1.38	1.4
Cysteine	1.09	1.03	0.87	0.80	0.67	1.4
Isoleucine	4.48	4.15	4.69	4.92	4.92	4.8
Leucine	7.71	7.40	8.11	8.40	8.38	8.1
Phenylalanine	4.87	5.07	5.27	5.44	5.24	5.1
Lysine	6.11	5.62	6.11	6.10	5.85	6.7
Tryptophan	1.4	1.4	1.4	1.4	1.4	1.4

1. Grams of amino acid/100 grams of protein
a: Reference pattern, Harper, 1989

Table 21. Percentage Difference of Each Essential Amino Acid in Differently Autoclaved SPCs from Reference Protein

	Autoclaving Time, min					
Amino Acid	Reference Protein	0	10	30	120	240
Threonine	4.1	- 9.27	- 12.44	+ 3.17	+ 3.66	+ 2.44
Tyrosine	3.9	- 6.92	- 6.15	- 2.56	+ 1.03	- 3.33
Valine	5.6	- 11.96	- 23.04	- 19.29	- 10.71	- 13.75
Methionine	1.4	- 3.57	- 11.43	- 0.05	+ 2.85	- 1.43
Cysteine	1.4	- 22.14	- 26.43	- 37.86	- 42.86	- 52.14
Isoleucine	4.8	- 6.67	- 13.54	- 2.29	+ 2.5	+ 2.5
Leucine	8.1	- 4.81	- 8.64	+ 0.12	+ 3.7	+ 3.46
Phenylalanine	5.1	- 4.5	- 0.59	+ 3.33	+ 6.67	+ 2.74
Lysine	6.7	- 8.81	- 16.12	- 8.81	- 8.96	- 12.69

probably due to an unexpectedly high value reported by company. The valine content reported by company was 5.6 g/100 g protein, while Waggle and Kolar (1978) reported a valine content of 4.9 g/100 g protein. The % difference of threonine, methionine, isoleucine and lysine in 10 min. autoclaved sample from those reference values were 12, 11, 13 and 16%. The higher deviations observed with these amino acids in the 10 min. autoclaved sample compared to other samples might be due to the general errors in procedure or difficulties in analyzing these amino acids.

Overall, the amino acid pattern showed that there was no decrease in amino acid content due to autoclaving except for cysteine. Previous investigators have demonstrated that severe autoclaving results in some heat destruction of amino acids. Significant amounts of cysteine, lysine and arginine and smaller amounts of tryptophan, histidine and serine may be destroyed by heat treatment (Riesen et al., 1947, Evans and Butts, 1951, Evans et al., 1961, rios Iriarte and Barnes, 1966, Taira et al., 1965). One of the reasons why there were apparently no losses of basic amino acids in this study may be attributed to the amount of carbohydrates in soy protein concentrate. Sucrose, stachyose, arabans, galactans and dextrin are the principal carbohydrates present in soybeans. Evans et al. (1951) reported that three types of heat inactivation of the basic amino acids and tryptophan occurred when soybean meal was autoclaved for 4 hours at 15 p.s.i.g.

pressure. The three types were 1) the actual destruction of amino acids caused by a reaction of the protein-bound amino acid with either sucrose or glucose formed from sucrose during autoclaving, 2) inactivation of protein bound amino acids by reaction with other constituents of the protein to form a linkage resistant to enzymatic hydrolysis 3) inactivation of protein bound amino acids by reaction with sucrose or glucose to form a linkage resistant to enzymatic hydrolysis. Lysine was inactivated by 1 and 2 type reactions. Arginine inactivation was by types 1 and 3. Histidine was by all three types and tryptophan was types 1 and 3. Since the linkages formed by types 2 and 3 can be hydrolyzed by acid, the difference in amino acid content before and after heat treatment would only account for destruction caused by Maillard Browning. Previous studies have investigated heat destruction of amino acids in defatted soybean flour and soybean oil meal. Considering the fact that SPC has a lower carbohydrate content than defatted soybean flour (see Table 1) and defatted soybean meal, there would tend to be less actual detruction of these basic amino acids in SPC than in defatted soybean flour or meal.

The relative ranking of essential amino acids in a protein is not really used in determining its nutritive value, since the efficiency of protein utilization depends primarily on the first limiting amino acid. Chemical score introduced by Mitchell and Block (1946) is a method to assess the

nutritive quality of a protein based on its constituent amino acids. They suggested that since all amino acids must be present in adequate amounts for protein synthesis to proceed, equal deficits of any two essential amino acids would limit protein synthesis to a comparable degree. The chemical score of each essential amino acid was calculated using a standard protein (whole egg protein). The chemical score for an individual protein is expressed as a fraction of unity, defined as: (mg of amino acid per g of test protein/mg of amino acid per g protein in reference protein) x 100. The essential amino acid limiting the nutritive efficiency of protein was the amino acid whose % deficit from that of the whole egg was the greatest.

Methionine is the first limiting amino acid in soy protein. However, cysteine becomes limiting in heated soy protein since it is sensitive to heat, while methionine content does not change due to heating (Rios Iriarte and Barnes, 1966). The chemical scores of SPC samples are shown in Table 22. Methionine was first limiting amino acid for the control, 10 and 30 min. samples. While cysteine became the limiting amino acid in 2 and 4 hr. autoclaved samples. Considering that methionine may be converted to cysteine but the reverse reaction does not occur, sulfur containing amino acids (methionine and cysteine) were considered as limiting in 2 and 4 hr. autoclaved samples.

The level of sulfur containing amino acids in autoclaved

Table 22. Chemical Score of Each Essential Amino Acid in Control and Autoclaved SPCs

	Chemical Score				
	Autoclaving Time, min				
Amino Acid	0	10	30	120	240
Arginine	94	94	115	119	109
Histidine	127	95	122	126	129
Lysine	85	78	85	85	81
Tyrosine	81	81	84	88	84
Phenylalanine	129	80	84	86	83
Tryptophan	93	93	93	93	93
Cysteine	45	43	36	33	28
Methionine	33	30	32	35	34
$M^1 + C^2$	38	35	34	34	32
Threonine	76	73	86	87	86
Leucine	84	80	88	91	91
Isoleucine	56	52	59	62	62
Valine	68	59	62	68	66

Amino acid pattern of whole egg protein was used as reference.
1. Methionine
2. Cysteine

SPC samples did not correlate well with PER (r = 0.68). This result was consistent with previous studies (Kakade et al., 1972, Lowe et al., 1985). However, the r value between cysteine content and PER was 0.85, indicating that cysteine content is one of the factors involved in determining the nutritive value of soybean protein. It is interesting to note that there was a sharp decrease in cysteine content of the 10 min. autoclaved sample. This result might indicate that cysteine content may drastically decrease even after mild heating. Therefore, soy manufacture should pay more attention to the cysteine content of heated soy protein.

5.9 PER Determined by Rat Bioassay

PER is the oldest and one of the easiest in vivo methods for evaluating protein quality. In a PER study, changes in the body weight of young rats fed diets for a 28 day period time are considered to reflect changes in protein utilization. The PER procedure is simple because it only requires the determination of weight gain and food intake to evaluate protein quality. However, PER has been criticized as a method for determining protein quality (Pellet, 1978). The validity of any animal assay for evaluating protein quality depends on how closely the nutritional quality of protein can be quantified as a characteristic of a protein (The United Nations University, 1980). The PER method does not measure

amounts of protein used for maintenance, consequently PER values are not directly proportional to protein quality (Bender, 1956), thus making the PER procedure less valid for quantifying the nutritional quality of proteins. Despite its weak points, the PER method has been extensively used for evaluating protein quality since introduced by Osborne et al. (1919) and it is the officially accepted method for regulatory purposes within the U.S.

Chapman et al. (1959) suggested that a PER value of 2.5 be assigned to ANRC casein and that experimental PER values be corrected by multiplying values by 2.5/PER of reference ANRC casein. The use of ANRC casein as internal reference standard reduces the variation in PER values found in different assays. However, Hegarty (1975) reported that a PER value of 2.5 for the casein standard as suggested by Chapman et al.(1969) should be re-evaluated since PER values of casein obtained in the literature were higher than 2.5. The PER value of casein obtained in this study was also higher than 2.5 before correction. Corrected PER values of SPC samples based on a casein value of 2.5 are shown in Table 23. The PERs of control, 10 and 30 min. autoclaved samples were 2.42, significantly higher than 1.83 and 1.79 for 2 and 4 hr. autoclaved samples.

The beneficial effects of a mild heat treatment on the nutritive value of soybean proteins have been well established. PER values greater than 2.0 have been reported

Table 23. The Effect of Autoclaving on PER and Food Intake of Soy Protein Concentrate as Determined by Rat Bioassay[1]

Treatment	PER	Corrected PER[2]	Food Intake (g)
Casein	$2.69^{a} \pm 0.50$	2.50^{a}	$49.59^{a} \pm 3.83$
Autoclaving Time (min)			
0	$2.60^{a} \pm 0.16$	2.42^{a}	$48.40^{a} \pm 3.16$
10	$2.60^{a} \pm 0.38$	2.42^{a}	$46.55^{ab} \pm 5.31$
30	$2.60^{a} \pm 0.28$	2.42^{a}	$48.54^{a} \pm 3.39$
120	$1.97^{b} \pm 0.26$	1.83^{b}	$43.45^{cb} \pm 3.82$
240	$1.93^{b} \pm 0.20$	1.79^{b}	$40.46^{c} \pm 3.05$

1. Data are reported as mean ± std. dev. of ten animals
2. PER was corrected based on casein value of 2.5.

abc. Means with the same superscript are not significantly different ($p < 0.05$)

for raw soybean protein after proper heat treatment. Reported PER values of 0.34 to 1.86 for raw SPCs increased impressively after mild heat treatment to between 2.02 and 2.10 (Longenecker et al., 1964). Several workers have determined the PER of excessively heated soybean protein. The average PER values for samples roasted to 170, 180, and 185° C end points were 1.70, 1.46 and 1.28, respectively (Badenhop and Hackler, 1971). In another study, the PER value of moistened soy flour autoclaved at 120° C for 2 hr. was 1.4, while that of soy flour autoclaved at 120° C for 15 min. was 2.8 (Rios Iriarte and Barnes, 1966). Similar results were reported by Hackler et al (1965). Cooking soy milk 1-6 hr. at 93° C had no adverse effect on PER. In contrast, cooking for 32 min. at 121° C significantly decreased PER of the soy milk. Along with these previous studies, the significantly decreased PER values obtained with the 2 and 4 hr. autoclaved samples point out that prolonged heating may have a detrimental impact on the nutritional value of soy protein.

The biological utilization of protein based on animal assay largely depends on digestibility and amino acid composition of dietary protein. Although the apparent digestibility and the contents of sulfur containing amino acids of the control SPC was higher than those of 10 and 30 min. autoclaved samples, the PER of three samples were same. In an ad libitum feeding experiment, more individual variation within diet groups was observed when the diet was

fed ad libitum than when a moderated restriction was imposed in feed intake (Harte et al., 1947). Mitchell and Beadles (1930) also reported that ad libitum feeding exaggerates large differences and obscures small differences in protein quality. Since PER values do not measure allowances for maintenance, identical PER values for the control, 10 and 30 min. autoclaved samples indicates that these three SPC samples are in the same rank order of growth-promoting value, but do not necessarily have the same biological value. The rats in the experimental group fed 2 hr and 4 hr. SPC samples had the lower PER than control, 10 and 30 min. samples. The lower PER of 2 and 4 hr. autoclaved samples are attributed to lower digestibility and destruction of cysteine. Since the digestibility values of 83% and 80% for the 2 and 4 hr. samples were reasonably good, the poor PER values of 1.83 and 1.79 were probably more influenced by the destruction of cysteine than by protein digestibility.

Biological assays using animals can only measure the biological value of proteins, but they do not explain why a given protein's biological value change after heat treatment even though its amino acid composition and digestibility remain unchanged. The nutritional value of food, especially heated protein depends not only on its pattern of essential amino acid but also on the extent and rate of amino acid liberation. For optimum utilization of food proteins all essential amino acids must not only be available for

absorption but also be liberated during digestion in vivo at rates permiting mutual supplementation (Melnick et al., 1946). Experiments with unheated, heat-processed and overheated soy meal demonstrated that lysine and methionine levels in portal blood depend not only on their relative amounts in food proteins but also on their digestive release and absorption (Guggenheim et al., 1960). It was also observed that the pattern of released amino acids from properly heated and overheated defatted soybean flour differed during different stages of digestion (Taira et al., 1969).

The protein solubility data (see Table 5) and SDS-PAGE electrophoretic patterns of unhydrolyzed SPC samples (see Figure 6) indicated that major conformational changes occured in soy protein after 2 and 4 hr. autoclaving. Also the decreased enzymatic hydrolysis rates (see Table 6) of 2 hr. autoclaved samples due to conformational change may explain the lower PER observed with 2 hr. autoclaved sample. Therefore the reduction in the PER values of 2 and 4 hr. autoclaved samples may also be due to the decreased rate of enzymatic hydrolysis, irregular release of amino acids and availability of amino acids for absorption.

Any toxicity of modified protein due to severe heating could also result in decreases in PER. Lysinoalanine is not only resistant to enzymatic hydrolysis but also is known to be toxic to rats (Woodard and Short, 1973, van Beek et al., 1974, de Groot, 1976). In the rat, ingestion of proteins

containing lysinoalanine is often accompanied by diarrhea, pancreatic hyperplasia and loss of hair. It is impossible to relate the hair loss observed with rats fed on 2 and 4 hr. autoclaved samples to the toxic effects of lysinoalanine, since actual amounts of lysinoalanine were not measured in this study. However, hair loss could be used as an indicator of impaired nutritional status. Also severe heat treatment produce isomers of amino acids (Provansal et al.,1975, Friedman et al., 1981). Since most D-amino acids have no nutritive value, the nutritional value of essential amino acid is reduced to about 50 % after racemization.

Osborne et al. (1919) and Mitchell (1927) recognized that growth rate was influenced by food intake. Food intake was determined by measuring differences in offered and consumed food. In this study, the amount of spilled food was also determined to correct for errors in the calculation of consumed food. The food intake of rats fed autoclaved SPC samples are shown in Table 23. The food intake of rats fed 2 and 4 hr. autoclaved samples was significantly less than the other SPC samples. This reduced dietary intake may have caused some additional changes in the nutritional status of rats fed these two diets. The consequences of reduced food intake are deficiences in energy as well as specific nutrients, leading in extreme cases to malnutrition. Rats fed the 2 and 4 hr. autoclaved SPC samples looked distintively smaller than rats in the other experimental groups. One rat

in the 2 hr. group and three rats in the 4 hr. group lost hair the last week of the study. Rats in both groups did not eat much, and were much less active.

Food intake of animals is reported to depend on the protein content and the amino acid pattern of the diet (Knipfel 1981). Food intake of animals is depressed if a diet contains an unbalanced pattern of amino acids, i.e., one or more essential amino acids is high or low (Harper and Peters, 1989). Considering the drastic decrease in cysteine in 2 and 4 hr. autoclaved samples, it is possibile that a deficiency in sulfur amino acids to below requirement levels may have reduced food intakes. Low protein intakes for 28 days may cause severe protein deficiency. In an attempt to overcome protein deficiency, all metabolic reponses work to conserve amino acids. This is accompanied by an immediate cessation of growth, which may have been observed in some of the rats fed on 2 and 4 hr. autoclaved samples. The weight gain of rats in each experimental group during the 28 day assay period is depicted in Figure 17. The weight gain of rats fed the 2 and 4 hr. autoclaved SPC samples was less than rats consuming other SPC samples during the first 14 days of the study. In a condition of protein deficiency, although turnover of body protein continues, the rate of muscle protein synthesis slows down, and muscle protein degradation occurs to release protein for obligatory metabolic needs. The overall emanciation of body muscle due to protein deficiency can result in impairment

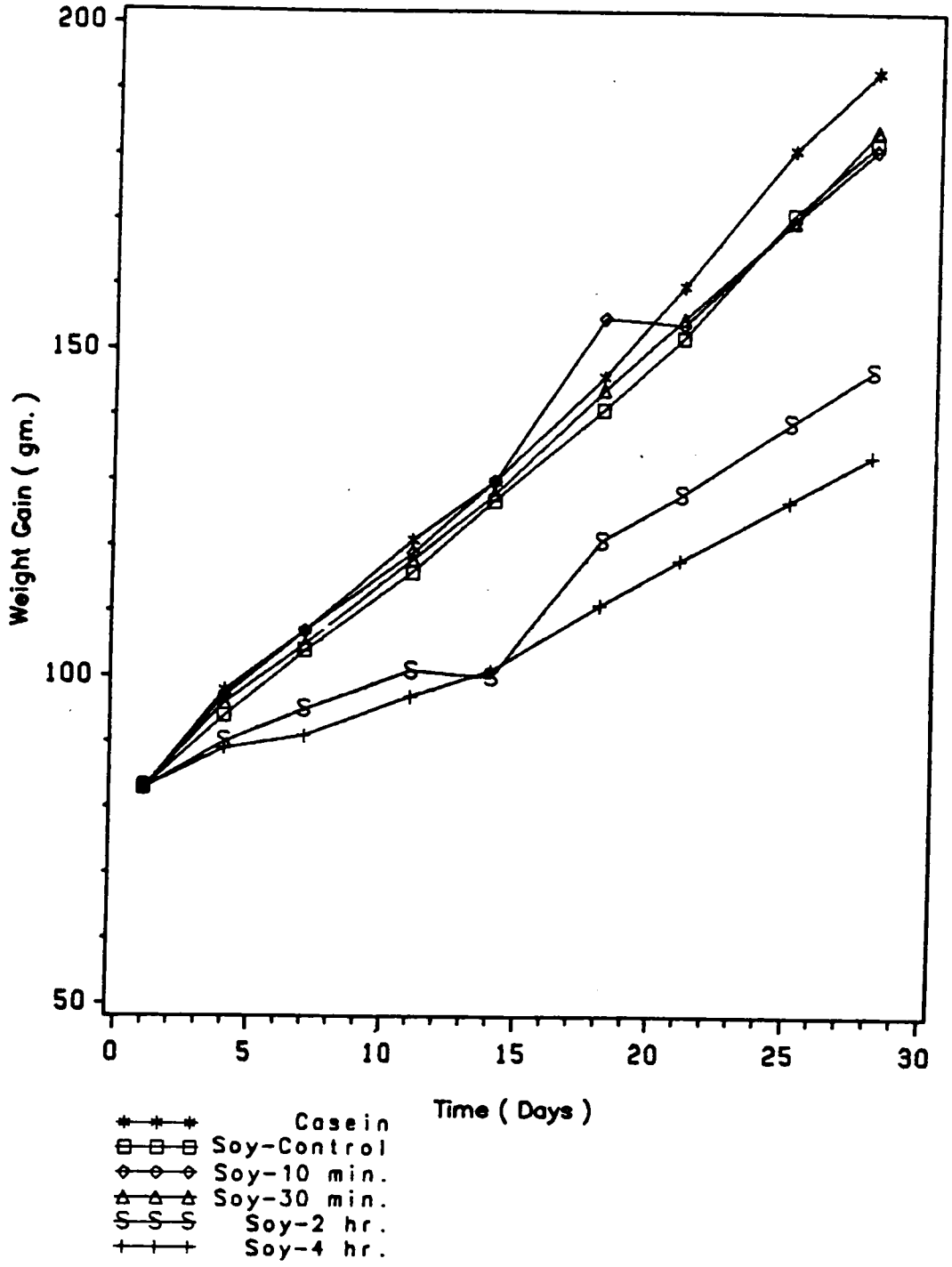


Figure 17. Weight Gain during 28 Days Rat Assay

of absorption in the small intestine. Therefore, it is possible that rats in the 2 and 4 hr. SPC groups suffered from a borderline protein deficiency, resulting in lower growth rates and significantly lower PERs.

5.10 C-PER

In recent years, FDA's nutrition labeling regulation have specified that labeling of the protein contribution of a product depends upon its quality as measured by PER. Since PER is a time-consuming and expensive bioassay procedure, there is demand for a cheap, rapid and reliable *in vitro* assay to replace the PER procedure.

The calculated protein efficiency ratio (C-PER) is a rapid assay developed by Satterlee et al. (1979). This assay combined *in vitro* protease digestibility obtained from pH drop method with essential amino acid composition to predict rat PER. PER was predicted for a variety of food proteins, and a high correlation coefficient, 0.90, was reported between C-PER and PER. Satterlee conducted a collaborative study of C-PER, which led to adoption of C-PER method by the AOAC in 1982 as official first action (Satterlee et al., 1982). *In vivo* digestibility obtained from pH drop (see Table 12) and essential amino acid composition (see Table 20) were used for the calculation of C-PER of autoclaved SPC samples. PER was predicted by adjusting the essential amino acid profile of the

sample protein and the reference casein for in vitro protein digestibility. Each essential amino acid is expressed as a percentage of the FAO/WHO (1973) standard. If each EAA percentage is less than 100% of the FAO/WHO standard, each amino acid percentage is multiplied by a constant. This constant increases in value as the sample EAA percentage relative to the FAO/WHO standard decreases. This gives more weight to those EAA that are most limiting. This calculation then formed a vector from which a harmonic mean was used to obtain the sample's average EAA score expressed as a percentage of the FAO/WHO standard EAA profile. The above-determined EAA score was then compared to a single, similarily computed value for the casein control. The PER of casein was arbitrarily adjusted to 2.5, and the sample was lineary extrapolated to a give a corrected PER assuming a casein PER of 2.5. Appendix C illustrates a computer printout showing 1) the EAA profile as the percentage of the FAO/WHO after correcting for protein digestibility 2) the various weights used for the sample 3) EAA; essential amino acid score 4) SPC; sample as the ratio of reference casein 4) Z; ratio of sample to casein (z = 2.5 x SPC) 5) C-PER; Calculated-PER

C-PER results are included in Table 24 along with the results of in vitro digestibility obtained from the pH drop method, apparent digestibility and rat PER. C-PER result was not able to show a 1 to 1 relationship with the PER assay; however, the two assays showed the same trend except for the

Table 24. C-PER, Rat PER, 4-Enzyme Digestibility and Apparent Digestibility of Control and Autoclaved SPCs

	Autoclaving Time, min				
Assay	0	10	30	120	240
Rat-PER	2.42	2.42	2.42	1.83	1.79
C-PER	2.5	2.33	1.94	1.93	1.70
App. Dig.,%	86.46	84.82	84.0	83.03	80.36
4-Enz. Dig., %	89.36	88.15	85.86	85.03	83.79

1: Digestibility was determined by pH drop method.

30 min. autoclaved SPC sample. The C-PER of 30 min. autoclaved sample was lower than 10 min. autoclaved sample, while rat PER did not show differences between the two samples. Calculated EAAs based on digestibility and amino acid composition of the 10 and 30 min. autoclaved samples were 73.46 and 72.71 in C-PER method. However, corresponding C-PER values for 10 and 30 min. autoclaved samples were 2.33 and 1.99, respectively. This result indicated that C-PER procedure might exaggerate the small differences in the nutritive value of proteins.

Even though PER is an official method and should serve as reference for any C-PER study, the PER assay has been criticized due to lack of precision (Hegarty, 1975, Pellett, 1978). Furthermore, C-PER values depend on the accuracy of the amino acid analysis. Satterlee et al. (1982) pointed out that the relative ranking of amino acids is not enough and emphasized the importance of an accurate measurement of the quantity of each amino acid. Therefore, it should not be expected that the PER and C-PER assays give the exact same results. However, the purpose of C-PER is as regulatory assay for protein quality. Thus, distinctions between good and poor quality protein should be sufficient for the regulatory purposes. In this study, control, 10 min. autoclaved samples were demonstrated to be better quality proteins than 30 min., 2 and 4 hour autoclaved samples by the C-PER method.

7.0 SUMMARY AND CONCLUSIONS

Protein solubility of 2 and 4 hr. autoclaved samples in potassium phophate buffer (about 27%) was higher than control, 10 and 30 min. autoclaved samples (about 12%). However, protein solubility of control, 10 and 30 min. autoclaved samples increased dramatically up to 60% when urea and beta-mercatoethanol were added to the buffer in increasing order, whereas the solubility of 2 and 4 hr. autoclaved samples changed very little remaining at 25 to 40% throughout. The lower protein solubility of 2 and 4 hr. samples than control, 10 and 30 min samples in potassium phosphate buffer by adding urea and beta-mercaptoethanol suggested that conformational change of soy protein(s) occurred after 2 and 4 hr. autoclaving.

Changes in protein structure due to autoclaving also had a significant effect on the digestibility of SPC. The mean % digestibility of control SPC determined by both *in vivo* and *in vitro* assays was significantly higher than those of autoclaved SPC samples. The mean % digestibility of 10 and 30 min. samples was significantly higher than SPC samples autoclaved for 2 and 4 hr. in *in vitro* assays. While, in *in vivo* assays, the mean % digestibility of 10, 30 min., and 2 hr. autoclaved samples was significnatly higher than 2 and 4 hr. samples. *In vitro* digestibility results were highly

correlated with apparent digestibility of the SPC samples. Correlation coefficients of *in vitro* digestibility determined by the TCA-soluble N measurement, SDS-PAGE and pH drop method were 0.96, 0.92 and 0.95, respectively.

SDS-PAGE was a useful tool for qualitative analysis; The effects of autoclaving on the change of soy protein components was demonstrated as SDS-PAGE patterns of control and autoclaved samples. The unique advantage of SDS-PAGE over other *in vitro* methods to determine protein digestibility was that a sequential process of protein digestion was visualized as SDS-PAGE gel pattern, and the molecular weight of digestive products on SDS-PAGE gel could be estimated. *In vitro* digestion in simultaneous dialysis for 8 hr. was not feasible in the evaluation of SDS-PAGE method as an *in vitro* digestion assay.

In addition to digestibility, there was significant change in the essential amino acid, cysteine, due to autoclaving; 6, 20, 27 and 39% of cysteine was destroyed after 10, 30 min., 2 and 4 hr. autoclaving, respectively. Except for cysteine, there was no actual difference in amino acid profile of control after 10, 30 min., 2 and 4 hr. autoclaving. The chemical score of SPC samples indicated that cysteine became the first limiting amino acid in 2 hr. sample.

PER value of the control did not change after 10 and 30 min. autoclaving. However, PER value of control significantly decreased after 2 and 4 hr. autoclaving, indicating

detrimental effects of 2 and 4 hr autoclaving on SPC. C-PER values of control, 10 min. samples were higher than 30 min., 2 and 4 hr. samples. and 30 min. autoclaved SPC samples. The PER of the 2 and 4 hr. autoclaved SPC samples was significnatly less than raw, 10 and 30 min. autoclaved samples. Decreased PER values of autoclaved SPC samples are likely due to i) decreased digestibility and rate of enzymatic hydrolysis ii) destruction of cysteine iii) decreased food intake.

SUGGESTIONS FOR FURTHER RESEARCH

1) Include a 1 hour autoclaved SPC sample in analyses.
2) Measure i) the number of free sulfhydryl groups and disulfide linkages in each of the SPC samples ii) hydrophobicity of SPC before and after autoclaving.
3) Measure PERs of rat groups fed with 2 and 4 hour autoclaved samples supplemented with cysteine.
4) Measure the activity of proteolytic enzymes vs SPC samples within the small intestine using a suitable technique.
5) Use a longer digestion period, at least 24 hours, in the simultaneous dialysis method.

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APPENDICES

Appendix A.

Reagents for acrylamide and buffer solutions for SDS-PAGE.

8-25% Acrylamide Gradient Resolving Gel (18 ml each)

	8%	25%
Acrylamide Bis Stock Solution	2.94 ml	9.18 ml
Resolving Gel Buffer	4.60 ml	4.60 ml
dd H2O	10.46 ml	4.22 ml
TEMED	6.0 ul	6.0 ul
1.6 (W/V)	0.4 ml	0.4 ml

Acrylamide Bis Stock Solution (100 ml)

Acrylamide	50.0 g
Bis-Acrylamide	1.35 g

Resolving Gel Buffer (100 ml)

Tris	36.33 g
SDS	0.40 g

Adjust pH to 8.80 with concentrated HCL.

5% Acrylamide Stacking Gel (10 ml)

Acrylamide Bis Stock Solution	1.0 ml
Stacking Gel Buffer	2.5 ml
dd H2O	6.3 ml
Ammonium Persulfate	10.0 ul
2.4% (W/V)	0.2 ml

Stacking Gel Buffer (100ml)

Tris	6.06 g
SDS	0.40 g

Adjust to pH 6.8 with concentrated HCl.

Sample Buffer (100 ml)

Tris	0.667 g
SDS	2.0 g
Urea	48.0 g
DTT	0.15 g
Pyronin Y	0.2 g

Adjust to pH 6.8 with concentrated HCl.

Running Buffer (5 l)

Tris	30.28 g
Glycine	71.31 g
SDS	5.0 g

Adjust pH 8.3 if necessary with glycine.

Appendix B

Calculations used to determine enzyme and sample weight used in pH drop.

Bacterial Protease

$$\frac{\text{x mg protease}}{\text{10 mg solution}} = \frac{\text{65 total units}}{\text{activity of protease (units/mg)}}$$

Trypsin

$$\frac{\text{x mg trypsin}}{\text{10 mg solution}} = \frac{\text{227,040 total units}}{\text{activity of trypsin (units/mg solid)}}$$

alpha-Chymotrypsin

$$\frac{\text{x mg alpha-chymotrypsin}}{\text{10 mg solution}} = \frac{\text{1,860 total units}}{\text{activity of alpha-chymotrypsin (units/mg solid)}}$$

Peptidase

$$\frac{\text{x mg peptidase}}{\text{10 mg solution}} = \frac{\text{0.520 total units}}{\text{activity of peptidase (units/g)}}$$

Sample weights

Caculation for determining weights of ANRC casein and SPC samples.

$$\text{sample wt.(g)} = \frac{\text{6.25 mg of protein/ml x 10 ml x 100}}{\text{\% protein of the sample}} \times \frac{\text{1g}}{\text{1000mg}}$$

Appendix C

Amino Acid Contents of SPC Control Expressed as % of Amino Acid Contents of Proteins of FAO/WHO Standard Protein Pattern after Adjustments with In-Vitro Digestibility and Weight Ratio.

Amino Acid	% FAO/WHO	Weight
Lysine	95.86	2.00
$M^1 + C^2$	61.28	5.66
Threonine	82.66	2.83
Isoleucine	100.53	1.00
Leucine	98.30	2.00
Valine	87.57	2.83
$P^3 + T^4$	126.59	1.0
Tryptophan	125.10	1.0

x = 0.225973725
y = 18.3199921
EAA = 81.0713348
SPC = 0.945769131
z = 2.36442280
C-PER = 2.86
1. Methionine
2. Cysteine
3. Phenylalanine
4. Tyrosine

Amino Acid Contents of 10 Min. Autoclaved SPC Expressed as % of Amino Acid Contents of Proteins of FAO/WHO Standard Protein Pattern after Adjustments with In-Vitro Digestibility and Weight Ratio.

Amino Acid	% FAO/WHO	Weight
Lysine	89.75	2.83
$M^1 + C^2$	57.93	8.00
Threonine	79.33	4.00
Isoleucine	92.56	2.00
Leucine	93.19	4.00
Valine	75.81	1.00
$P^3 + T^4$	127.82	1.00
Tryptophan	123.41	1.00

x = 0.331815898
y = 24.8299866
EAA = 74.8305969
SPC = 0.872965395
z = 2.18241310
C-PER = 2.33
1. Methionine
2. Cysteine
3. Phenylalanine
4. Tyrosine

Amino Acid Contents of 30 Min. Autoclaved SPC Expressed as % of Amino Acid Contents of Proteins of FAO/WHO Standard Protein Pattern after Adjustments with In-Vitro Digestibility and Weight Ratio.

Amino Acid	% FAO/WHO	Weight
Lysine	95.23	2.00
$M^1 + C^2$	53.97	8.00
Threonine	88.01	2.83
Isoleucine	100.89	1.0
Leucine	98.13	2.00
Valine	77.27	4.00
$P^3 + T^4$	130.22	1.00
Tryptophan	120.20	1.00

x = 0.299448490
y = 21.8299866
EAA = 72.9006348
SPC = 0.850450695
z = 2.12612629
C-PER = 1.94
1. Methionine
2. Cysteine
3. Phenylalanine
4. Tyrosine

Amino Acid Contents of 2 Hour Autoclaved SPC Expressed as % of Amino Acid Contents of Proteins of FAO/WHO Standard Protein Pattern after Adjustments with In-Vitro Digestibility and Weight Ratio.

Amino Acid	% FAO/WHO	Weight
Lysine	94.31	2.0
M[1] + C[2]	53.45	8.0
Threonine	91.41	2.0
Isoleucine	104.16	1.0
Leucine	103.25	2.0
Valine	85.03	2.83
P[3] + T[4]	131.80	1.0
Tryptophan	119.04	1.0

x = 0.261323094
y = 18.8299866
EAA = 72.0563354
SPC = 0.840601146
z = 2.10150242
C-PER = 1.93
1. Methionine
2. Cysteine
3. Phenylalanine
4. Tyrosine

Table 30. Amino Acid Contents of 4 Hour Autoclaved SPC Expressed as % of Amino Acid Contents of Proteins of FAO/WHO Standard Protein Pattern after Adjustments with In-Vitro Digestibility and Weight Ratio.

Amino Acid	% FAO/WHO	Weight
Lysine	89.88	2.83
$M^1 + C^2$	47.88	11.31
Threonine	87.98	2.83
Isoleucine	102.64	1.00
Leucine	100.55	1.00
Valine	83.79	2.83
$P^3 + T^4$	125.68	1.00
Tryptophan	117.31	1.0

x = 0.369811118
y = 23.7999725
EAA = 64.3571014
SPC = 0.750782728
z = 1.87695599
C-PER = 1.70
1. Methionine
2. Cysteine
3. Phenylalanine
4. Tyrosine

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