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HEAT INDUCED INTERACTIONS BETWEEN SOY (7S and 11S) AND MILK PROTEINS

by

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ABSTRACT

HEAT INDUCED INTERACTIONS BETWEEN SOY (7S and 11S) AND MILK PROTEINS

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Interactions of selected milk fractions and soy water extracted acid precipitated proteins (APP) were examined at pH 7.0, ionic strength (μ) 0.1. Two major proteins of soy APP, the 7S and 11S fractions, failed to demonstrate sulfhydryl group participation in heat induced alterations. However, use of a fluorescent probe, 8-anilino-1-naphthalene sulfonate (ANS) indicated hydrophobic interaction and/or hydrogen bonding participation.

Soy 7S and 11S protein mixtures with sodium caseinate, whey proteins and β-lactoglobulin revealed the independent nature of reactivity for all soy-milk protein combinations. The divalent cation, calcium (II), greatly reduced the 11S fraction solubility characteristics, which also was demonstrated when combined with various milk fractions.

Lysinoalanine (LAL) was detected in defatted soybeans. Increased alkali treatment of the APP fraction favored LAL formation while combination with sodium caseinate reduced LAL formation.

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INTRODUCTION

The utilization of proteins to extend the versatility and acceptance of food systems is widely known. These added proteins should impart specific sensory, tactual, and functional properties to the new simulated product. Protein sources commonly utilized have been dehydrated milk or milk components. However, within the last ten years a variety of other proteins have become economically acceptable and commercially available. The vegetable proteins, soybeans in particular, have found new life and compete with the more expensive milk proteins as protein supplements.

Soy proteins have been incorporated into products ranging from breads and cookies to comminuted meat products. Extensive research has been carried out to determine the compatibility of soy as a foaming agent, fat binder, emulsifier, etc., in non-fluid systems. Fluid foods such as soy milks have been developed, but not generally accepted in Western cultures. Soy milk is commercially available in the Orient, yet has received little attention in this country, due to the characteristic flavor and odor associated with the product.

In the United States there is a great incentive to incorporate soy into fluid products due to the rising costs of dehydrated milk products. Formulation on an empirical basis has resulted in two successful products using soy proteins--a nutritional infant formula and an inexpensive, high quality protein food for underdeveloped nations.

Little, if any, experimental results are available on the interaction of individual soy proteins with milk or other animal proteins. Research on milk proteins has been centered in the Western Hemisphere, whereas, the chemical and physical properties of soy have been examined most extensively in Japan, Korea, and to a lesser extent, in this country. Minimal research at the molecular level is available on interactions of the two proteins in a pure or simple model system. This type of work is needed to understand the effect of processing parameters on the stability and functional properties of foods containing these proteins.

LITERATURE REVIEW

History of Soybeans

The use of soybeans as a food stuff dates back to ancient times, originating in the Far East. Various simple processes developed for the food use of the bean include cooking, grinding, extracting and fermentation.

Early use of soybeans in the Western world centered around the extraction of soybean oil for human consumption and use of the meal for animal feed. Interest in the United States for soybean production was minimal prior to 1920; however, the demand for an inexpensive oil spurred the building of the first oil extracting facility in 1922 (Wolf and Cowan, 1971). The cake which remained after extraction was of lesser importance and was used as an inexpensive protein source for animal feed. The first food grade defatted soy flour was introduced in the 1930 decade, but development was slow due to the competitive prices of milk and animal protein at that time.

Increased interest for soy as a food protein centers around the level of the food chain from which the protein is obtained. For example, an acre of land may produce 58 lbs of protein from beef versus 508 lbs of protein from soybeans. Milk production averages 97 lbs of protein per acre (Anton, 1976). Food grade soybeans range from 26¢/lb protein for soy flour to 71¢/lb protein for soy isolate and is often

competitive with the cyclical prices for dehydrated milk proteins.

Nutritional Aspects

The soybean contains substances which have been found to retard growth in rats. Booth et al. (1960) reported that poor growth, low food efficiency and pancreatic hypertrophy resulted from feeding raw soybean meal to rats. Rackis et al. (1963) found that autoclaving the meal significantly improved these deficiencies. The acid precipitated protein (precipitate of the water extracted protein after adjustment to pH 4.6) showed no differences when heat treated or untreated, which suggested the antinutritional factors were associated with the whey fraction (soluble protein at pH 4.6). Heat treatment of soy whey proteins restored growth and protein efficiency ratio (PER) levels to heated whole soybean. Trypsin inhibitors (Eldridge et al., 1966; Rackis et al., 1962; Rackis and Anderson, 1964) and hemagglutinins (Leiner and Pallansch, 1952) have been found in soy whey fractions and thought to be the cause of the antinutritional symptoms.

Recently questions have been raised whether soybean trypsin inhibitors and hemagglutinins were the only factors that caused low PER values. Liener (1976) showed that soy protein with trypsin inhibitor removed still caused 40% growth depression in rats. He postulated that poor growth and pancreatic hypertrophy were related to incomplete enzymatic digestion in the gut. Fukushima (1968), while investigating the internal structure of the globular 7S and 11S soy proteins, determined that the proteins were not hydrolyzed by proteinase prior to disruption of the internal structure.

Reduced nutritional values of heat-treated soy protein may result from loss of the first limiting essential amino acid, methionine (Shemer and Perkins, 1975). Lysine, although not a limiting amino acid, may become biologically unavailable due to non-enzymatic browning (Jokinen et al., 1976) or lysinoalanine formation during treatment at high pH (Sternberg et al., 1975a; DeGroot and Slump, 1969).

Fluid Products

Soy protein has been used extensively for its functional properties, such as whippability, water absorption, fat absorption, emulsifiability, and viscosity characteristics (Pagington, 1975; Wolf, 1970; Wolf and Cowan, 1971). The use of soy proteins for fluid products, such as soy milk, was first developed in the Far East. In modern society, soy based fluid products are used in feeding infants who may be allergic to cow's milk. A problem early encountered in the preparation of foods containing soy proteins was the off flavor resulting from lipoxidase-catalyzed lipid oxidation. By extracting the beans at 80-100°C for 10 minutes, denaturation of the lipoxidase enzyme was sufficient to reduce much of this off flavor (Wilkens et al., 1967). Other flavor problems, possibly resulting from the proteins themselves or their hydrolyzed products, have not received sufficient attention.

Fluid soy products have recently been reviewed by Johnson (1975) and Jonas (1975). At present, most products are designed for use in areas of the world which suffer from lack of protein. For example, SCM (a mixture of corn, soy, and milk proteins) was developed by the USDA laboratories for use in food support programs to foreign nations while

a fortified commercial soy milk, Vitasoy, was first manufactured in Hong Kong during 1973 (Johnson, 1975). With the probable long range increase in the cost of milk proteins, soy proteins may serve as an acceptable replacement and protein extender if flavor and stability problems can be corrected.

Soybean Proteins

A large portion of seeds from legumes consist of protein. With the increased use of food grade soy protein, isolation and characterization of the protein components are necessary to understand the possible interactions among themselves and with other protein sources.

The soybean is a complex mixture of fat, protein, carbohydrates, and mineral. Soybeans, as do other legumes, segregate certain of their proteins into small packets called protein bodies or aleuron grains. Dieckert and Dieckert (1976) presented an excellent review on the proposed synthesis of legume proteins, the packaging of those proteins in Golgi bodies, and finally, the formation of the vacuole-like structure within the cell. The vacuoles play an important role for storage of the reserve proteins, which are used as a protein nitrogen source during termination of the seed (Hill and Breidenback, 1974; Catsimpoolas et al., 1968).

Extensive investigations have been performed to determine if the proteins in the protein bodies and water extractable proteins (WEP) are similar. Koshiyama (1972a), Saio and Watanabe (1966), and Catsimpoolas et al. (1968) used the techniques of gel filtration, isoelectric focusing, analytical ultracentrifugation and immunoelectrophoresis and have shown similarities exist between the WEP and proteins which comprised the

protein bodies. Tombs (1967) estimated that 60-70% of the total protein of the seed was stored in the aleuron grain and in all cases were of large molecular weight and a heterogenous mixture. Dieckert and Dieckert (1976) suggested the large molecular weight was nature's way of reducing the osmotic pressure the cell must bear upon dehydration.

Smith et al. (1966) demonstrated that protein from soy was extractable with water or dilute salt solutions. Extraction was performed at ambient temperature or slightly higher, by soaking or mechanical agitation of the soybeans. The undenatured soluble protein was separated from the insoluble residue by centrifugation or filtration.

The soluble protein after adjustment to pH 4.6 and centrifugation was referred to as the soy whey proteins and consisted of such proteins as trypsin inhibitors, hemagglutinins and enzymes (Rackis et al., 1959). Catsimpoolas and Leuthner (1969) observed six different fractions by gel filtration on Sephadex G-200, illustrating the diversity of the soy whey fraction.

Acid Precipitated Proteins (APP) - Isolation Method and Chemical-Physical Properties

The precipitate that results from adjustment to pH 4.6 and centrifugation are referred to as the acid precipitated proteins (APP). These proteins are insoluble at their isoelectric point in water, hence are referred to as globular proteins. The nomenclature which describes the protein constituents of the APP are based on immunological characteristics (Catsimpoolas, 1969a) and the more favored system which utilizes sedimentation constants (Wolf, 1969).

The ease of separating the APP allows great potential for characterizing the various functional properties of these proteins. However, due to the

complexity of the proteins present, little definitive information about the reaction mechanisms is attainable from such a group separation.

The APP was composed of four proteins as determined by analytical ultracentrifugation when analyzed in phosphate buffer (pH 7.6, ionic strength (μ) of 0.5, made to 10 mM with mercaptoethanol) (Wolf and Briggs, 1959). The four proteins, 2S, 7S, 11S, 15S comprised 11, 33, 48, and 7%, respectively, of the APP fraction (Roberts and Briggs, 1963). The 7S protein existed at two different molecular weights, which depended on the ionic strength (Roberts and Briggs, 1965) and was determined to be heterogenous (Thanh et al., 1965a; Thanh et al., 1975b; Hasegawa et al., 1963). The complexity of the APP fraction was further heightened by aggregate formation through the sulfhydryl amino acids (Briggs and Wolf, 1957) and hydrophobic interaction (Fukushima and VanBuren, 1970; Hashimoto and Yokotsuka, 1974).

Catsimpoolas et al. (1970) studied the heat treatment of soy APP at various concentrations and in the presence of selected chemical additives to gain insight on possible modes of interaction. Soybean globulin dispersions greater than 8% were able to form gels when heated. Such gels broke down when treated above 125°C. The authors decided to use gel structure and breakdown as criteria of protein alteration to investigate the effect of various chemical additives. The disulfide splitting agent, mercaptoethanol, had a marked effect on the reduction of gel integrity. Hydrogen bonding was also a critical factor, since urea incorporation prevented gel formation. Therefore, the authors suggested the protein alteration involved sulfhydryl-disulfide interchange and hydrogen bonding. Wolf et al. (1963) showed a marked increase in protein solubility of freeze-dried samples when the buffer contained mercaptoethanol, again

suggesting thiol-disulfide interaction. The direct involvement of the cysteine residue was demonstrated by Nash et al. (1971) when alkylation of the -SH spared the protein from denaturation.

Soy 7S-Isolation Methods and Chemical-Physical Reactions

The 7S and 11S proteins were found to represent over 70% of the globular APP fraction (Wolf and Briggs, 1959). These two proteins have been isolated and previously characterized.

The 7S was first investigated by Naismith in 1955, after being purified by salting out procedures with ammonium sulfate. The purified product demonstrated a reversible transformation when the ionic strength was altered. The 7S molecule underwent a 7S ->9S conformational change when the ionic strength was lowered from 0.5 to 0.1 at pH 8.0 in the analytical ultracentrifuge. The initial 7S protein preparation of Naismith still contained impurities and Wolf in 1956 attempted purification by differential solubility with salt solutions and varying temperature. He also noted a 7S ->9S transformation. Roberts and Briggs (1965) obtained the first preparation with greater than 90% purity. The procedure used cooling and ammonium sulfate fractionation, yet lacked usefulness due to the low yield of protein (about 4%).

Gel filtration has been attempted, but proved inadequate to remove the often found 11S contaminant (Koshiyama, 1969b). Koshiyama (1965) took advantage of the 11S sensitivity to the divalent calcium ion and selectively precipitated the protein with 0.025 N $CaCl_2$. Differential solubility in buffers of varying pH, ionic strength, and temperature have proved moderately successful. Koshiyama (1972b) noted the 7S was soluble at low pH (pH 2) when the ionic strength was high (0.6 M NaCl). The small

2S impurity that remained was easily removed by gel filtration on Sephadex G-200. Similarly, Thanh et al. (1975a) used differential solubility and noticed the 7S was soluble in 63 mM Tris-HCl buffer, pH 6.6, at 2°C, and used this in initial steps of purification.

The 7S protein was found to be the only glycoprotein in the globular APP fraction. Koshiyama (1966) determined the carbohydrate content to be 4%. Hexosamine contributed 1.2% of the carbohydrate while mannose composed the remainder. Three years later Koshiyama (1969a) was able to isolate the peptide which contained the carbohydrate moiety and found a single 50 sugar polysaccharide. The two sugars, glucosamine and mannose, were found attached to aspartic acid (Yamauchi et al., 1976).

Roberts and Briggs (1965) noticed that a reduction of ionic strength from 0.5 to 0.1 caused the 7S ->9S transformation. Sedimentation analysis proved the 9S to be the dimer of the 7S monomer. The 7S protein as examined by the analytical ultracentrifuge was suspected about its existence as a single polypeptide. The 7S, being composed of smaller molecular weight fractions, was suggested since n-octyl benzene and urea caused slow moving boundaries by sedimentation analysis (Roberts and Briggs, 1965). Koshiyama (1968a) supported subunit formation and found at least nine N-terminal amino acids. The mechanism of monomer formation was also questioned since Roberts and Briggs (1965) were unable to show appreciable involvement of sulfhydryl-disulfide interchange in the 7S molecule. Koshiyama (1968a) failed to detect any cysteine groups, yet did find four 1/2 cystine residues. Forces other than sulfhydryl-disulfide interaction were suggested in monomer formation since nine subunits were known to be involved and only four disulfides were detected. Koshiyama (1971) investigated the soy protein with ultraviolet spectrophotometry and optical

rotatory dispersion and concluded that hydrophobic interactions and hydrogen bonding were the main contributors of monomer assembly.

The 7S molecule, when investigated by optical rotatory dispersion, was found to consist of little α-helix arrangement and increasing amounts of antiparallel and disordered forms (Fukushima, 1967). Fukushima (1968) concluded the molecule was a fairly compacted structure with a polypeptide arrangement that resulted in a water impermeable, hydrophobic internal core. The native molecule failed to be hydrolyzed by action of proteinase until the internal structure was disrupted. Later, Koshiyama (1973) determined the α-helix, β-structure, and random coil to be 5, 35, and 60%, respectively, for both soy 7S and 11S proteins.

Sulfhydryl-disulfide interchange was thought to be the main mechanism of soy protein interaction. Saio et al. (1971) compared the changing sulfhydryl concentration of soy 7S and 11S as affected by heat treatment. The soy 7S protein showed less variation in sulfhydryl concentration compared to the soy 11S. They also examined protein aggregation by disulfide formation in tofu, an Oriental cheese-like product made from soy. Tofu was made from an enriched 7S preparation that previously had the available sulfhydryl groups blocked by alkylation with N-ethylmaleimide. The tofu that resulted showed identical characterizations to the non-derivatized form. It was suggested the involvement of sulfhydryl-disulfide interaction was of minimal influence.

Saio et al. (1969) examined the effect of calcium on tofu made from a 7S-enriched preparation. Tofu involves the use of calcium and heat in its manufacture. By incorporation of different protein preparations, for example the 7S, the overall characteristics were greatly affected. The curd of tofu prepared from the 7S fraction was much softer when

compared to a water extractable soybean tofu. Upon electron microscopic examination the 7S-tofu appeared as dispersed aggregates, which contributed little to the final texture. Later Saio et al. (1973) investigated the calcium sensitivity of the two major globular proteins on a molecular level. The 7S required three times the calcium concentration to cause flocculation when compared to the 11S protein. Appu Rao and Narasinga Rao (1976) determined the 7S protein bound 10 moles of calcium per mole of soy protein. This correlated with the histidine content of the molecule and was, therefore, thought to be the site of binding. Calcium, as did other divalent cations, decreased the stability of the 7S protein to heat treatment. However, monovalent cations such as sodium, were found to compete for the calcium binding sites and aided in overall stability.

Soy 11S-Isolation Methods and Chemical-Physical Reactions

The 11S molecule comprised the greatest amount, approximately 48%, of the proteins in the APP fraction. The first attempt to purify the protein was by Naismith (1955) who used ammonium sulfate fractionation. Wolf and Briggs (1959) obtained better results by acid precipitation of whole soybeans and fractional extraction of the APP with sodium chloride. The protein which resulted was 87% pure and determined to have a molecular weight of 350,000 daltons. Briggs and Mann (1950) first noticed that upon cooling the water extractable protein, a white precipitate, resulted, which was rich in the 11S fraction. Wolf et al. (1962) used the cold insoluble fraction (CIF) and obtained greater than 90% purity by salt fractionation. The authors observed polymerization of the protein through the sulfhydryl groups and prevented this reaction by

blocking the thiol groups with N-ethylmaleimide. Previously Briggs and Wolf (1957) noticed sulfhydryl oxidation when exposed to atmospheric oxygen.

Chromatographic isolation of the 11S protein was first attempted by Hasegawa et al. (1963). Gel filtration of the water extractable soy protein on Sephadex G-200 resulted in Fraction 3 being a heterogenous protein mixture, with the 11S the major component. Further attempts to purify the 11S by gel filtration proved tedious (Mitsuda et al., 1964; Koshiyama, 1972c). Catsimpoolas et al. (1967) was able to increase the purity of the ammonium sulfate preparation of Wolf et al. (1962) on DEAE Sephadex A-50. Kitamora et al. (1974) recognized the 7S was a glycoprotein and selectively removed it from the 11S by affinity chromatography on Conconavalin A followed by clean-up procedures utilizing gel filtration.

Eldridge and Wolf (1967) utilized differential solubility of the APP fractions in buffers with varied temperatures and ionic strength. The procedure resulted in 94% purity and a 15% yield of protein.

Conformational analysis of the 11S molecule was found to be quite similar to the 7S protein. Koshiyama (1973) found values of 5, 35, and 60% for α-helix, β-structure and random coil contents, respectively. Fukushima (1968) also noted the internal structure contained an appreciable amount of random coil and existed as a very tightly packed molecule. Hydrophobic interactions provided the integrity to the core structure and explained the inability of proteinase to hydrolyze the protein before disruption of the stabilizing forces occurred. Similar conclusions about the 11S conformation were found by Fukushima (1967) and Koshiyama (1972c).

Shvarts and Vaintraub (1967) reported the 11S protein contained 48 moles of 1/2 cystine per molecule based on a molecular weight of 360,000. The environment or type of physical treatment were found to markedly affect the quantity of reduced sulfhydryl groups present. Briggs and Wolf (1957) were among the first investigators to show the importance of reducing agents or sulfhydryl blocking agents during purification and characterization of the protein. Examination of the CIF in the presence of a disulfide splitting agent, mercaptoethanol, resulted in a large increase of the 11S protein during sedimentation analysis. The time for reaction was less than one hour after addition of the mercaptoethanol.

The 11S, similar to the 7S protein, was found to contain subunits. Mitsuda et al. (1964) first showed the presence of subunits by N terminal analysis. Catsimpoolas et al. (1967) treated the 11S with 6 M guanidine-HCl and found 12 subunits per mole 11S protein. Quantitative analysis by paper chromatography of the N terminal amino acids revealed 8 moles of glycine, 2 moles of leucine (or isoleucine), and two moles of phenylalanine per mole 11S protein. Later with aid of electron microscopy Catsimpoolas (1969b) reported the 11S was composed of two annular-hexagonal structures, one resting on top of the other. Saio et al. (1970) found similar results and supplied an electron micrograph as proof.

The 11S molecule showed great sensitivity to calcium and other divalent cations. Saio et al. (1973) showed the majority of the 11S protein precipitated at 0.4 mM calcium when 1.56 mg of protein N per 10 ml was present. This corresponded to one-third the calcium needed to precipitate the 7S protein. Saio et al. (1969) used tofu to examine how calcium affected the overall physical properties of the final product.

Crude preparations of 7S and 11S were used and processed by a standard procedure found in tofu manufacture. The 11S caused a harder, tighter texture compared to the 7S-tofu. Electron micrographs compared the structure that resulted from the two tofu preparations and revealed a highly aggregated texture from the 11S-tofu. The authors concluded the tight texture was caused by the sensitivity of the 11S to calcium, while the soft gel structure was attributed to the 7S protein. Appu Rao and Narasinga Rao (1975) determined the 11S molecule bound calcium with the molar ratio being equivalent to the histidine content. Monovalent cations such as sodium caused inhibition of binding and reduced the sensitivity of the protein to precipitation by calcium.

Briggs and Wolf (1957) suggested the 11S reacted mainly through its sulfhydryl and disulfide bonds (this thought still exists today). To study such reactions reagents which were specific for the sulfhydryl or disulfide groups were incorporated into the buffer system and the changing physical ramifications that result were noted. Wolf et al. (1962) determined the 11S possessed great stability when stored as a solution in the presence of mercaptoethanol. Hashizume et al. (1971) used purified 11S and showed that freezing and subsequent thawing caused the protein to precipitate. Addition of mercaptoethanol, which reduced any disulfides present, caused the aggregate to disappear with the appearance of the 11S protein. Alkylation of the available sulfhydryl groups prior to freezing failed to show any changes upon thawing. Saio et al. (1971) was able to substantiate sulfhydryl-disulfide involvement by preparation of tofu with an enriched 11S fraction and examination of the texture that resulted. Alkylation of the thiol groups with N-ethylmaleimide produced a tofu that was less hard, less

cohesive, less springy and more adhesive compared to the non-derivatized 11S fractions. The authors concluded from tofu preparations with soy APP, 7S and 11S, that the 11S was the major contributor of firmness to tofu and was caused by sulfhydryl-disulfide interactions.

Thermal denaturation of proteins was considered due to alterations in functional characteristics. Catsimpoolas et al. (1969) examined thermal denaturation of the 11S by light scattering. Temperatures above 70°C caused turbidity, while at 90°C precipitation occurred. Similar results were obtained by immunological examination (Catsimpoolas and Meyer, 1968; Catsimpoolas et al., 1971). Wolf and Tamura (1969) used the analytical ultracentrifuge to determine heat effects on the 11S proteins. Temperatures above 70°C caused the formation of soluble subunits (3-4S) and other lower molecular weight peptides, with the potential to form soluble aggregates and eventually insoluble aggregates. The formation of the insoluble aggregates (precipitate) was prevented by blocking the available sulfhydryl groups with N-ethylmaleimide. Catsimpoolas et al. (1970) observed aggregate formation at 70°C even in the presence of 0.2 M mercaptoethanol. Wolf and Tamura (1969) concluded that the formation of the insoluble precipitate occurred only when the 11S molecule contained free sulfhydryl groups. Hydrophobic interaction was suggested to occur simultaneously with thiol interaction in formation of the insoluble aggregate.

Stability was also imparted to the 11S by high protein concentration. Cumming et al. (1973) (who incorrectly identified the 7S and 11S) extruded a 70% suspension of defatted soybean meal and found that the heated meal contained undenatured 11S protein after heating to 204°C.

Milk Proteins-Chemical and Physical Reactions

The isolation of the different milk fractions will not be mentioned due to the many papers and review articles available (McKenzie, 1967; McKenzie, 1971; Yaguchi and Rose, 1972). However, the various interactions of the caseins and whey proteins are important in light of their possible reaction with the soy acid-precipitated proteins.

Milk is a complex mixture of proteins, carbohydrates, fat, and salts distributed in solution, colloidal suspension, or bound to membranes. The conformation of the various milk proteins are susceptible to heat, calcium ions, pH and various denaturants. These factors, which determine the overall protein characteristics, are important considerations in the stability of a fluid product.

Milk Caseins

The caseins are composed of three main components, α_s-, β-, κ-casein, with many minor components, the origin of which are unknown. Studies involving each component separately and in combination with the other major casein subunits may aid in the understanding possible interactions of milk caseins with soy proteins.

The caseins were found to bind calcium at the pH of milk, the degree varying little among the three main components (Rose, 1965). Demott (1968) used a calcium sensitive electrode and determined the free calcium concentration to be 1.4 to 2.5 mM. Waugh (1971) reported 30 mM calcium in skimmilk of which 10 mM was diffusible, 20 mM bound to casein and 2-3 mM existed in the free ionized state. The bound calcium, which was approximately 2/3 of the total calcium, existed as a phosphoserine ester in milk caseins (Rose and Calvin, 1966). The diffusible salt,

approximately one-third of the total calcium, existed as a phosphate salt (Puri and Parkash, 1965). The salt was referred to as colloidal calcium phosphate and was an important component in calcium equilibrium between the three physical states.

The importance of calcium for micelle integrity was examined by Jenness et al. (1966). Whole casein when treated with a chelating agent resulted in the formation of soluble casein. It was found the shifting of the calcium equilibrium caused a new distribution of casein protein, from a colloidal suspension to a soluble state.

The relative amounts of each calcium form affected the storage stability and heat coagulation of the caseins. Fox and Hoynes (1975) and Rose (1962) showed that the reduction in the level of colloidal phosphate resulted in increased heat stability of skimmilk. Muldoon and Liska (1972) heat treated skimmilk at different temperatures and determined decreased levels of ionized calcium present with increasing heat treatment. Similarly, Aoki and Imamura (1974), who studied the storage stability of high temperature short time sterilized skimmilk, noticed a decrease in ionic calcium in the ultracentrifuged whey when compared to the control. In all instances, the calcium salt equilibrium gradually shifted, with a demonstratable change in casein stability. Aoki and Imamura (1975) examined the changing calcium equilibrium in sterilized concentrated skimmilk. Storage of the product resulted in an increase of soluble casein. The result again suggested the involvement of the calcium equilibrium. The authors suspected citrate, a chelating agent, of forming a soluble calcium salt which reduced calcium bound to casein. This resulted in reduced micelle integrity and finally the increased soluble casein.

The casein fraction of milk contains no sulfhydryl groups while one disulfide group was associated with the κ-casein fraction (Jenness, 1971). The potential for reactivity through the disulfide bonds was discovered when the κ-casein-β-lactoglobulin complex was formed during heating (El-Negoumy, 1974; Tessier and Rose, 1964). Zittle et al. (1962) noted addition of N-ethylmaleimide to a mixture of β-lactoglobulin and κ-casein was able to prevent complex formation. The complex also failed to be formed when β-casein was substituted for κ-casein. Sawyer (1969) reviewed the β-lactoglobulin-κ-casein complex and its method of formation, yet suggested the involvement of only thiol-disulfide interaction was not conclusive.

Krescheck et al. (1964) examined heat treated whole casein by light scattering. Temperatures up to 90°C caused little effect on the molecular weight of whole casein. When each fraction was examined separately, only κ-casein showed instability to heating at 90°C. Zittle (1969) examined the effect of sulfhydryl reducing agents, such as mercaptoethanol, on stability of κ-casein. When the disulfide group of the κ-casein was reduced and alkylated, heat lability was not affected when compared to the native form. Also, addition of mercaptoethanol failed to demonstrate any change in susceptibility of the κ-casein to heat. Morr (1965) examined heated skimmilk at 28°C for 10 minutes and failed to detect any change in particle size of the casein micelles, in chemical composition of the micelles or any alterations detectable by free boundary electrophoresis. However, Aoki et al. (1975), using a concentrated "milk" free of whey protein heated at 135-140°C for 15 seconds, noticed increased levels of soluble casein. The authors showed nearly half of the soluble casein was composed of κ-casein.

β-lactoglobulin

The protein, β-lactoglobulin, comprises 65% of the whey proteins and represents approximately 3 g/l in milk. The various conformational forms of β-lactoglobulin as influenced by heat, pH, ionic strength and denaturants have been reviewed by McKenzie (1971).

Briggs and Hull (1945) were the first to investigate the influence of temperature on stability of β-lactoglobulin. Two different reactions were noticed on exposure to heat when examined by free boundary electrophoresis. Sawyer (1968) used analytical ultracentrifugation and starch gel electrophoresis to study the effects of heat treatment on β-lactoglobulin. Denaturation was determined to occur in two steps, and found to involve sulfhydryl groups and non-specific aggregation. The primary reaction was favored at 97.5^{o}C and resulted in aggregates with a sedimentation value of 3.7S (compared to 2.6S for the native molecule). The protein in the presence of N-ethylmaleimide or reduced with mercaptoethanol and heat treated showed the reaction involved intermolecular disulfide bonds. The secondary reaction, which was favored at a lower temperature, resulted in a larger aggregate of 29S. This aggregate was shown not to involve thiol-disulfide interaction since N-ethylmaleimide addition failed to alter starch gel electrophoretic patterns. Therefore, the secondary reaction was concluded to involve nonspecific aggregation. Lyster (1970), who studied the kinetics of heat denaturation for β-lactoglobulin, determined the rate constant to be second order in skimmilk and markedly affected by addition of reagents specific for thiol groups. The author concluded, as have others, that cysteine and cystine amino acids were involved in heat-initiated aggregation.

Whey Proteins

The whey proteins are comprised of β-lactoglobulin, α-lactalbumin, bovine serum albumin, and immunoglobulins at concentrations of 3, 0.7, 0.3, and 0.6 g/l milk, respectively (Jenness, 1971). Two proteins of whey contain sulfhydryl groups; β-lactoglobulin, which has previously been discussed and bovine serum albumin which contains 0.7 mole -SH/mole protein (Jenness, 1971).

The heat stability of the whey protein has been shown to be dependent on the method of preparation. Guy et al. (1967) used cottage cheese whey and determined heat treatment at 91°C for 30 minutes denatured 80% of the protein. The stability was found to increase by higher levels of total solids. Morr and Josephson (1968) studied acid precipitated whey solutions dialyzed against skimmilk. Samples heated at 90°C for 10 minutes resulted in 25% destabilized protein. However, addition of N-ethylmaleimide and whole casein to the whey proteins resulted in complete protein stability. When the whey proteins were dialyzed against phosphate buffer (pH 6.8, μ=0.1), loss of stability of only 4.3% when heated at 90°C for 10 minutes resulted. The results suggested a three step reaction occurred during heat treatment. The first reaction involved the reversible denaturation of whey proteins by breaking of hydrogen and hydrophobic bonds followed by irreversible denaturation by sulfhydryl-disulfide interaction. The second reaction was calcium dependent and resulted in intermediate sized aggregates. The final process of heat denaturation of the whey proteins was large-scale aggregation and was determined to be dependent on the calcium concentration.

Investigation of Hydrophobic Interaction by Fluorescence of 8-anilino-1-naphthalene Sulfonate (ANS)

Compounds may be inserted into proteins and used to monitor various conformational alteration by spectroscopic measurements. A wide array of organic compounds for measuring protein hydrophobicity were produced in the 1950 decade.

In 1954, Weber and Laurence noticed that polycyclic aromatic compounds which failed to fluoresce in aqueous buffers became highly fluorescent in a nonpolar environment. For example, several anilinonaphthalene sulfonates and anilinoacridine derivatives exhibited fluorescence when bound to serum albumin. Weber and Young (1964) used 8-anilino-1-naphthalene sulfonate (ANS) to examine the globular fragments which resulted from pepsin digestion of bovine serum albumin. Due to the hydrophobic nature of certain proteins, Naik et al. (1975) determined ANS was useful for quantitative determination of serum albumin.

The true advantages of ANS were demonstrated by Stryer (1965) for the hydrophobic interaction of apomyoglobin and apohemoglobin with the heme moiety. Apomyoglobin had previously been shown to involve nonpolar interactions in the binding of the heme to the globular protein. ANS was found to bind stiochiometrically (1:1) with both apoproteins. The hydrophobic ANS-apoprotein complex was disrupted by addition of the heme moiety and shown to replace the ANS molecule in the binding site. The heme bound proteins, myoglobin and hemoglobin, failed to bind ANS. Stryer also investigated the relative fluorescence of ANS in various organic solvents. Fluorescence increased with the hydrophobicity of the solvent examined (e.g., methanol < ethanol < n-propanol < n-octanol). Besides increased fluorescence, a shift in the wavelength of maximum

emission towards the blue wavelengths was detected. Stryer (1968) discussed in a comprehensive review of fluorescent hydrophobic markers important factors involved in energy transfer, various rules to be followed, and theory of fluorescence for ANS and analogous fluorescent markers.

ANS fluorescence as an indication of hydrophobic interactions was used marginally for milk caseins. Sugimoto et al. (1974) used ANS to investigate the hydrophobicity of the three major casein subunits. ANS was found to bind to all three protein fractions, α_s-, β-, and κ-casein, while κ-casein showed the greatest binding. The hydrophobic nature of α_s and κ-casein complex was suggested in micelle structure and integrity, since 1.7 mM ANS was needed to disassociate the complex. Creamer and Wheelock (1975) studied α_s- and β-casein interactions by ANS fluorescence. Increased aggregation of the two proteins enhanced the fluorescence, thus indicating hydrophobic interactions. Addition of $CaCl_2$ (at 37^oC) showed increased fluorescence of α_s-β casein mixture and suggested the involvement hydrophobic interactions in calcium aggregation. Similar results were found by turbidity measurements.

Hashimoto and Yokotsuka (1974) used ANS fluorescence to study the protein which precipitated during soy sauce manufacture. Hydrophobic interaction was suggested by the shift of maximum wavelength of the emission spectra in the blue wavelength direction when fluorescence of acetate buffer was compared to buffer which contained the protein sediment. Determination of hydrophobic interaction was performed by adding sodium chloride to the protein solution and determination of fluorescence. The increased salt concentration would decrease protein-solvent interaction and if hydrophobic interaction occurred, would be detected by increased

fluorescence. The fluorescence was found to increase which suggested the involvement of hydrophobic interaction.

Lysinoalanine (LAL)

Lysinoalanine is an unnatural amino acid which forms in some proteins on treatment with alkali at elevated temperatures. Alkali treatment is used frequently in the food industry for lye peeling of fruits and vegetables and in fiber spinning of soy isolate and other textured food products.

Bohak (1964) detected the loss of lysine and 1/2 cystine residues when ribonuclease A was treated at pH 13 and acid hydrolyzed. Analysis by ion exchange detected six of a possible 10 lysine residues and one out of a possible 8 1/2 cystine amino acids for the sequenced protein. The author found a new peak upon amino acid analysis and determined by chemical and enzymatic methods the compount to be N^{ε}(DL-2-amino-2-carboxyethyl)L lysine or lysinoalanine. Formation of the new amino acid depended on the protein source, for example, lysozyme and papain formed LAL, whereas pepsin and pepsinogen failed to produce any detectable LAL. The author suggested LAL was formed by adjacent -cys-lys- residues.

Simultaneously, Patchornik and Sokolovshy (1964) also isolated LAL in modified bovine pancreatic ribonuclease and proposed the involvement of the amino group of lysine and cystine amino acids. The authors showed the involvement of cys 40, 65, and 110 of ribonuclease in LAL formation.

The formation of new amino acids upon acid hydrolysis was not a new idea. Asquith and Carthew (1972) studied the mechanisms of alkaline

degradation of cystine in proteins. They suggested alkali treatment hydrolyzed the disulfide bond, which resulted in dehydroalanine and cysteine. Various derivatives were formed upon reaction of dehydroalanine with different nitrogen groups, the result being a β-N-alkyl amino acid. For example, ammonia yielded β amino alanine (Asquith et al., 1969), cysteine formed lanthione (Horn et al., 1941) and lysine resulted in LAL (Bohak, 1964). Whiting (1971) also suggested the involvement of glycosidically bound serine in the protein-polysaccharide complex of cartilage after alkali treatment.

DeGroot and Slump (1969) exposed several proteins (milk casein, soy isolate, and soybean meal) to pH 12.2 at 40°C for 4 hours. LAL formation occurred with subsequent decrease in cysteine and lysine amino acids. Rats fed soy protein isolate treated at high pH values showed no clinical or histological abnormalities, except increased degree of nephrocalcinosis in females, which was relieved upon feeding dietary calcium. However, Woodward and Short (1973) reported renal lesions in rats that were fed a modified soy protein (Alfa protein) and soy protein subjected to severe alkali treatment in 0.1 N NaOH at 60°C for 8 hours.

DeGroot et al. (1975) fed rats alkali treated protein which contained an equivalent of 0.14% LAL. No clinical or pathological changes were detected. When the rats were fed diets which contained either synthetic LAL at levels equaled to 0.02% (and above) or soy protein that was alkali treated followed by acid hydrolysis, severe tubular nephrosis occurred and was characterized by necrosis, regeneration and cytomeglia of the epithelial cell. Excretion of LAL from the body of rats fed alkali treated protein was predominately in the feces, while synthetic LAL was excreted in the urine.

In 1976, DeGroot et al. performed a more comprehensive study of free and bound LAL. Rats fed at levels of 100 ppm synthetic LAL demonstrated renal changes, which he characterized as nephrocytomeglia. No changes were observed when up to 6,000 ppm of protein bound LAL was ingested after treatment in 0.1 N NaOH for 8 hours at 60°C. However, if the alkali treated protein was acid hydrolyzed, similar results of synthetic LAL were found in rats. Synthetic LAL fed at levels of 1,000 ppm to mice, hamsters, rabbits, quail, dogs, or monkeys failed to produce any renal changes. The authors suggested species specificity of rats to the free, unbound LAL present in alkali treated protein.

EXPERIMENTAL PROCEDURES

Isolation Methods

To study the chemical and physical properties of soy and fluid milk, the following protein fractions were isolated for the subsequent work.

Preparation of Soybean

Corsoy variety soybeans (1975 crop) were conditioned to the proper moisture to facilitate cracking and ease of hull removal. The beans were cracked in a Waring blender and passed through a U.S. No. 4 sieve to remove uncracked beans. An air cyclone was used to remove hulls from the cotyledon. The beans were ground in a Waring blender followed by extraction with n-pentane in a Soxhlet extractor for 10 to 12 hours. After removal of excess solvent, the beans were passed through 20, 40 and 60 size screens in a Wiley Mill. The ground beans were extracted with 30% ethanol in diethylether in a ratio of 1:10 (w/v) beans to solvent for one hour at $0^{o}C$. Suction filtration to remove the solvent was followed by washing the meal twice with ether ($0^{o}C$) to remove the residual ethanol. Excess solvent was allowed to evaporate.

Preparation of Acid Precipitated Protein (APP)

Water extraction of the defatted beans was carried out as follows: A 1:10 mixture (w/v) of soy flour to water was stirred for one hour with a propeller type stirrer with adjustment to pH 7. The defatted soybean

extract was centrifuged to separate the supernatant from the undissolved precipitate followed by filtering the soluble fraction through four layers of cheese cloth. The water extract was acidified to pH 4.6 with 1 N HCl to precipitate the soy APP fraction. The APP was centrifuged and collected, washed twice with water, followed by centrifugation after each washing. The precipitate was dissolved to the original volume of water with 1 N NaOH and adjusted to pH 7.6. Centrifugation was used to remove the "acid sensitive fraction". The proteins were reprecipitated at pH 4.6 and washed twice with water as described above. The final precipitate was dissolved in water to the original volume of the extract, adjusted to pH 7.6 and dialyzed against phosphate buffer, pH 7.6, μ=0.5, in 10 mM mercaptoethanol (2.6 mM NaH_2PO_4, 32.5 mM Na_2HPO_4, 0.4 M NaCl, 10 mM mercaptoethanol).

Preparation of the Soy 7S

Isolation of the 7S component was performed by a combination of methods from Thanh et al. (1975a), who found differential solubilities of the 7S and 11S proteins in 63 mM Tris-HCl buffer at two different pH levels and Koshiyama (1972b), who observed that only the 7S molecule was soluble in 0.01 N HCl, 0.6 M NaCl buffer. It was observed that a better yield of the 7S fraction was obtained by using the supernatant following removal of the cold insoluble fraction (CIF).

A mixture of meal and water (1:5 w/v) was stirred for one hour at pH 7. The slurry was centrifuged at 2,000 rpm in an IEC Model K centrifuge for not less than 20 minutes. The supernatant was poured through four layers of cheese cloth. The water extract was placed in an ice bath and allowed to stand overnight. The slurry was centrifuged at 15,000 x g for

30 minutes at 2°C and the supernatant collected (the precipitate, the cold insoluble fraction, was saved for 11S purification). The supernatant was recooled to 0°C, allowed to stand overnight and centrifuged. The precipitate was discarded and the procedure repeated once more. The final supernatant was adjusted to pH 6.6 and dialyzed overnight at 2°C against 63 mM Tris-HCl buffer, pH 6.6. The white precipitate that formed was centrifuged at 12,000 x g for 20 minutes at 2°C and discarded. The supernatant which contains the 7S protein, whey proteins and some 11S protein was warmed to room temperature, adjusted to pH 4.8 with 1 N HCl and centrifuged at 12,000 x g for 20 minutes, leaving only the reserve proteins in the precipitate. Since a minor 11S impurity was still present, the precipitate was slurried with 0.01 N HCl plus 0.6 M NaCl at a concentration of one part of precipitate to 10 parts of high ionic strength buffer (w/v). This slurry was stirred for 45 minutes (prolong exposure will denature the 7S). The slurry was centrifuged at 12,000 x g for 20 minutes and the supernatant, which contained the 7S fraction, was made to pH 7.6 with 1 N NaOH and dialyzed against phosphate buffer (pH 7.6, μ=0.5, in 10 mM mercaptoethanol).

Preparation of the Soy 11S

The procedure selected was described by Eldridge and Wolf (1967) with one modification. The CIF, obtained from the 7S preparations, was made to 0.2% in acetate, pH 4.6, μ=0.5 buffer (100 ml of 1 M NaOH, 215 ml of 1 M acetic acid, 23.4 g NaCl per liter, 10 mM mercaptoethanol) and stirred gently for 3-4 hours at room temperature. The resulting sol was centrifuged at 15,000 x g for 30 minutes and the supernatant cooled overnight at 0°C in an ice-water slurry. The white precipitate which formed

was removed by centrifugation at 15,000 x g for 30 minutes. The supernatant, which contained the 11S, was then diluted from μ=0.5 to μ=0.3 by addition of 10 mM mercaptoethanol in water at 2°C. The precipitate of purified 11S was picked up in phosphate buffer (pH 7.6, μ=0.5 in 10 mM mercaptoethanol) and dialyzed against the buffer overnight.

Preparation of Sodium Caseinate

Whole milk was obtained from the Michigan State University dairy herd. After centrifugal separation to remove cream, the casein was precipitated at pH 4.7 from the skimmilk while heated at 30°C. The casein was collected in a strainer, washed with water and redissolved in water by adjusting to pH 7. The above procedure was repeated twice and the purified sodium caseinate was freeze-dried at 100°F, 5 μ absolute pressure.

Preparation of β-lactoglobulin

The isolation of β-lactoglobulin was carried out according to Aschaffenburg and Drewry (1957) from mixed herd milk. The only point of departure was the collection of the oil instead of crystals. The oil was collected and dissolved by addition of small amounts of sodium chloride. The pH was adjusted to pH 5.2 and redialyzed against distilled water to precipitate the β-lactoglobulin oil. The oil was dialyzed against phosphate buffer, pH 7.0, μ=0.1, (20.2 mM NaH_2PO_4, 26.6 mM Na_2HPO_4) and stored under toluene at 2°C.

Preparation of Milk Whey Proteins

Mixed herd milk obtained from the university herds was separated at 35°C and the casein removed by precipitation at pH 4.7 at 30°C.

The precipitated casein was removed by using cheese cloth followed by filtration of the whey through filter paper. The pH was adjusted to pH 7 and saturated with solid ammonium sulfate. After standing overnight at 2-4°C, the whey proteins were collected as a precipitate by centrifuging at 15,000 x g for 30 minutes at room temperature. The precipitate was collected, dialyzed against phosphate buffer (pH 7.0, μ=0.1) and stored under toluene at 2°C.

Analytical Procedures

Protein Determinations

Kjeldahl. Total nitrogen was determined by a micro-Kjeldahl technique utilizing SeO_2 as a catalyst. Details are to be found in the Appendix.

Biuret. Colorimetric determination by the biuret method was carried out according to Layne (1957) with slight modifications. See Appendix.

Lowry. Colorimetric determination by the Lowry method was carried out according to Lowry et al. (1951). See Appendix.

Sulfhydryl Determinations

Colorimetric determination of cystein was modified from procedures of Ellman (1959) and Beveridge et al. (1974). See Appendix.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis in glass tubes was carried out by a modification of Melachouris (1969) procedure. For systems not containing milk caseins as a component, the stock solution of 10% Cyanogum 41 (Fischer) in Tris-HCl buffer, pH 8.9, (4.6 g Tris to 100 ml made to pH 8.9

with HCl) was utilized. The gels, 5, 6, and 7%, were made by taking the proper amount of stock 10% Cyanogum 41 in Tris-HCl buffer, pH 8.9, and Tris-HCl buffer, pH 8.9, for a total of 16 ml. To this was added 20 µl of TEMED (N,N,N',N'-tetramethylethylenediamine, Bio-Rad Laboratories). To initiate polymerization, 0.1 ml of a 5% solution of ammonium persulfate was used. The gel solution was put in the gel tubes with the aid of a syringe to a depth of 6.5 to 7 cm. Water was layered on top of the gel to prevent inhibition of polymerization. Polymerization was carried out for 30-45 minutes after which the gels were mounted in a Bio-Rad Model 150 Gel Electrophoresis Cell. The electrode buffer was Tris-glycine buffer, pH 8.3, (5.6 g Tris and 28.8 g of glycine per liter of water). The samples had a few grains of reagent grade sucrose added to increase the density and one drop of Bromophenol Blue (1% in phosphate buffer, pH 7.0, μ=0.1). A 5 µl sample was layered on top of the gel under the electrode buffer solution. Running conditions were 2.5 mA/tube, constant current, with a Bio-Rad 400 Power Supply for approximately 50 minutes. The gels were stained for 5 minutes in Amido Black solution (2 g of Amido Black in 250 ml of water, 250 ml of methanol, and 50 ml of glacial acetic acid) followed by destaining with 7% acetic acid in a diffusion destainer.

The samples containing milk caseins were examined in 6 M urea-10% Cyanogum 41 gels. The gel buffer was Tris-HCl buffer, pH 8.9 (mentioned previously) containing 6 M urea (36.0 g/100 ml of stock solution). The gels were 10% final concentration and were prepared and run as mentioned previously.

The samples from the heat studies (0.5% concentration) were placed directly on the gel after sucrose and Bromophenol Blue solution had been

added. It was found that satisfactory results were obtained without changing the buffer to 62 mM Tris-HCl, pH 6.7, as was recommended by Melachouris (1969). Samples containing sodium caseinate were made to 6 M urea and one percent mercaptoethanol by adding 0.36 g of urea and 10 μl of mercaptoethanol to 0.7 ml of sample.

Analytical Ultracentrifuge

Samples were dialyzed against either phosphate buffer (pH 7.6, μ=0.5 in 10 mM mercaptoethanol) or phosphate buffer (pH 7.0, μ=0.1). Protein concentrations were determined by the Lowry method if mercaptoethanol was present or by the biuret method in the absence of mercaptoethanol.

Sedimentation velocity experiments were carried out using a Beckman-Spinco Model E Analytical Ultracentrifuge. The speed was 52,640 rpm with a bar angle of 65°, at room temperature. Time zero was defined as 2/3 maximum speed and pictures were taken at eight minute intervals.

Only S (sedimentation coefficient) was determined with no attempt to extrapolate to zero concentration for $S_{20,w}$ or examine concentration effects on S.

The equations of interest are:

$$S_{20,\,app} = \frac{2.303}{w^2 .60} \times \text{slope}$$

when slope is:

$$\text{slope} = \frac{\log(x + \frac{X_2}{M}) - \log(x + \frac{X_1}{M})}{t_2 - t_1}$$

where:

x = distance from center of rotor to reference line

M = magnification factor

X_n = distance from reference line to peak at t_n

t_n = time of picture, minutes

$w = \frac{2 \tilde{\pi} \text{ rpm}}{60}$

Heat Studies-Methods

Time-Temperatures Used for Investigation

Initial studies to determine the extent of denaturation and subunit formation of soy APP by heat were determined by heating buffered aliquots for 5 minutes over a temperature range of 50 to 110°C. Soy APP was dialyzed against phosphate buffer (pH 7.0, μ=0.1) at 2°C overnight with two changes of new buffer. The APP, in pH 7.0, μ=0.1 buffer, was centrifuged at 15,000 x g for 30 minutes and adjusted to 1% final concentration. Heating was carried out in a circulating bath for temperatures under 100°C. For 100°C a boiling water bath was used and for temperatures greater than 100°C, a mineral oil bath was used on a magnetic stirrer-hot plate. Two ml samples were placed in 1.2 x 10.0 cm screw cap pyrex tubes. Once heating was accomplished, the samples were cooled in an ice-water slurry and centrifuged at 20,000 x g for 30 minutes. The supernatant was used for analysis.

Soy-milk protein combinations were heated in a 1:1 ratio at three temperature levels: 63°C to examine the relative effect of low temperature pasteurization with minimal heat treatment, 74°C for intermediate heat treatment sufficient to "activate" sulfhydryl containing proteins,

and 121°C to evaluate ultra-high temperature processing affects.

Samples to be heated were dialyzed against the appropriate buffer (phosphate buffer, pH 7.0, μ=0.1 or for study with calcium, using cacodylate buffer, pH 7.0, μ=0.1: 5 mM cacodylate, 0.095 M NaCl made to pH 7.0 with HCl) for a minimum of 12 hours at 2°C with frequent changes. The final concentration of the protein solution was made to 0.5% as determined by the biuret method.

Two to four ml samples were placed in 1.3 x 9.7 cm test tubes and heated as follows: control (no heat treatment), 63°C for 5, 10 and 30 minutes and 74°C for 5, 10 and 15 minutes. Heating at 121°C for 5, 10 and 15 seconds was accomplished in 1.2 x 10 cm screw cap pyrex tubes. The "come-up" time was determined with a thermometer submerged in 3 ml of water in a sealed screw cap tube. Heating time was recorded after the thermometer read 121°C. Following heat treatment the tubes were removed and cooled to room temperature in an ice-water slurry. The samples were centrifuged at 20,000 x g for 30 minutes. The supernatant was used for analysis.

The soy proteins were examined at different calcium levels, 1, 3 and 8 mM calcium, singularly and in combination with milk proteins (1:1, w/w). The samples, dialyzed against cacodylate buffer (pH 7.0, μ=0.1), were adjusted to 0.5% concentration. A known amount of stock $CaCl_2$-$2H_2O$ was added to a stirred protein sol to yield a final calcium concentration of 1, 3, or 8 mM calcium. The solution was stirred for an additional 60 minutes and allowed to stand quiesent for 30 minutes before heat treatment. Heating and centrifugation was carried out as previously mentioned. The supernatant was examined by protein solubility and gel electrophoresis following centrifugation.

Examination with 8-anilino-1-naphthalene Sulfonate (ANS) for Hydrophobicity

The sodium salt of 8-anilino-1-naphthalene sulfonate (ANS) was purchased from Eastman Chemical Company. The green-gray powder was twice crystallized from water and subsequent washing with cold water.

Preliminary experiments indicated marked increase in fluorescence of ANS in buffer containing protein compared to aqueous buffer. To examine the effect of heat treatment on the 7S and 11S hydrophobicity, the proteins were made to 1.0 and 0.6 mg/ml, respectively. To this was added ANS to a level of 5×10^{-5}M. Heating was continued for 5 minutes in a water or oil bath at temperatures from 50 to 100°C in 10°C increments. The relative fluorescence of the cooled samples was measured at 365 nm excitation wavelength and scanning emission wavelength from 400 to 600 nm. The instrument, an Amicon Bowan Spectrophotofluoremeter, was standardized daily with 1 µg/ml of quinine sulfate at 365 nm excitation and 475 nm emission wavelengths.

Proteins which represented soy acid precipitated proteins were examined for various types of interactions as induced by heat treatment. Method of analysis involved adding reagents indicative of sulfhydryl-disulfide interchange, hydrogen bonding, and/or hydrophobic interaction. Heat treatments were: control (no heat treatment), 63°C for 5, 10 and 30 minutes, 74°C for 5, 10, and 15 minutes and 121°C for 5, 10, and 15 seconds, as described previously.

The 7S and 11S were made to 0.9 and 0.6 mg/ml, respectively. Sample trials involved addition of the selective reagent(s), allowing one hour for equilibration, addition of ANS to 5×10^{-5}M level, and heating for the required time followed by determination of the relative fluorescence.

The following treatments were used: (1) no chemicals added; (2) making the protein solution to 2 M urea; (3) making the protein sol 10 mM with respect to mercaptoethanol; (4) preparing a protein sol in a combination of 2 M urea and 10 mM mercaptoethanol; and (5) alkylation of sulfhydryl groups in the sol with N-ethylmaleimide (NEM) as described by Briggs and Wolf (1957). The latter procedure involved taking the protein solution, making it 10 mM with respect to mercaptoethanol and adding solid N-ethylmaleimide to 0.011 M while stirring, and continuing for an additional 30 minutes. The alkylated protein was recovered by precipitating at pH 4.6 and centrifuging at 16,000 x g for 20 minutes. The precipitate was picked up in phosphate buffer (pH 7.0, μ=0.1) and dialyzed against this buffer. Protein concentrations were adjusted to 0.9 mg/ml for soy 7S and 0.6 mg/ml for soy 11S.

To examine the effect of added calcium, similar protein concentrations were made to 0, 1, 3, and 8 mM calcium for the 7S protein and 0, 0.5, 1.0, and 1.5 mM calcium for the 11S in cacodylate buffer (pH 7.0, μ=0.1). The samples were equilibrated for 40 minutes, ANS added to a level of 5×10^{-5} M, and heat treated. Heating was carried out at: no heat treatment, 63°C for 30 minutes, 74°C for 15 minutes, and 121°C for 5 seconds. Finally, the samples were analyzed by fluorescence.

Additional information on hydrophobicity was obtained by use of increasing amount of salt to suppress hydrogen bonding and ionic effects. The 7S and 11S fractions were made to 0.9 and 1.0 mg/ml, respectively, in phosphate buffer (pH 7.0) with increasing ionic strength from 0.1 to 3.0 with sodium chloride. Once prepared, ANS was added to 5×10^{-5} M level and allowed to equlibrate for 30 minutes. Relative fluorescence determinations were then carried out.

Lysinoalanine (LAL)

Lysinoalanine was quantitated on a Beckman Amino Acid Analyzer Model 120C according to the procedure of Moore et al. (1958) with the exception that a 9 cm column was selected and eluted with citrate buffer, pH 5.28 (Koch, unpublished).

Details of hydrolysis, recovery and elution parameters may be found in the appendix.

The first sample examined was the APP fraction (dialyzed against deionized water and lyophilized). Four samples, 1% concentration, were divided into two lots, one adjusted to pH 6.8 and the other to pH 10.0. For each pH, half the sample was heated at 50°C for 30 minutes while the other sample remained at room temperature for 30 minutes. Following incubation the samples were frozen in dry ice-ethanol bath (the pH 10.0 samples were adjusted to ca pH 7 prior to freezing) and lyphilized at ambient temperature.

To examine if LAL was inherent to the bean, a sample of defatted soybean meal was also analyzed. A 1% sodium caseinate sol and a mixture of 1% APP-sodium caseinate were treated at pH 6.8, 50°C for 30 minutes to ascertain if LAL also was formed in the presence of sodium caseinate.

Protein determinations for all samples were performed by conversion of nitrogen as determined by micro-Kjeldahl analysis to protein (N x 6.25).

RESULTS

Analytical Ultracentrifugation

Analysis of soy APP fraction revealed four peaks (Figure 1a). Calculation of $S_{20,app}$ was determined for only the first three peaks due to the small amount of protein present and poor resolution of the fastest peak (Table 1). During the first part of the run, small spikes in the pattern were noticed and attributed to insoluble aggregates.

The modified procedures of Thanh et al. (1975a) and Koshiyama (1972a) for the isolation of the 7S protein showed minor contamination from the 2S component (Fibure 1b). The 7S monomer predominated in the high ionic strength phosphate buffer (pH 7.6, μ=0.5 in 10 mM mercaptoethanol). Polymerization of the 7S fraction at low ionic strength was shown to occur with phosphate buffer (pH 7.0, μ=0.1). This resulted in a $S_{20,app}$ value of 9.6 (Figure 1c). The 11S protein isolated according to the procedure of Eldridge and Wolf (1967) was shown to be pure by analytical ultracentrifugation (Figure 1d). Since no reports have indicated polymerization at low ionic strength, the 11S protein was examined only in high ionic strength phosphate buffer (pH 7.6, μ=0.5 in 10 mM mercaptoethanol).

Table 1. Sedimentation Coefficient (apparent) Obtained by Analytical Ultracentrifugation for Various Soy Proteins.

Sample	Buffer	Protein Concentration	$S_{20,app}$
APP	phosphate, pH 7.6 μ= 0.5 10 mM mercaptoethanol	1.25%	2.0 6.8 10.7 -15S-
7S	phosphate, pH 7.6 μ=0.5 10 mM mercaptoethanol	0.9%	2.0 6.8
7S(9S)	phosphate, pH 7.0 μ=0.1	1.0%	1.8 9.6
11S	phosphate, pH 7.6 μ=0.5 10 mM mercaptoethanol	0.9%	11.1

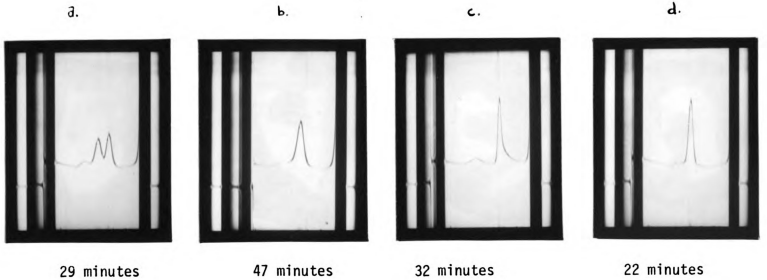


Figure 1. Ultracentrifuge patterns of various components of Corsoy (1975) soybeans. (a) APP (1.25%) in phosphate buffer, pH 7.6, μ=0.5 in 10 mM mercaptoethanol; (b) 7S (0.9%) in phosphate buffer, pH 7.6, μ=0.5 in 10 mM mercaptoethanol; (c) 7S-dimer (1.0%) in phosphate buffer, pH 7.0, μ=0.1; (d) 11S (0.75%) in phosphate buffer, pH 7.6, μ=0.5 in 10 mM mercaptoethanol. Centrifuge speed was 52,640 rpm and pictures taken as indicated.

Effect of Heat on Soy Protein Systems

Identification of Gel Electrophoretic Bands

The identification of soy proteins on polyacrylamide gels was determined by application of a single purified preparation (7S and 11S) on 5% gels and noting the patterns that resulted. Soy 7S corresponded to three (possibly four) bands on Cyanogum 41 gels. The 11S molecule appeared as a single band, with slightly higher mobility than the 7S bands. The 2S component, due to its low molecular weight, moved with the marker dye, while the large molecular weight 15S remained on top of the gel (Figure 2).

Effect of Heat on Proteins as Revealed by Gel Patterns

A 1% solution of soy acid-precipitated protein was heated for 5 minutes over a temperature range of 50 to 110°C (Figure 2). The gels corresponding to the control, 50 and 60°C, showed no alterations. At 70°C, the first indication of 7S aggregation was noted by diminished intensity of the 7S bands. At 80°C, further decrease in band intensity occurred, while at 90°C and above, little if any 7S protein remained detectable. The 11S band faded slightly at 80°C and at 90°C and above vanished completely. Disappearance of the 11S was accompanied by new band formation with higher mobility. The 2S protein faded slightly at 90, 100 and 110°C temperatures. The fate of the 15S was inconclusive.

Effect of Heat on Soy 7S and 11S Components in Presence of Milk Proteins

Polyacrylamide gels were used to demonstrate the difference in heat stability of soy 7S and 11S fractions following heat treatment at 63, 74, and 121°C (Figure 3a and 3b). The bands representing the 7S began to

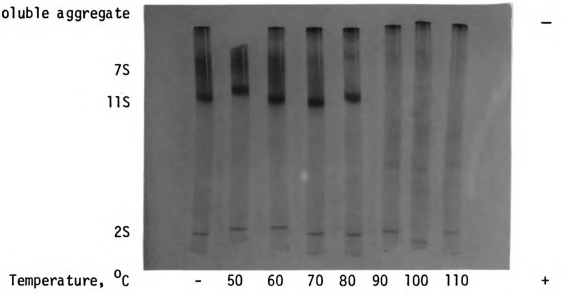


Figure 2. Effect of temperature on gel electrophoresis patterns of soy APP. Heating time: 5 minutes; 5% gels.

disappear after heating 30 minutes at 63°C, with complete disappearance at 74 and 121°C. Although the three bands representing the 7S decreased, an increased amount of protein staying on top of the gel was noticed. Determination of protein in the supernatant following centrifugation at 20,000 x g for 30 minutes, showed no decrease in solubility at any time-temperatures examined. Therefore, the protein not entering the gel was referred to as the 7S soluble aggregate. The soy 11S (Figure 3b) was stable at 63 and 74°C, while at 121°C (for 5, 10, and 15 seconds) the band disappeared with the formation of a faster moving band.

The free (available) and total (exposed by urea treatment) sulfhydryl content for components 7S and 11S were examined to ascertain the involvement of these groups in aggregation (Figure 4). The 7S contained 3.4 free and 3.5 total μM -SH/g protein in the unheated samples. With increasing heat treatment the sulfhydryl values rose slightly, followed by a decrease at 121°C - 15 seconds to 1.4 μM -SH/g protein. The 11S control contained 2.5 and 6.5 μM/g protein for free and total sulfhydryl concentration, respectively. Upon heating, both values decreased to zero and remained there for subsequent time-temperature treatments.

Soy-milk protein mixtures were examined to determine the relative stability of one in the presence of the other. The 7S, when combined with milk proteins, showed little change in solubility at the three temperatures of interest (Figure 5). The 7S/sodium caseinate mixture was slightly more soluble than combinations of 7S with β-lactoglobulin or whey proteins. Similarly, at 63 and 74°C, the 11S combined with the milk fractions resulted in no decrease in protein stability. However, at 121°C, the solubility of 11S/β-lactoglobulin and whey protein mixtures dropped below 80%. The 11S/sodium caseinate mixture remained soluble above 90%

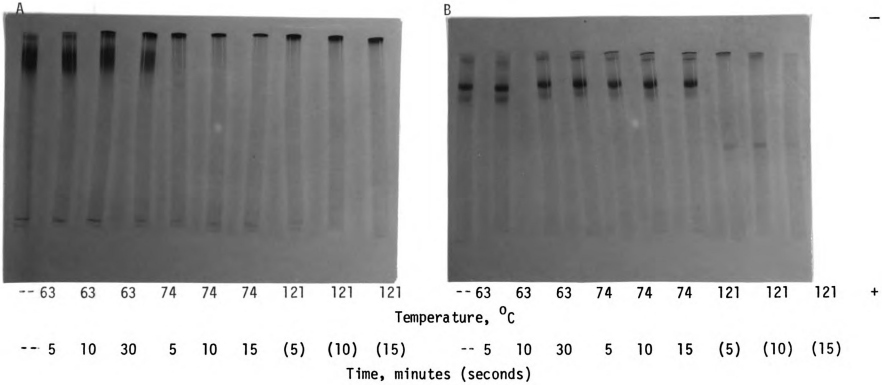


Figure 3. Effect of heat treatment on gel electrophoretic patterns of soy 7S (A) and 11S (B). Gel concentrations: A - 5%; and B - 7%.

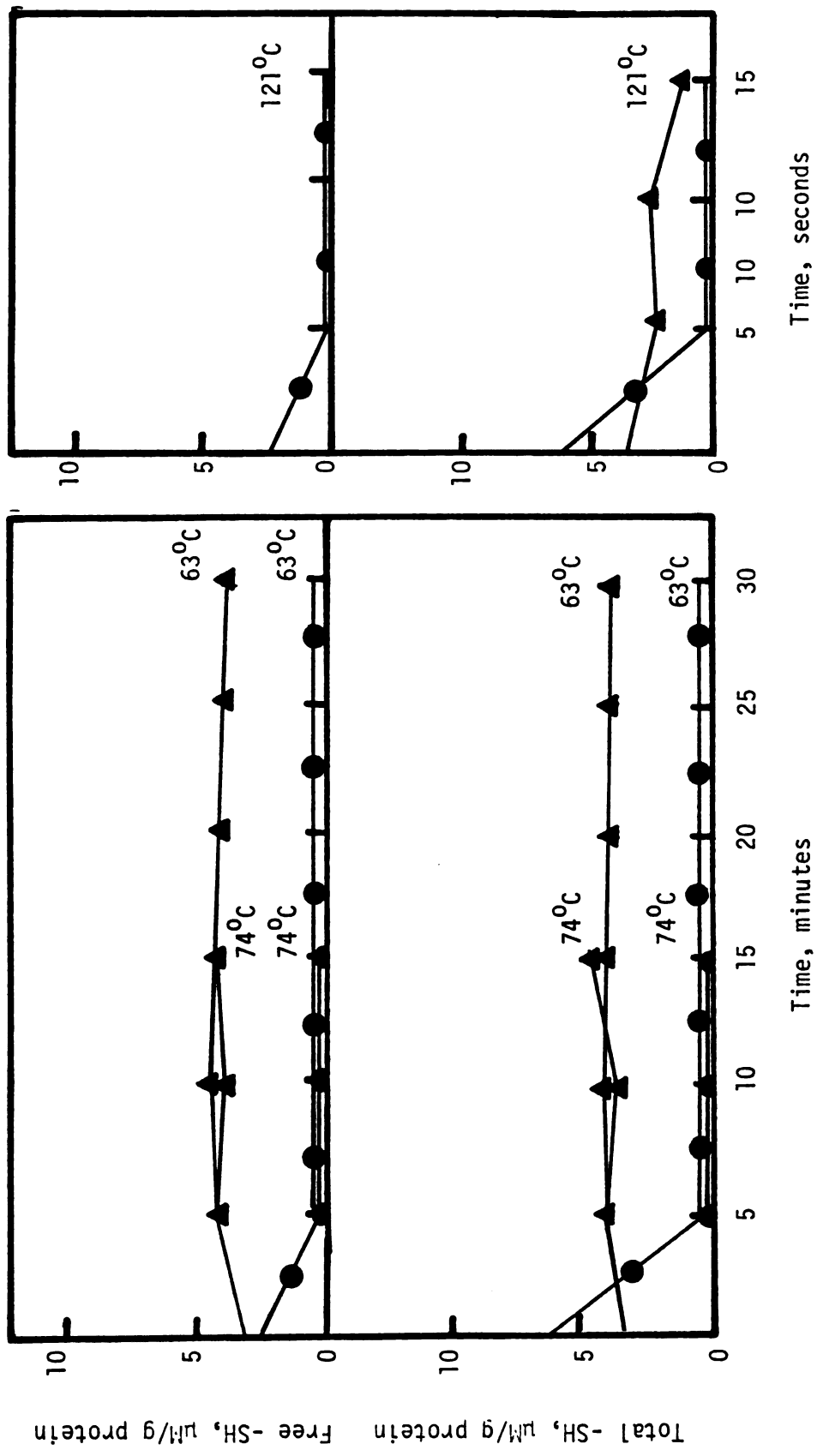


Figure 4. Effect of time-temperature heat treatment on free and total sulfhydryl content of soy 7S (▲—▲) and 11S (●—●).

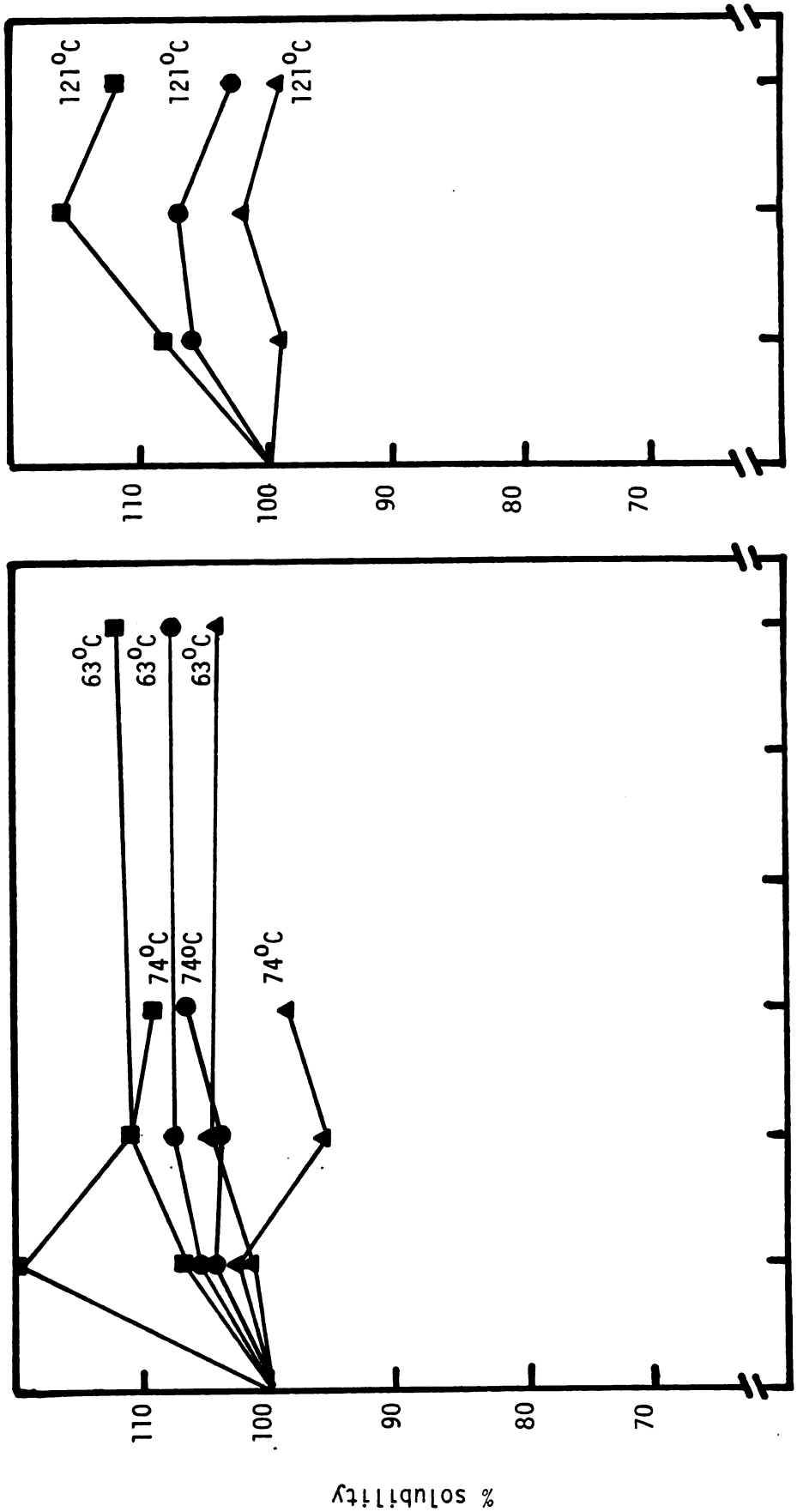


Figure 5. Effect of time-temperature on solubility of mixtures of 7S protein with milk proteins. Legend: 7S/sodium, caseinate (■—■), 7S/β-lactoglobulin (●—●), and 7S/whey proteins (▲—▲).

(Figure 6).

Polyacrylamide gel electrophoresis was used to examine the effect of heat treatment on mixtures of 7S protein with sodium caseinate and β-lactoglobulin (Figure 7 and 8). The gel bands corresponding to the 7S molecule diminished in intensity at 63°C - 30 minutes and vanished at higher temperatures. An increase in the amount of protein not entering the gel was noted with the disappearance of the 7S protein bands, even in the presence of the two milk proteins.

The soy 11S component was found to move independently of the milk proteins in gel electrophoresis when combined with β-lactoglobulin (Figure 10) or sodium caseinate (Figure 9). In both cases the 11S was stable at 63 and 74°C, while at 121°C subunit formation was noticed as minor band formation increased in the non-urea gels. Sodium caseinate in combination with soy 11S showed little decrease in the two major 11S subunit bands in urea polyacrylamide gels (Figure 9).

To examine the influence of heat treatment on the total sulfhydryl content, the soy 7S and 11S proteins were combined with a sulfhydryl containing protein, β-lactoglobulin (Figure 11). The curves for 7S/β-lactoglobulin and 11S/β-lactoglobulin mixtures were approximately midway between the curves representing the sample constituents.

Effect of Heat and Calcium on Soy Components (7S and 11S) in Combination with Milk Proteins

The 7S and 11S fractions make up the majority of the reserve soy proteins and thus greatly affect solution characteristics when one or both are altered. For this reason the influence of calcium on the two soy proteins was examined (Figure 12). The 7S was found to be stable up to 8 mM calcium concentration when heated at 63 and 74°C. However,

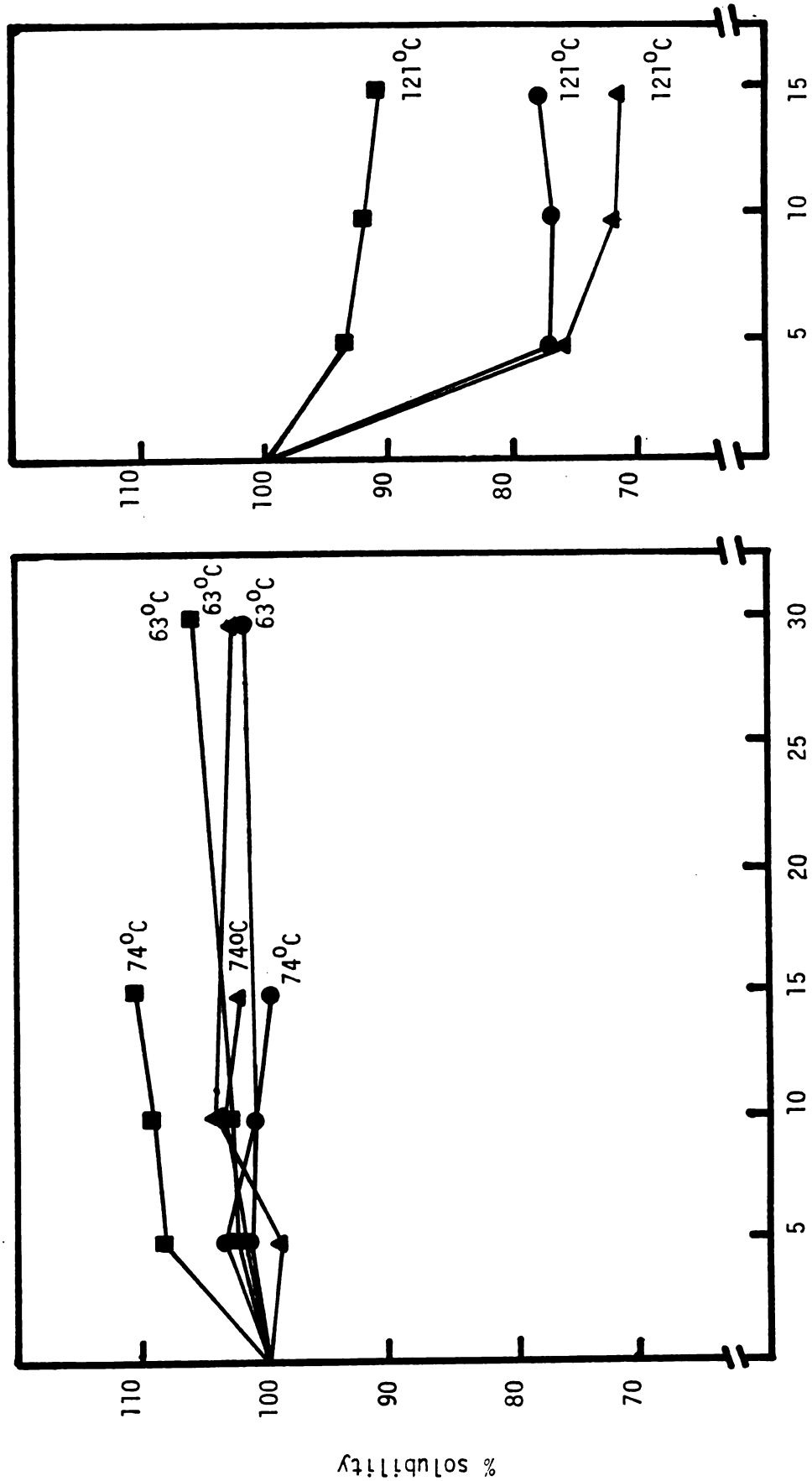


Figure 6. Effect of time-temperature treatments on solubility of mixtures of 11S with milk proteins. Legend: 11S/sodium caseinate (■—■), 11S/β-lactoglobulin (●—●), and 11S/whey proteins (▲—▲).

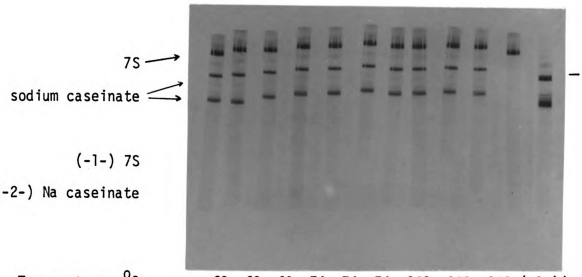


Figure 7. Effect of time-temperature treatments on PAGE patterns of a mixture of 7S protein and sodium caseinate. Gel concentration: 10%-urea gels.

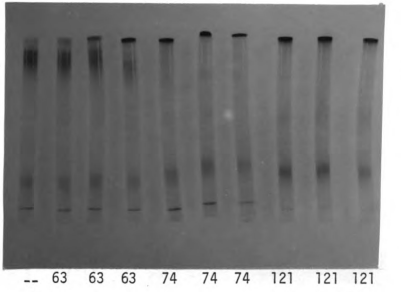


Figure 8. Effect of time-temperature treatments on PAGE patterns of a mixture of soy 7S and β-lactoglobulin. Gel concentration: 6%.

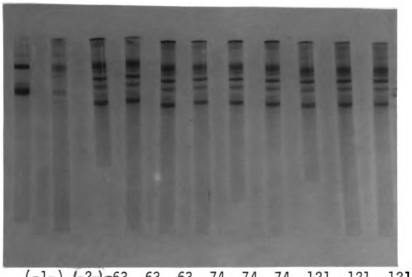


Figure 9. Effect of time-temperature treatments on PAGE patterns of mixtures of soy 11S and sodium caseinate. Gel concentrations: 10%-urea gels.

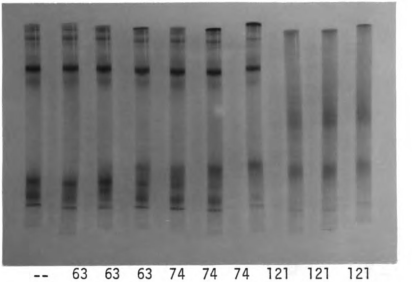
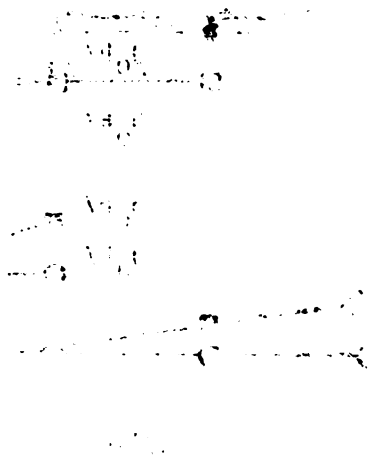


Figure 10. Effect of time-temperature treatments on PAGE patterns of a mixture of soy 11S and β-lactoglobulin. Gel concentrations: 7%.



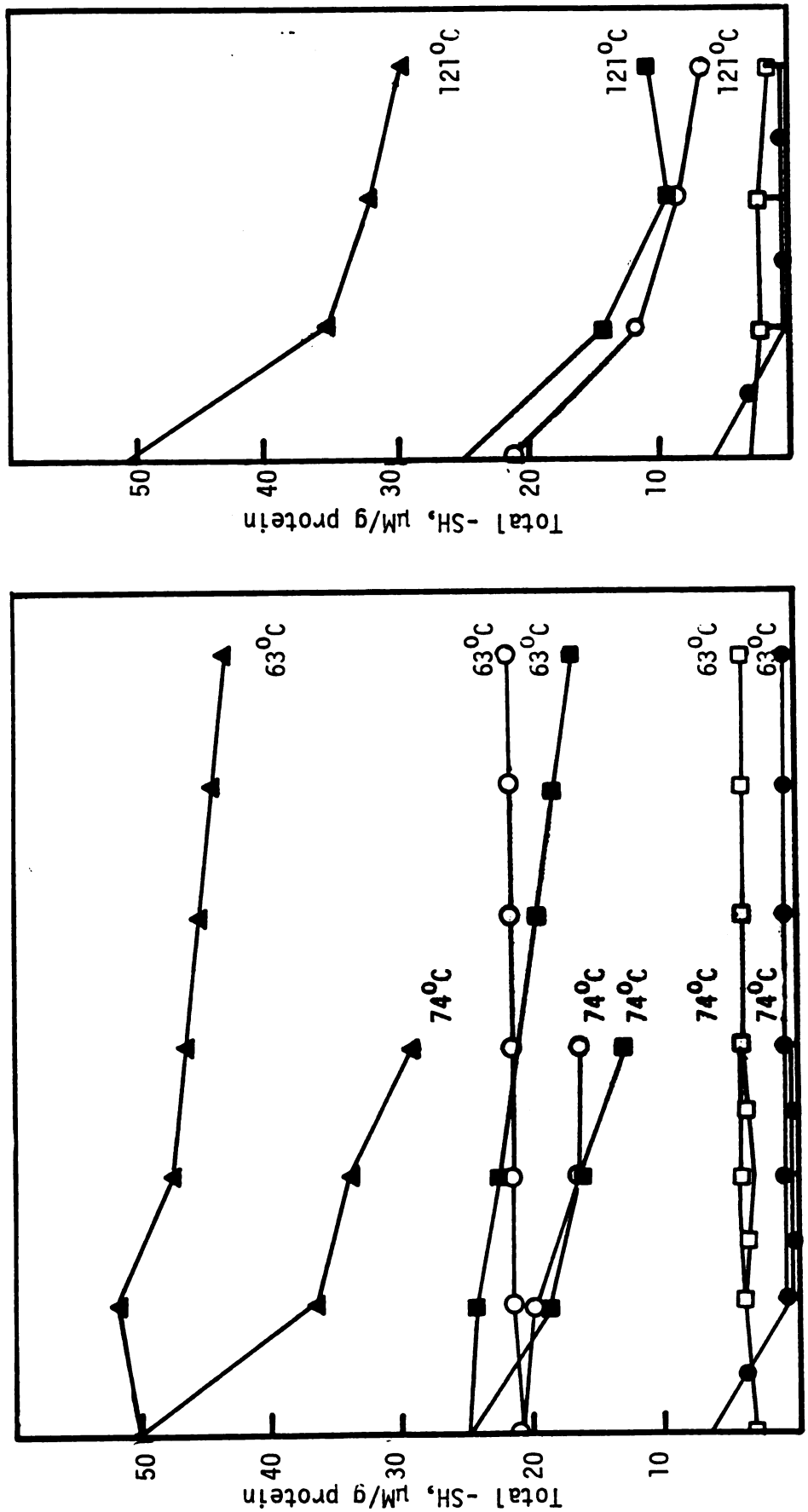
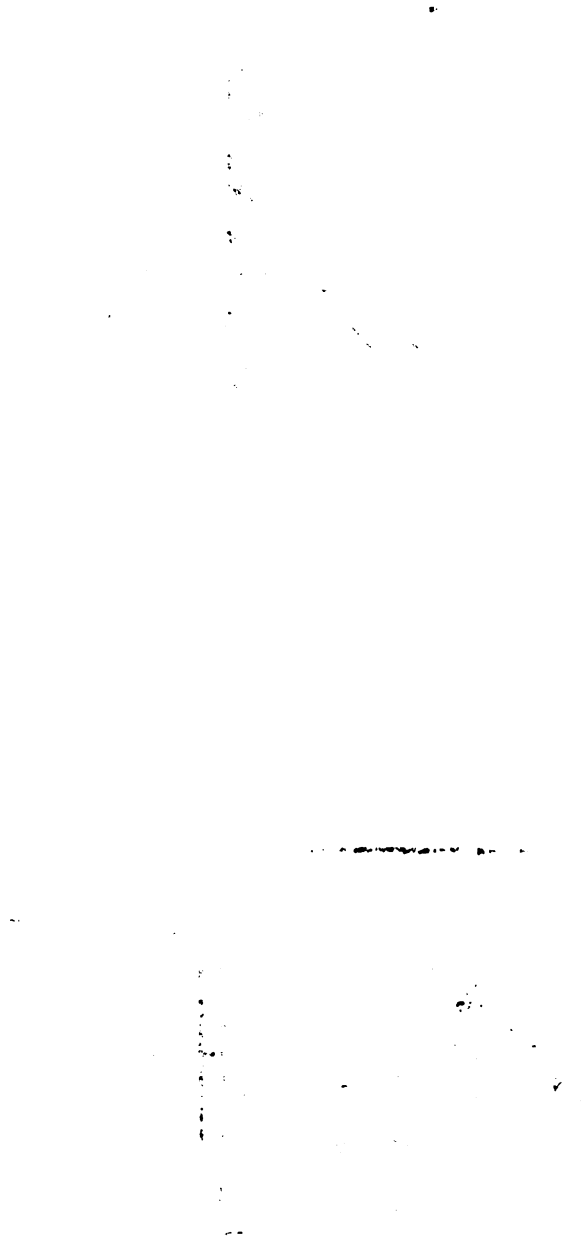


Figure 11. Effect of time-temperature on total sulfhydryl content of β-lactoglobulin combined with soy 7S and 11S. Legend: β-lactoglobulin (▲—▲), 7S (□—□), 11S (●—●), 7S/β-lactoglobulin (○—○), and 11S/β-lactoglobulin (■—■).



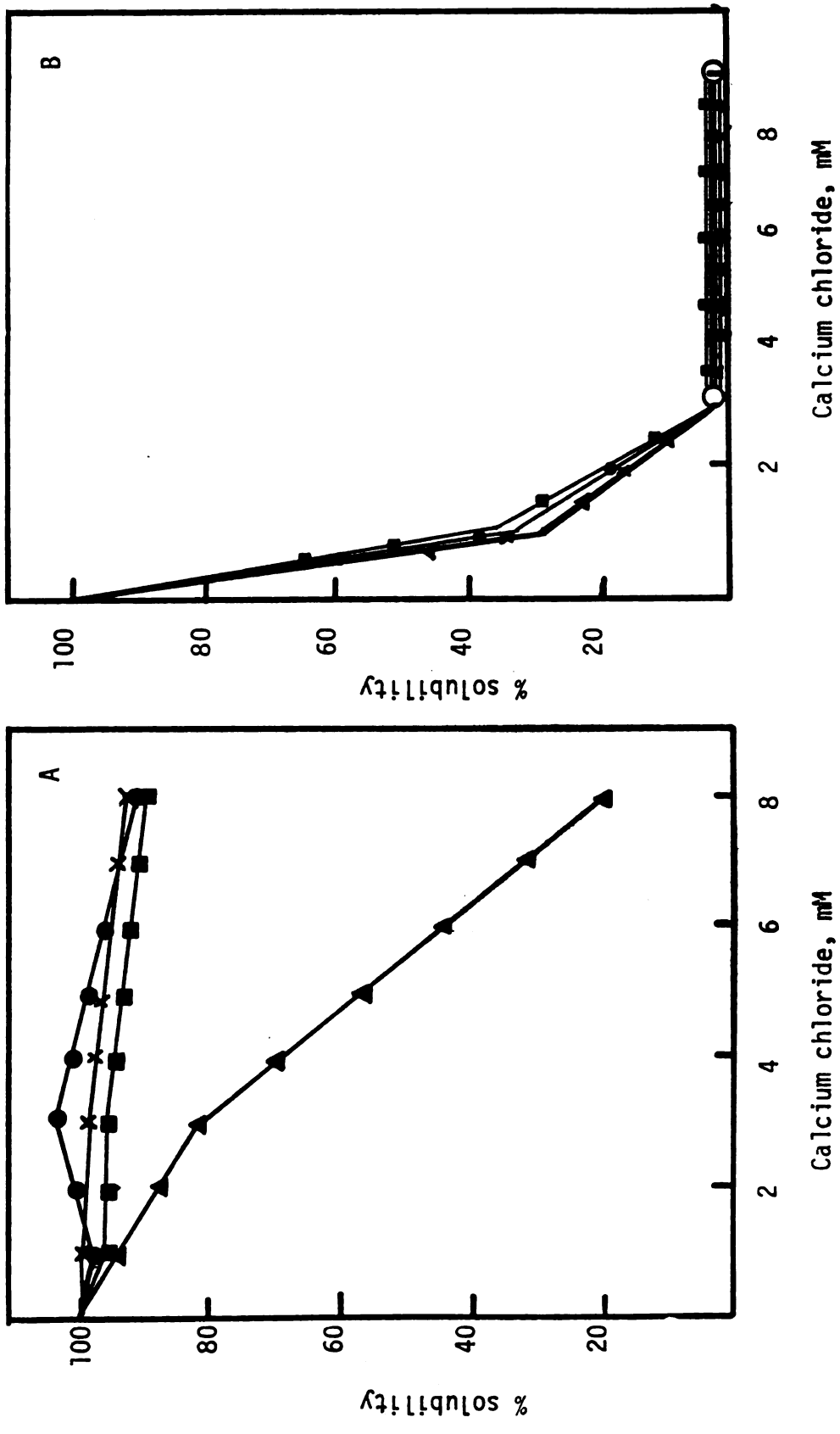


Figure 12. Effect of calcium and heat treatment on soy 7S (A) and 11S (B). Legend: no heat treatment (✱—✱), 63°C - 30 minutes (●—●), 74°C - 15 minutes (■—■), and 121°C - 5 seconds (▲—▲).

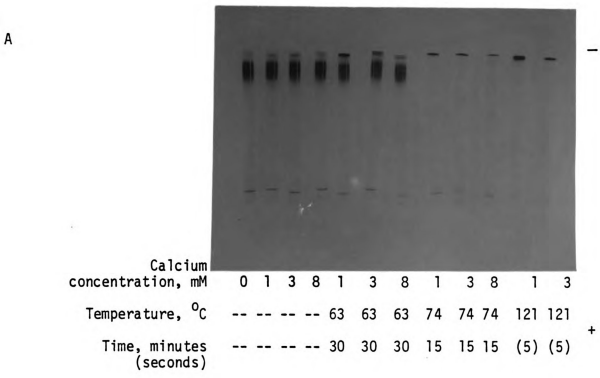
an appreciable decrease in protein stability was noticed when this protein was treated with 8 mM Ca^{++} heated at 121°C for 5 seconds. Gel electrophoresis revealed the formation of a soluble aggregate at 74 and 121°C (Figure 13a).

The 11S showed great sensitivity to calcium with or without heat treatment (Figure 12b). One mM calcium, with no heat treatment of the protein, resulted in a 70% loss of solubility following centrifugation at 20,000 x g. Similarly, heating of the protein sol at 63, 74, and 121°C in the presence of 1 mM Ca^{++} reduced solubility from 66 to 71%. The next higher concentration of calcium, 3 mM, resulted in complete loss of 11S solubility at all temperatures examined. These results were substantiated by gel electrophoresis (Figure 13b).

Similar studies with one-to-one ratios of soy/milk protein combinations in the presence of calcium suggested the predominating influence on 11S soy protein stability was calcium. The 7S/milk protein combination had higher stability than the corresponding 11S/milk protein mixtures at all four temperatures examined (Figure 14 and 15).

The 7S when combined at ambient temperature with any of the milk proteins used in this research and heated at 63°C - 30 minutes showed solubility greater than 85%. With the greater heat treatment of 74°C - 15 minutes or 121°C - 5 seconds, the stabilizing effect of sodium caseinate was noted. Although calcium concentration ranged from 1 to 8 mM calcium the 7S was always present when examined by gel electrophoresis (Figure 16 and 17).

The 11S was the more sensitive soy protein with regard to calcium content, singularly and in combination with milk proteins. The effect of increasing heat treatment on solubility of this component was shown



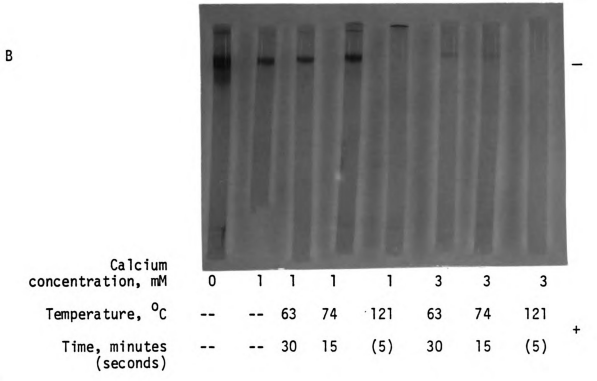


Figure 13. Effect of calcium and time-temperature treatment on PAGE patterns of soy 7S (A) and 11S (B). Gel concentrations: A - 6%; B - 7%.

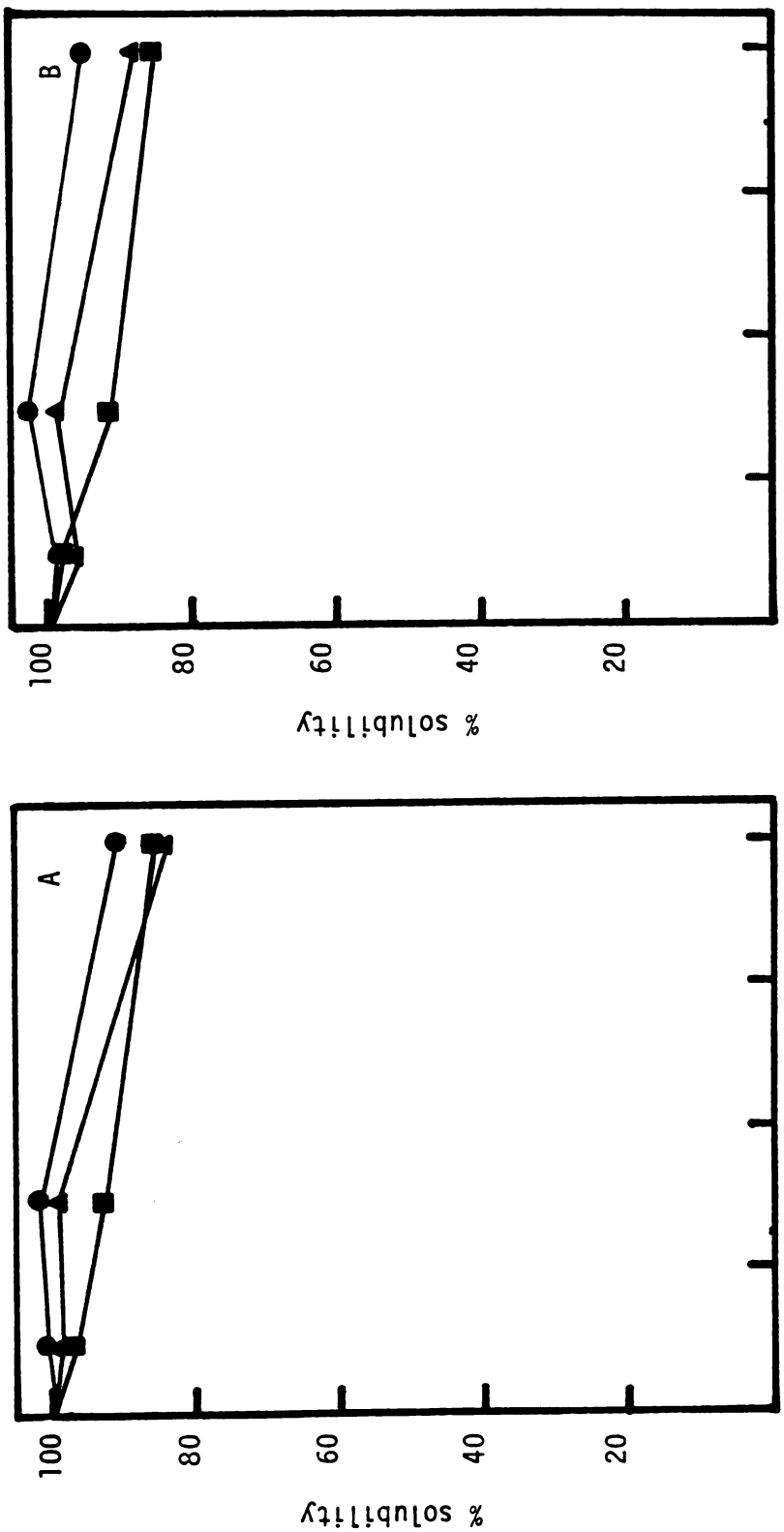


Figure 14. Effect of calcium on stability of heat treated 7S-milk protein mixtures. Identification of graphs: (A) no heat treatment; (B) 63°C - 30 minutes; (C) 74°C - 15 minutes; (D) 121°C - 5 seconds. Legend: 7S-sodium caseinate (■—■); 7S-β-lactoglobulin (●—●); and 7S-whey proteins (▲—▲).

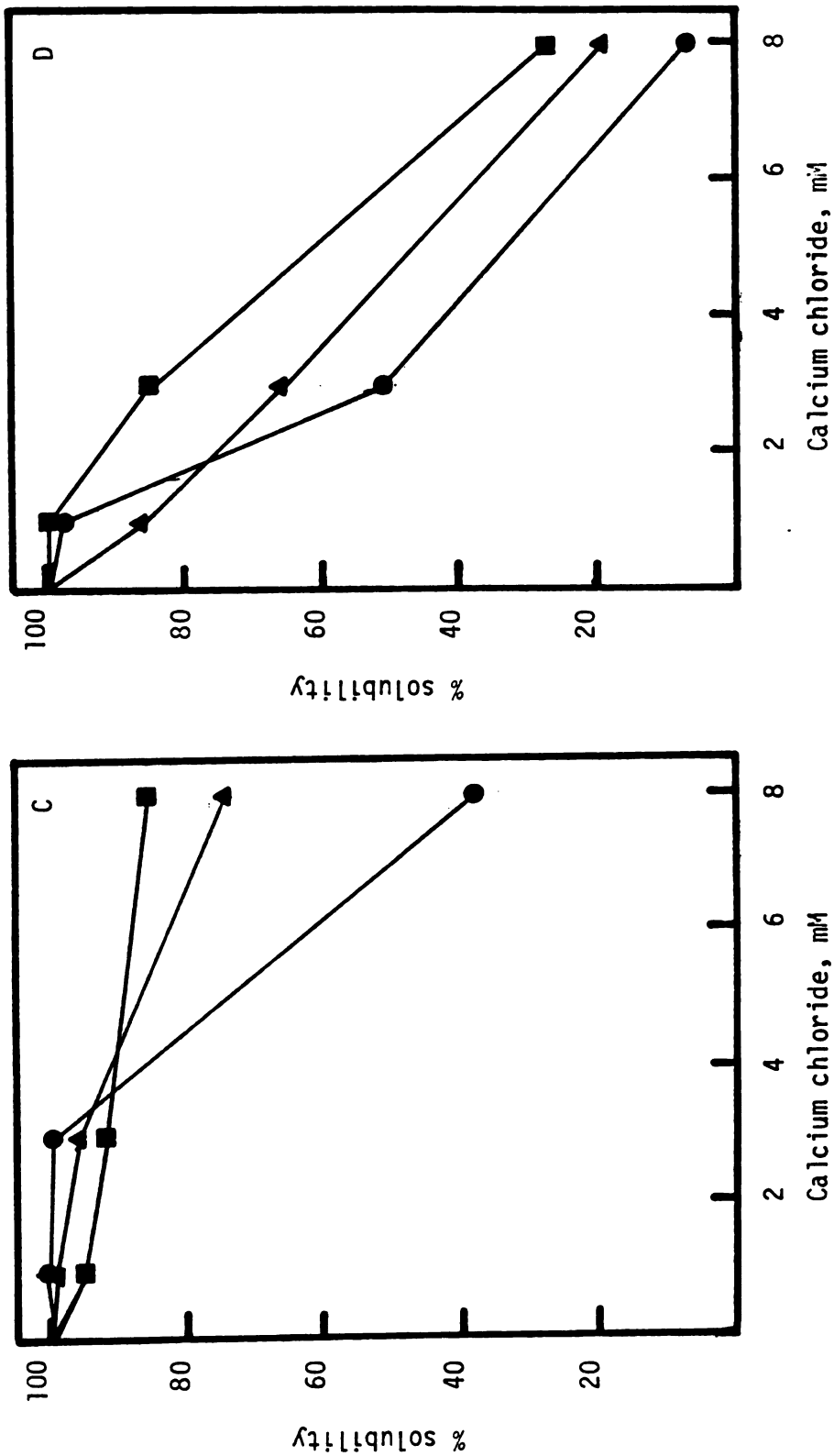


Figure 14 (continued)

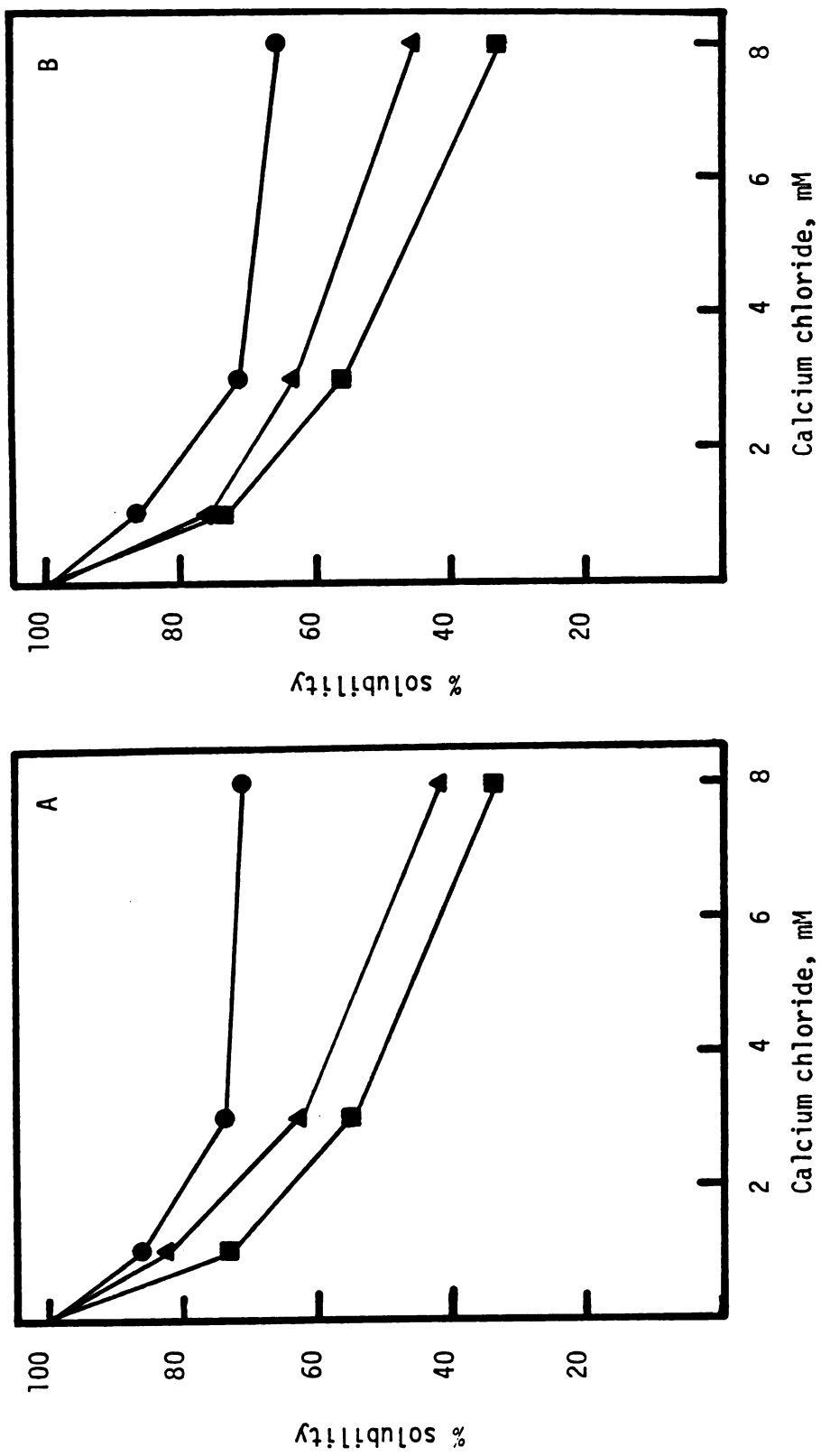


Figure 15. Effect of calcium on stability of heat treated 11S protein combinations with milk proteins. Identification of graphs: (A) no heat treatment; (B) 63°C - 30 minutes; (C) 74°C - 15 minutes; and (D) 121°C - 5 seconds. Legend: 11S-sodium caseinate (■—■); 11S-β-lactoglobulin (●—●); and 11S-whey proteins (▲—▲).

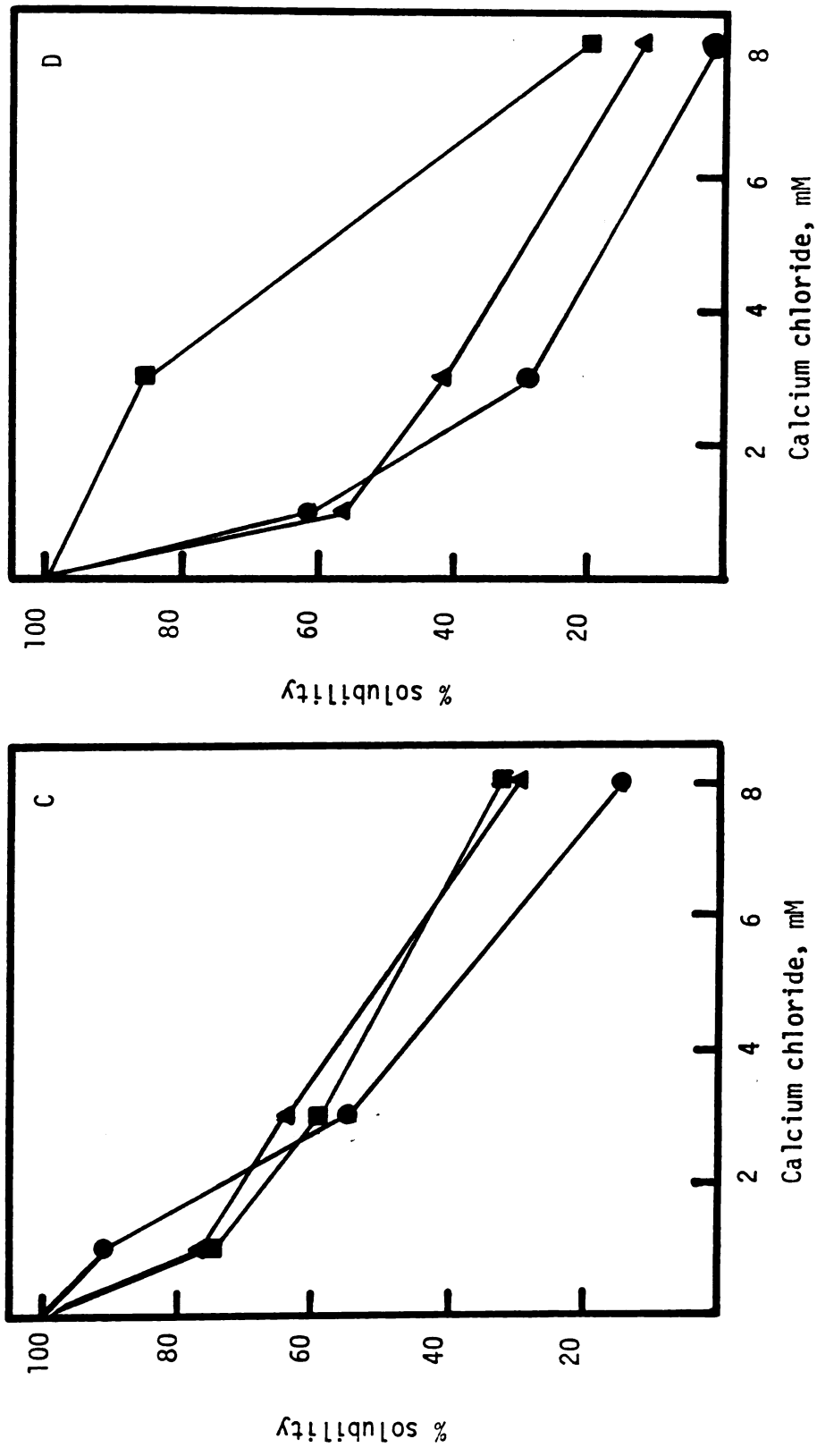
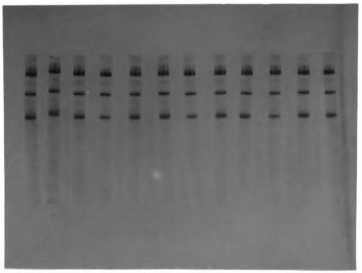
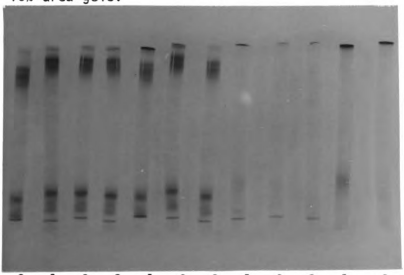


Figure 15 (continued).



Calcium concentration, mM	1	1	3	8	1	3	8	1	3	8	1	3
Temperature, ^{o}C	--	--	--	--	63	63	63	74	74	74	121	121
Time, minutes (seconds)	--	--	--	--	30	30	30	15	15	15	(5)	(5)

Figure 16. Effect of calcium and time-temperature treatment on PAGE patterns of soy 7S/sodium caseinate mixtures. Gel concentrations: 10%-urea gels.



Calcium concentration, mM	0	1	3	8	1	3	8	1	3	8	1	3
Temperature, ^{o}C	--	--	--	--	63	63	63	74	74	74	121	121
Time, minutes (seconds)	--	--	--	--	30	30	30	15	15	15	(5)	(5)

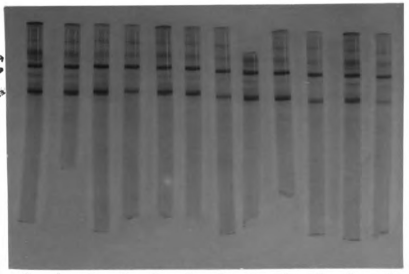
Figure 17. Effect of calcium and time-temperature treatment on PAGE patterns of soy 7S/β-lactoglobulin protein mixtures. Gel concentrations: 6%.

in Figure 15. Mixtures of soy 11S and whey proteins or β-lactoglobulin heated at 63°C for 30 minutes had the greatest solubility in the 1 to 8 mM calcium range. However, at higher temperatures and higher calcium concentrations, 11S/sodium caseinate mixtures were most soluble, particularly noticeable at 121°C - 5 seconds. The 11S/sodium caseinate mixture when examined by urea gel electrophoresis revealed a marked decrease in the major subunit representing the 11S as calcium concentration increased. However, the 11S was still present in samples treated with 8 mM calcium (Figure 18). In Figure 19, the 11S, when combined with β-lactoglobulin, showed complete loss of the 11S at 8 mM calcium, while a faint band was noticed at 1 and 3 mM calcium.

Investigation of Hydrophobicity of Soy Protein by use of 8-anilino-1-naphthalene Sulfonate (ANS)

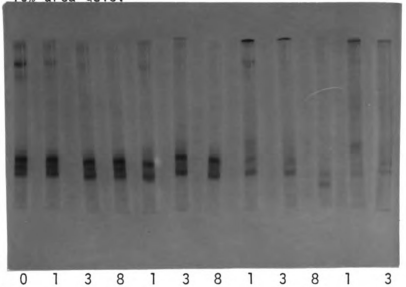
ANS was used to investigate hydrophobic interactions in soy 7S and 11S molecules. Heat, ionic strength, calcium and selective reagents for hydrogen bonding and sulfhydryl-disulfide interchange were used to determine if conformational changes occurred and possibly what caused such transformations. In phosphate buffer (pH 7.0, μ=0.1) the fluorescent probe revealed a low relative fluorescence (RF) of 2 units with a maximum emission wavelength of 525 nm. When soy 7S and 11S were incorporated into the system, the RF increased with a corresponding shift of the maximum emission wavelength in the blue direction (Figure 20).

Soy 7S and 11S were investigated by ANS fluorescence to determine heat effects on protein conformation. The 7S protein (Figure 20a) with no heat treatment resulted in 25 RF units. Increasing the heat treatment resulted in an increase of RF and a blue shift in the maximum emission wavelength. Heating at 74°C - 15 minutes resulted in the highest RF with



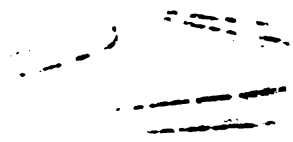
Calcium concentration, mM	0	1	3	8	1	3	8	1	3	8	1	3
Temperature, °C	--	--	--	--	63	63	63	74	74	74	121	121
Time, minutes (seconds)	--	--	--	--	30	30	30	15	15	15	(5)	(5)

Figure 18. Effect of calcium and time-temperature treatments on PAGE patterns of soy 11S/sodium caseinate mixtures. Gel concentrations: 10%-urea gels.



Calcium concentration, mM	0	1	3	8	1	3	8	1	3	8	1	3
Temperature, °C	--	--	--	--	63	63	63	74	74	74	121	121
Time, minutes (seconds)	--	--	--	--	30	30	30	15	15	15	(5)	(5)

Figure 19. Effect of calcium and time-temperature treatment on PAGE patterns of soy 11S/β-lactoglobulin mixtures. Gel concentrations: 7%.



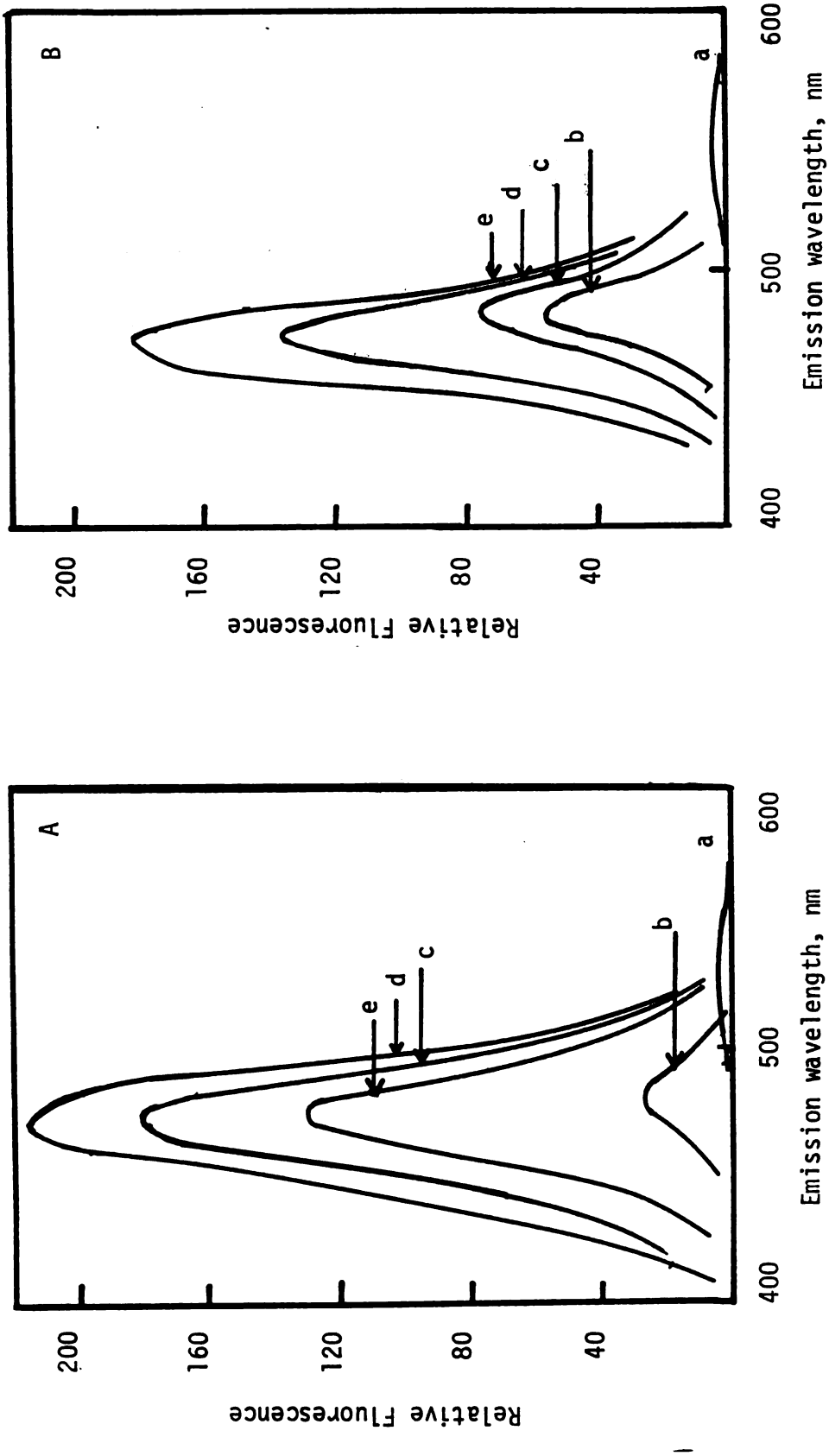


Figure 20. Effect of time-temperature treatment on ANS fluorescense of 7S (A) and 11S (B). Legend: (a) phosphate buffer - no protein; (b) no heat treatment; (c) 63°C for 30 minutes; (d) 74°C for 15 minutes; (e) 121°C for 5 seconds. Protein concentrations: 7S - 0.9 mg/ml; 11S - 0.6 mg/ml.

a value of 215 units, while 121°C - 5 seconds showed the second highest value of 179 units. The 11S protein also increased the RF value and shifted the maximum emission wavelength in the blue direction. The relative fluorescence increased in the same order as the temperature of heat treatment. The 11S treatment at 121°C - 5 seconds resulted in the highest value of 179 RF units, while no heat treatment resulted in a RF of 54 units.

ANS was used to investigate the critical temperature of conformational change that occurred in purified soy 7S and 11S (Figure 21). The 7S had two transitions; the first occurred at 70°C and the second at 90°C. The 11S was more stable to heat treatment as measured by ANS fluorescence and showed a large increase in hydrophobicity at 80°C with subsequent rapid decrease at 100 and 110°C.

Cacodylate buffer (pH 7.0, μ=0.1) was used to examine the effect of calcium on soy 7S and 11S hydrophobicity. Heating times and temperatures were similar to those previously mentioned. The soy 7S, which was shown to be more stable to calcium, was examined at 1, 3, and 8 mM concentrations (Figure 22a). The relative fluorescence remained constant for samples receiving no heat treatment and for those heated at 63°C - 30 minutes. Fluorescence following treatment at 74°C - 15 minutes was highest, ranging from 228 to 261 RF units with increased calcium concentration. The 7S treated at 121°C - 5 seconds exhibited slightly less fluorescence when treated at 74°C - 15 minutes and ranged from 180 to 253 RF units with increased calcium. A similar temperature profile of relative fluorescence for the 7S protein was seen in the absence of calcium (Figure 20a). Due to the sensitivity of the 11S protein to calcium, a lower concentration of calcium was selected--0 to 1.5 mM calcium (Figure 22b). Samples treated without heat and heated at 63°C - 30 minutes were identical,

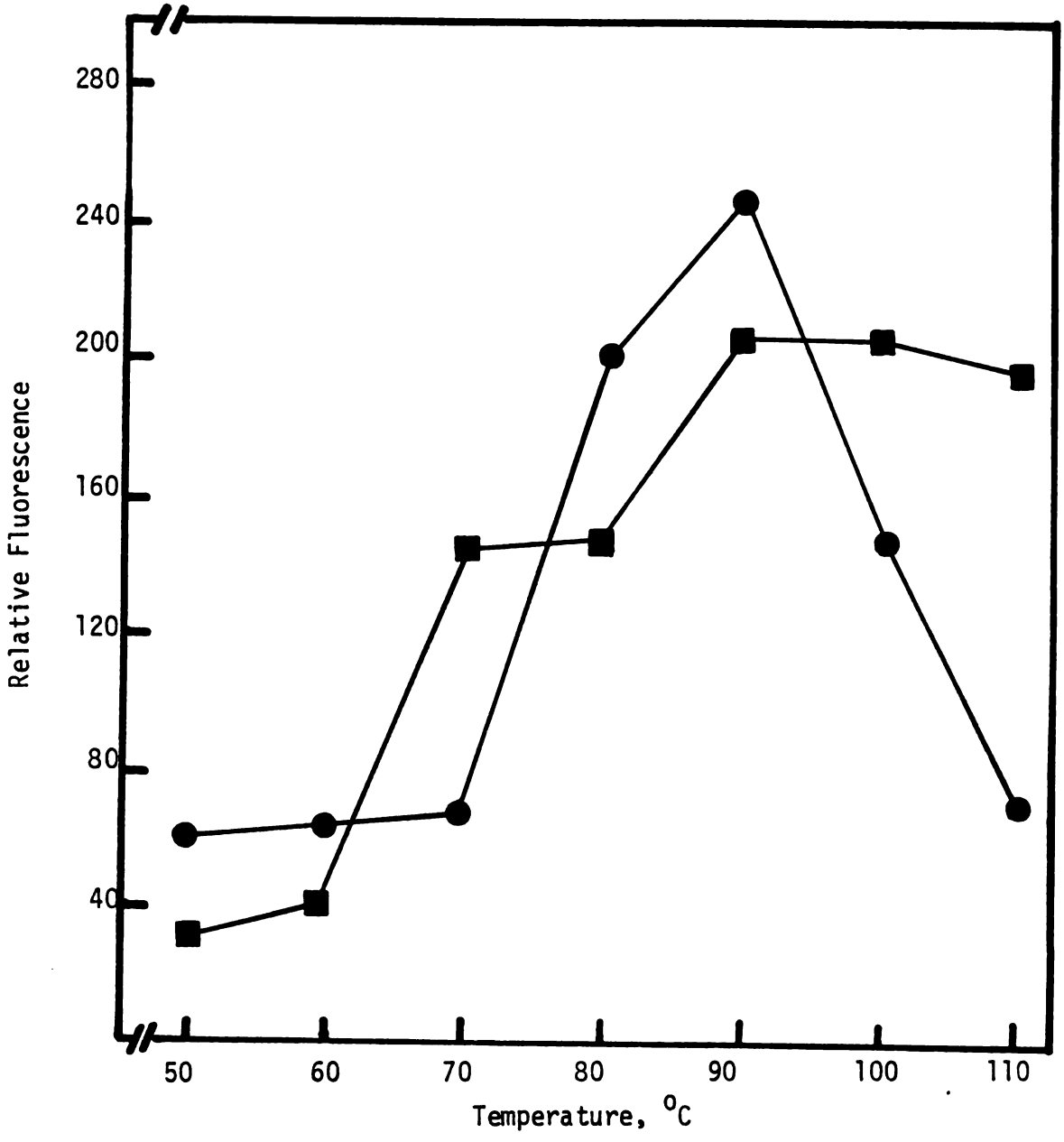


Figure 21. Effect of temperature on ANS fluorescence in the presence of 7S (■—■) and 11S (●—●). The concentrations: 7S - 1.0 mg/ml; 11S - 0.6 mg/ml; ANS - 50 μM. Heating time: 5 minutes.

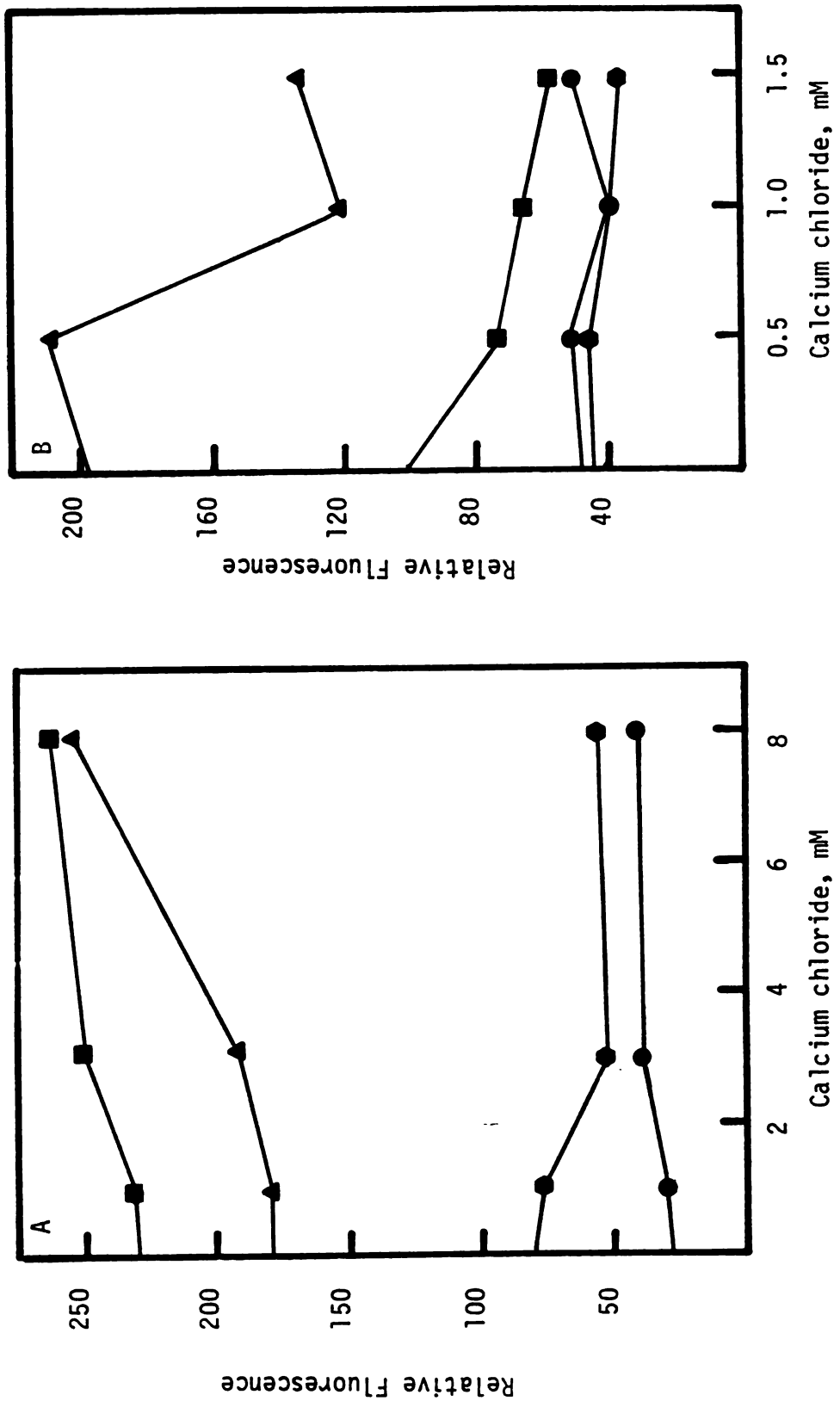


Figure 22. Effect of calcium on ANS fluorescence of soy 7S (A) and 11S (B) as influenced by various heat treatments: no heat (●—●); 63°C for 30 minutes (⬢—⬢); 74°C for 15 minutes (■—■); and 121°C for 5 seconds (▲—▲). The concentrations: 7S - 0.9 mg/ml; 11S - 0.6 mg/ml; and ANS - 50 µM.

ranging from 39 to 51 RF units. Heating at 74°C - 15 minutes caused a decrease in hydrophobicity with increased calcium; RF units fell from 196 with zero calcium to 135 RF units with 1.5 mM calcium. The higher calcium concentration was found to cause a slight turbidity in the system.

Possible hydrophobic interactions of soy proteins have been suggested because of heat stability in the presence of sodium caseinate and the negligible involvement of sulfhydryl groups. To further examine this possibility the purified 7S and 11S soy proteins were treated in the presence of various reagents to determine which would cause the greatest alteration in ANS fluorescence. The proteins were examined following equilibration with solutions of: no reagents (control); 10 mM mercaptoethanol; 2 M urea; a combination of 2 M urea and 10 mM mercaptoethanol; and, finally, reduced and alkylated with N-ethylmaleimide.

The 7S fractions were examined following heat treatment at ambient temperature, 63, 74, and 121°C (Figure 23). At 63°C - 30 minutes, RF values lower than the control were noted in samples made to 2 M urea and in samples made to 2 M urea - 10 mM mercaptoethanol. The reduced RF value following these two treatments were more noticeable when samples were heated at 74°C - 15 minutes and 121°C - 5 seconds. In all heat treatments the samples with 10 mM mercaptoethanol were similar to those of the control.

The 11S, when treated with 2 M urea and 2 M urea-10 mM mercaptoethanol mixture, reacted similar to the 7S protein at 121°C - 5 seconds. The lower temperatures resulted in a random arrangement of curves with no marked deviation from the control (Figure 24).

Additional evidence was found for the hydrophobic nature of the soy

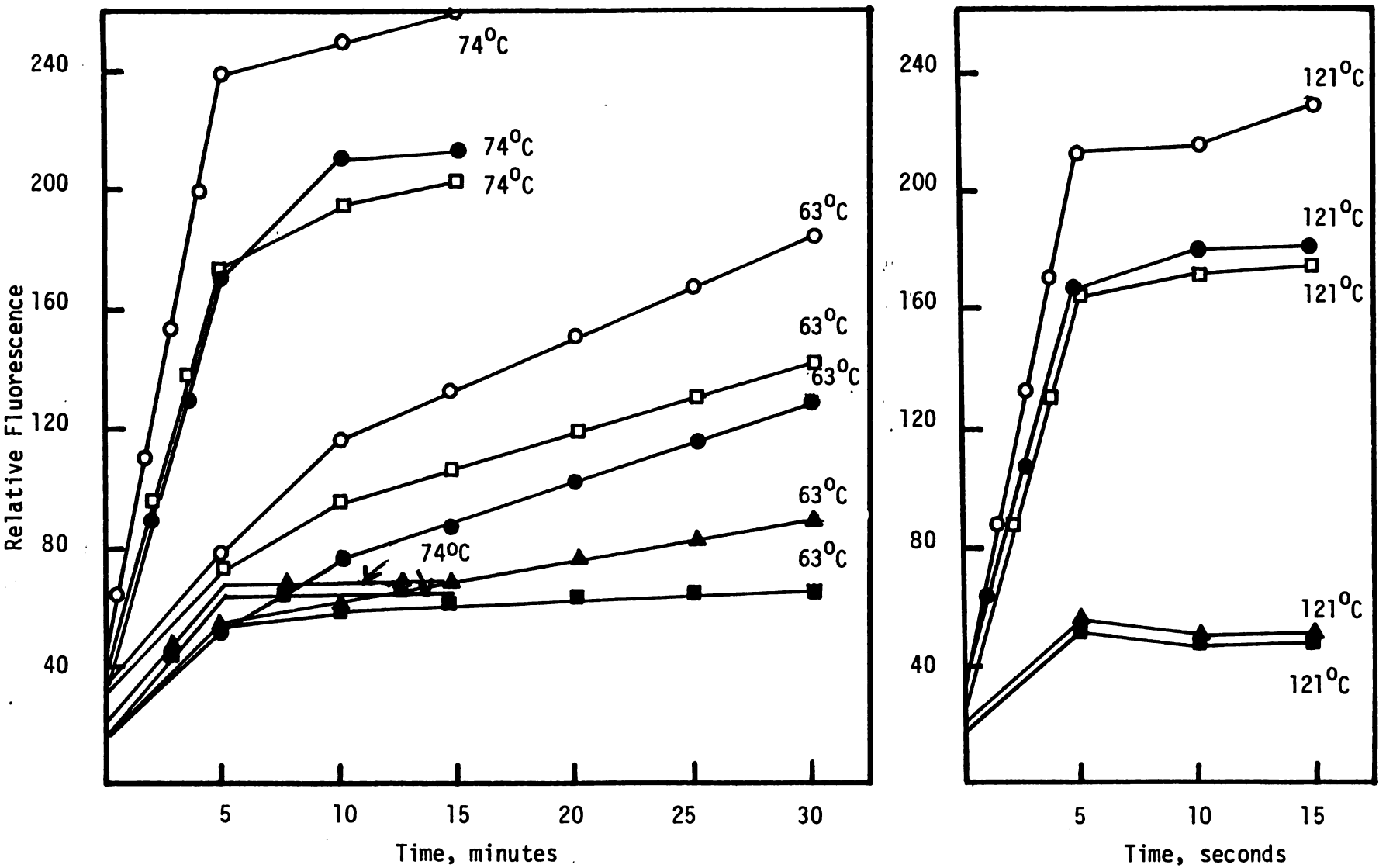


Figure 23. Effect of various reagents on ANS fluorescence of the 7S protein heated at 63, 74, and 121°C. Reagents are: control (●—●); 10 mM mercaptoethanol (□—□); 2 M urea (▲—▲); combination of 2 M urea and 10 mM mercaptoethanol (■—■); 7S-NEM (○—○). The concentrations: 7S - 0.9 mg/ml; ANS - 50 μM.

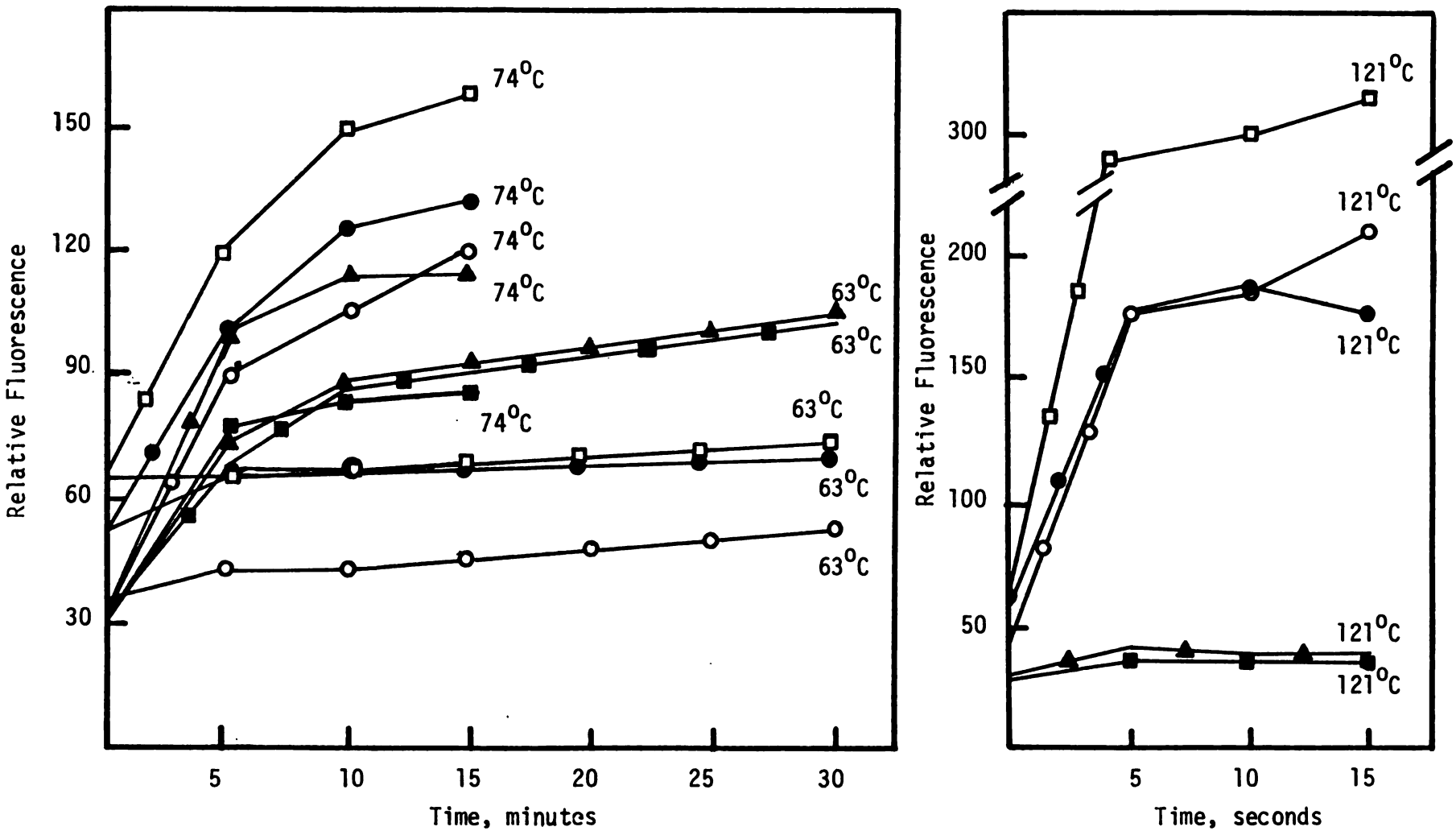


Figure 24. Effect of various reagents on ANS fluorescence of the 11S protein heated at 63, 74 and 121°C. Reagents are: control (●—●); 10 mM mercaptoethanol (□—□); 2 M urea (▲—▲); combination of 2 M urea and 10 mM mercaptoethanol (■—■); 11S-NEM (○—○). The concentrations: 11S - 0.6 mg/ml; ANS - 50 μM.

7S and 11S fractions by examination of the effect of increased ionic strength on relative fluorescence. In Figure 25, relative fluorescence increased as ionic strength increased from 0.1 to 3.0.

Lysinoalanine (LAL)

Lysinoalanine was quantitated from a Beckman Amino Acid Analyzer Model 120C, equipped with a basic column, by eluction with citrate buffer, pH 5.28. The initial resolution of LAL from lysine and the amino sugars was difficult. By increasing the length of the column from 6 to 9 cm, LAL was eluted from the column at 27.2 minutes compared to 29.9, 32.1 and 39.9 minutes for glucosamine, galactosamine and lysine, respectively (Figure 26).

Naturally occurring LAL in soybean was suggested when 305 μg LAL/g protein was found in defatted soybean meal. Treating a 1% solution of APP in water at two pH values and temperatures also resulted in LAL formation (Table 2).

The influence of sodium caseinate on LAL formation was examined using a 1% mixture of APP/sodium caseinate (57:43) at pH 6.8 for 30 minutes at 50°C. Formation of LAL was confirmed in the presence of sodium caseinate and determined to be 706 μg/g protein mixture. Since the sodium caseinate showed no LAL formation when examined singularly, the soy APP accounted for 1239 μg LAL/g protein. This was a 19% reduction from the 1% APP solution treated at pH 6.8, 50°C for 30 minutes.

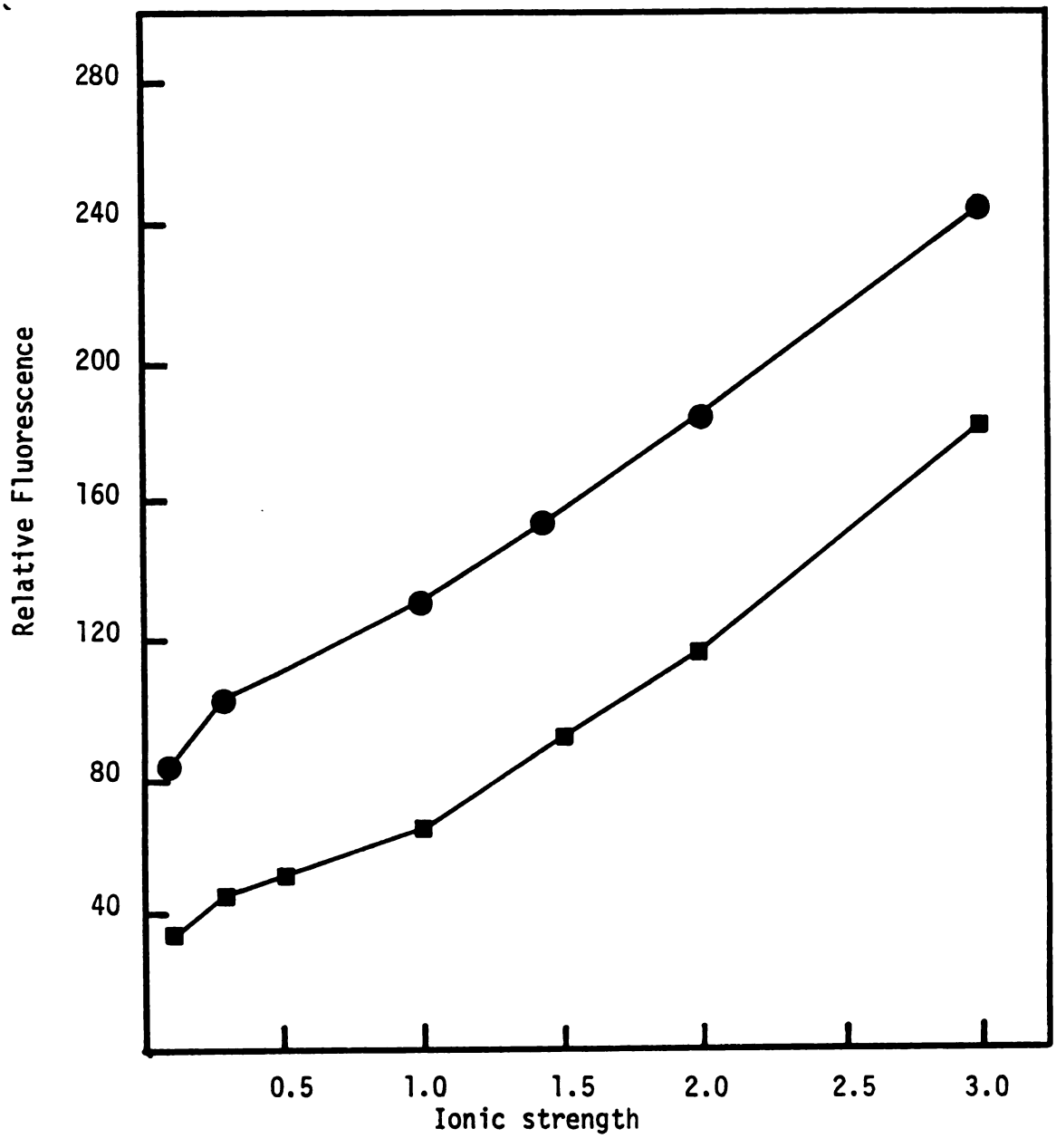


Figure 25. Effect of ionic strength on ANS fluorescence in presence of 7S (■—■) and 11S (●—●). The concentrations of proteins: 7S - 0.9 mg/ml; 11S - 1.0 mg/ml; and ANS - 50 μM.

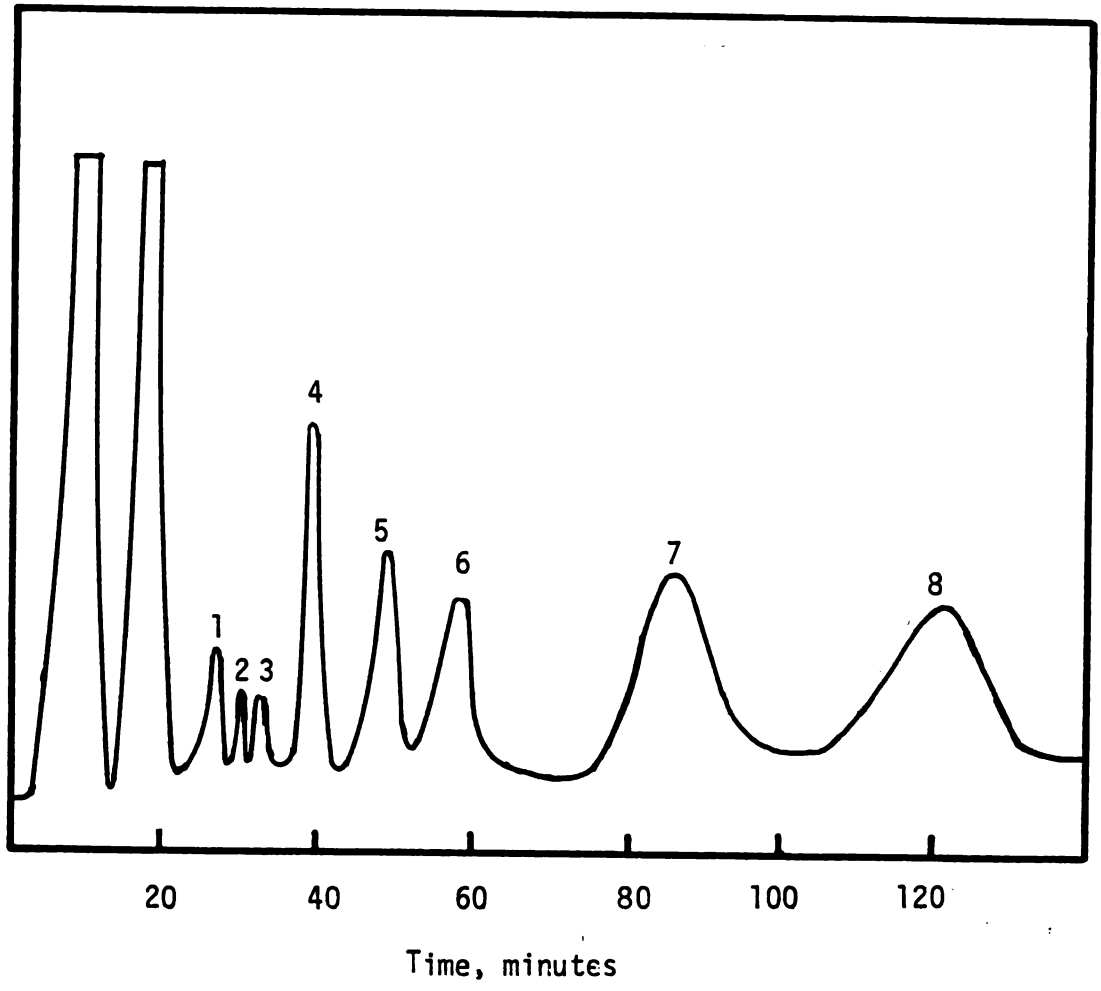


Figure 26. Elution profile of a standard mixture of amino acids, including LAL on a 9 cm basic column with citrate buffer, pH 5.28. Legend: (1) LAL; (2) glucosamine; (3) galactosamine; (4) lysine; (5) histidine; (6) NH_3; (7) arginine; and (8) internal standard.

Table 2. Lysinoalanine Formation in Soy Protein

Sample	Treatment	LAL µg/g Protein
defatted soybean meal	pentane and 30% ethanol diethyl ether extracted	305
1% APP	pH 6.8 no heat 30 minutes	1,447
1% APP	pH 6.8 50°C 30 minutes	1,524
1% APP	pH 10.0 no heat 30 minutes	1,738
1% APP	pH 10.0 50°C 30 minutes	1,760
1% APP-sodium caseinate (57:43)	pH 6.8 50°C 30 minutes	706
1% sodium caseinate	pH 6.8 50°C 30 minutes	-0-

DISCUSSION

Analytical Ultracentrifugation

The purpose of the analytical ultracentrifugation was two fold: (1) to compare purified soy protein with literature values; and (2) to determine if the 7S molecule in phosphate buffer (pH 7.0, μ=0.1) existed as a monomer or dimer.

The APP fraction showed four sedimentation peaks (Table 1). The values reported correlated well with the approximate whole numbers used for sedimentation value identification (Naismith, 1955; Wolf, 1969). The inability to resolve and determine the fastest peak was due to the low protein concentration, 1.25% whereas, most literature sedimentation patterns utilized 2% protein or higher. For this reason, the 15S peak was arbitrarily assigned to the fastest moving peak.

The two major proteins of APP were isolated and examined by sedimentation analysis for purity. The 7S molecule showed a slight impurity which consisted of the 2S component. The 11S protein, the more difficult protein to isolate, was found to be homogenous by sedimentation analysis. The $S_{20,app}$ values of the 7S and 11S proteins were 6.8 and 11.1, respectively (Table 1). Roberts and Briggs (1965) noticed the dramatic effect protein concentration had on sedimentation values, which explained any slight variations from literature values.

The 7S preparation dimerized to the 9S conformation in low ionic strength phosphate buffer, pH 7.0, μ=0.1 (Table 2). Koshiyama (1968b), who studied conformational changes of the 7S protein at pH values above the isoelectric point, also found the 9S protein predominated at an ionic strength of 0.1. The molecular weight of the dimer was determined as 370,000 daltons. Therefore, it was assumed the 7S protein existed in the 9S conformation for subsequent heat studies in pH 7.0, μ=0.1, buffers.

Polyacrylamide gel electrophoresis revealed three (possibly four) bands on 5% Cyanogum 41 gels, which demonstrated the heterogenity of the 7S molecule. Thanh et al. (1975a) and Thanh et al.(1975b) also demonstrated the 7S heterogenity. In addition, the soy 7S protein probably existed in the 9S conformation due to the low ionic strength gel buffer (0.38 M Tris-HCl, pH 8.9, where the pK_a of Tris is 8.21) employed during gel electrophoresis. The molecular weight of the 9S conformation determined by Koshiyama (1968b) was higher than 345,000 daltons of the 11S molecule found by Wolf and Briggs (1959). This molecular weight difference possibly accounted for the lower mobility of the 7S protein compared to the 11S in gel electrophoresis.

Effect of Heat on Soy Protein Systems

Effect of Heat on Soy APP

The effect of heat on soy APP was examined by gel electrophoresis (Figure 2) and on soy 7S and 11S proteins by ANS fluorescence (Figure 21). The gel patterns showed a loss of the 7S protein at 70^{o}C, with a resultant increase in protein not entering the gel. Also, ANS fluorescence of the

7S protein (Figure 21) detected a noticeable increase in relative fluorescence (RF) at 70°C. Therefore, increased hydrophobic interaction of the 7S protein at 70°C resulted in aggregation as noticed by the protein not entering the gel. However, the protein not entering the gel remained soluble after centrifugation at 20,000 x g for 30 minutes. Subunit formation was not detected at the higher temperatures as demonstrated by fluorescence which formed a plateau when heated from 90 to 110°C and by gel electrophoresis which failed to detect formation of any minor bands. Hashizume et al. (1975) also noted 7S conformational change at 70°C, with complete disappearance of the 7S bands above 80°C for a water extracted soybean mixture.

The soy 11S protein was also susceptible to heat treatment as determined by gel electrophoresis (Figure 2) and ANS fluorescence (Figure 21). The first indication of conformational change was detected at 80°C by diminished band intensity and increased fluorescence by ANS. At 90°C the gel band representing the 11S protein disappeared completely, with subsequent subunit bands formation. The intensity of subunit bands never equalled the original intensity of the 11S protein. ANS fluorescence demonstrated a maximum relative fluorescence of the 11S (Figure 21) at 90°C followed by a radical decrease at 100 and 110°C. The increased fluorescence at 80 and 90°C correlated with a conformational change due to hydrophobic interaction while the decreased fluorescence was attributed to subunit formation which may have lead to protein aggregation. Hashizume et al. (1975) showed the importance of ionic strength on the 11S stability while heated at 80°C. Decreased ionic strength resulted in a protein that was more susceptible to heat denaturation. The instability of the protein to low ionic strength was noted in preliminary

work in this research. Precipitation of the 11S protein was detected when the buffer was changed from high to low ionic strength and on prolonged exposure in the reduced ionic strength. The latter contributed to increased susceptibility to heat treatment.

Electrophoretic mobility of soy 7S and 11S were examined at 63, 74, and 121°C (Figure 3). The 7S protein retained its conformation at 63°C for 5 and 10 minutes. However, after 30 minutes the bands faded slightly and disappeared completely at 74 and 121°C. The protein not entering the gel remained soluble even though centrifuged at 20,000 x g and was referred to as the soluble aggregate. The 11S protein retained its integrity at 63°C and 74°C but formed subunits at 121°C.

Influence of Sulfhydryl Groups Versus Hydrophobic Interaction on Soy Protein Aggregation

Phosphate and cacodylate buffers (pH 7.0, μ=0.1) were used to approximate the environment of fluid milk. It was important to investigate the sulfhydryl and hydrophobic interactions in this environment to better understand the cause of protein denaturation and aggregation, since low ionic strength showed marked effects on soy protein stability.

Changes in free and total sulfhydryl groups of the soy 7S and 11S appeared to be of little influence in possible sulfhydryl interactions (Figure 4). The unheated 7S protein resulted in 0.60 and 0.65 moles free and total -SH/mole protein, respectively, for a molecular weight of 186,000 daltons (Koshiyama, 1968a). This was in conflict with results by Koshiyama (1968a), who found no cysteine residues and only four 1/2 cystine amino acids. However, Saio *et al.* (1971) reported one free mole -SH per mole 7S protein at ambient temperature and when heated above 70°C the value rose to approximately 2 moles -SH/mole 7S protein.

In this study sulfhydryl groups were present, yet the change of sulfhydryl content was not appreciable except at 121°C-15 seconds where 0.3 mole of total -SH/mole 7S occurred. A large decrease in -SH would be expected if some type of intra- or inter-molecular sulfhydryl interaction occurred during various heat treatments, particularly at 74°C where gel electrophoresis has previously shown conformational changes.

Further examination of possible sulfhydryl involvement was determined when soy proteins were combined with β-lactoglobulin, a sulfhydryl containing protein. First, total sulfhydryl content was determined on the individual 7S and β-lactoglobulin proteins, both at 5 mg/ml concentration, followed by the determination of a 1:1 mixture (2.5 mg/ml of each component) of the 7S protein and β-lactoglobulin. In Figure 11, the combination of the two proteins (each 2.5 mg/ml) resulted in values midway between that of the individual constituents (5 mg/ml). If massive sulfhydryl interaction occurred, the values of the mixture should greatly be depressed from the intermediate values due to sulfhydryl conversion to disulfides. Therefore, the 7S appeared to interact at various time-temperature relations by means other than sulfhydryl-disulfide interaction.

The behavior of the 11S protein was in marked contrast to the 7S protein. The initial unheated values resulted in 0.9 and 2.3 moles free and total -SH/mole 11S, respectively (Figure 4), which assumed a molecular weight of 345,000 (Wolf and Briggs, 1959). Shvarts and Vaintraub (1967) showed a possible 48 1/2 cystine residues per protein molecule. It was concluded the majority of the sulfhydryl groups from the 11S protein was in the disulfide form. Upon heating at 63, 74, and 121°C the values of both free and total -SH decreased to zero (Figure 4). Methods other than sulfhydryl-disulfide interchange were suggested for the conformational

change noted at 121°C, since no thiol groups were detected.

Soy 11S protein combined with β-lactoglobulin revealed total sulfhydryl concentrations intermediate of that found for the sample constituents (Figure 11). Once again the samples examined singly were 5 mg/ml and the mixture was 1:1 soy:milk protein, each 2.5 mg/ml. Since the sulfhydryl content of the mixture was not reduced substantially from values midway between the samples constituents, involvement of the sulfhydryl-disulfide interchange was ruled out.

Since sulfhydryl-disulfide interactions appeared to be of minimum influence, the role of hydrophobic interaction was examined. Stryer (1965) used the fluorescent properties of 8-anilino-1-napthalene sulfonate (ANS) to investigate the hydrophobic nature of the heme binding sites in apomyoglobin and apohemoglobin. Preliminary results with soy protein suggested the involvement of hydrophobic interactions when heat treated. Therefore, the following was assumed for subsequent investigation: Increased ANS fluorescence was an indication of increased hydrophobicity and may be used to indicate hydrophobic interactions in soy protein systems.

The 7S molecule was treated with various reagents that demonstrated possible hydrogen bonding and/or reactivity through the thiol group. Reactivity was expected at temperatures where conformational changes occured. At 63°C, 30 minutes, 74 or 121°C decreased fluorescence was detected by treatment with 2 M urea and the combination of 2 M urea and 10 mM mercaptoethanol. However, equilibration of the 7S protein with mercaptoethanol failed to cause a deviation from the control. Therefore, the compound attributed to reducing hydrophobic interactions of the 7S molecule was urea, which involved alteration of hydrogen bonding and/or hydrophobic interaction. Investigation of hydrophobic interaction involved

reducing hydrogen bonding with increased levels of sodium chloride. The increased ionic strength, from sodium chloride, would reduce intra- and inter-molecular hydrogen bonding which will cause an increase of RF if hydrophobic interactions of the protein existed. The hydrophobic nature of the 7S protein was suggested since increased fluorescence of ANS occurred with increasing ionic strength (Figure 25).

The 11S molecule showed similar results as the 7S molecule (Figure 24), however, most dramatically at 121°C. The 11S soy protein was stable when heated at 63 and 74°C (Figure 3B and 21) and revealed no dramatic decrease of fluorescence for any treatments (Figure 24). However, at 121°C reduced ANS fluorescence of the 11S fraction was noted when heated with 2 M urea and a combination of 2 M urea and 10 mM mercaptoethanol. Because soy 11S protein treated with mercaptoethanol failed to show deviation from the control, the effect of urea on hydrogen bonding and/or hydrophobic interaction was demonstrated. Hydrophobic interaction was confirmed in 11S aggregation when increased ionic strength resulted in increased ANS fluorescence. Hashimoto and Yokotsuka (1974) also showed the involvement of hydrophobic interaction among protein molecules in sediment that resulted from manufacture of soy sauce.

Effect of Heat on Soy-Milk Protein Combinations

The combination of the two soy proteins with various milk fractions and subsequent stability was a major concern. The 7S soy protein was combined with sodium caseinate, β-lactoglobulin, and whey proteins and stability examined by gel electrophoresis and protein solubility following centrifugation of 20,000 x g for 30 minutes. Combination of soy 7S

with the various milk fractions failed to cause any reduction in solubility. Gel electrophoresis was used to determine the conformation of the various proteins, particularly the 7S combined with sodium caseinate (Figure 7) and β-lactoglobulin (Figure 8). In Figure 8 a non-urea gel the native 7S molecule was present at 63°C for 5 and 10 minutes. The 7S/β-lactoglobulin mixture heated 30 minutes at 63°C resulted in diminished 7S bands while at 74 and 121°C vanished completely. At the higher temperatures the soluble 7S aggregate was noticed. The 7S combined with sodium caseinate was examined by urea gel electrophoresis. Two subunits derived from soy 7S were used to indicate the presence of soluble 7S protein following centrifugation at 20,000 x g. At all time-temperatures examined the 7S was present, with band intensity similar to the control. Therefore, the 7S sensitivity to heat treatment singularly and combined with milk proteins appeared the same.

The 11S combined with various milk fractions were also examined at 63, 74, and 121°C. At 63 and 74°C the 11S protein combined with sodium caseinate, β-lactoglobulin, and whey proteins showed no decrease in solubility (Figure 6). However, when heated at 121°C the 11S combined with β-lactoglobulin and whey proteins was reduced to 70-80% solubility. The 11S sodium caseinate mixture was highly soluble, 92%, compared to the ambient temperature control.

Gel electrophoretic patterns of the soy protein combined with β-lactoglobulin and sodium caseinate revealed similar 11S transitions as treated singularly (Figure 9 and 10). The non-urea gels, 11S/β-lactoglobulin mixture, showed the 11S band present at 63 and 74°C. However, at 121°C the band that represented the 11S was absent with a faster moving band apparent. The urea gel system of 11S/sodium caseinate mixture showed the

11S and casein protein present at all time-temperatures examined. Again, the independent nature of the soy 11S protein towards heat treatment was recognized in the presence of milk proteins.

A general trend was observed with both soy proteins. The 7S and 11S, when combined with sodium caseinate, resulted in higher solubility than soy/β-lactoglobulin or soy/whey protein mixtures. Previously, soy 7S and 11S were shown to involve hydrophobic interactions. Stability by hydrophobic interaction with sodium caseinate was suggested since the casein constituents α_s-, β-, and κ-caseins possess higher than average hydrophobicity (Mercier et al., 1971; Ribadeau-Duman et al., 1972; Mercier et al., 1973).

Effect of Calcium on Soy-Milk Proteins

Effect on Soy Proteins

A marked difference of solubility of soy 7S and 11S was noticed when treated with 1-8 mM calcium. The 7S showed a maximum 10% reduction in solubility in the presence of calcium while heat treated at ambient temperature, 63°C - 30 minutes and 74°C - 15 minutes (Figure 12A). Only when the fraction was heated at 121°C - 5 seconds in the presence of 8 mM calcium was there greater than a 20% reduction in solubility. In fact, electrophoretic examination of the 7S heat treated with and without calcium present revealed similar patterns (Figure 3A and 13A). Koshiyama (1965) also recognized the 7S stability in the presence of calcium on attempted purification of the soy glycoprotein.

Increased calcium caused decreased stability of the 7S molecule when the protein level was lowered to 0.9 mg/ml and examined by ANS

fluorescence (Figure 22A). At 74°C and 121°C increased hydrophobicity was evident with increased calcium. However, whether with or without calcium present, the highest fluorescence after heat treatment was 74°C - 15 minutes, followed by 121°C - 5 seconds. Since this temperature effect predominated, and only a very high ratio of calcium to protein caused conformational alteration, the principal effect demonstrated was that of temperature rather than calcium concentration.

The sensitivity of the soy 11S protein to calcium was in sharp contrast compared to the 7S protein. In Figure 12B the effect of calcium at ambient temperature--63, 74 and 121°C--was examined. Almost independent of temperature, the reduced solubility was noticed with increased calcium concentrations. At 3 mM calcium the 11S resulted in less than 5% soluble protein. Gel electrophoresis (Figure 13B) showed the dramatic effect of calcium on 11S band intensity, even at temperatures where in the absence of calcium the 11S was soluble (Figure 3B). Also, evidence of calcium aggregation prior to thermal denaturation was noted at 121°C heat treatment by no subunit formation in the presence of calcium.

ANS fluorescence in the presence of reduced levels of calcium, 0 to 1.5 mM, was also examined (Figure 22B). At 74°C and 121°C decreased fluorescence was observed with increased calcium. This suggested possible calcium induced aggregation instead of hydrophobic interaction. It was concluded the 11S molecule, when in the presence of calcium, appeared to be affected by calcium concentration more than by the heat treatment.

Effect on Soy/Milk Protein Interactions

As noted in the section which discussed soy-milk interactions without calcium, the reactivity or rather the disappearance of the soy

protein on polyacrylamide gels, was dependent on time and temperature of heat exposure, regardless of the type of milk protein present. However, mixtures of sodium caseinate and soy proteins were found to have the greatest overall stability.

Soy-milk protein mixtures heated in the presence of calcium also showed the independent reactivity of the soy proteins. The 7S protein combined with the three milk protein systems demonstrated higher overall solubility than the 11S/milk protein systems (Figure 14 and 15). At low heat treatment, ambient temperature and 63°C - 30 minutes, all 7S/milk protein combinations were greater than 84% solubility. This was in sharp contrast with the 11S/milk protein combinations where solubility ranged from 34-70%. At temperatures of 74°C - 15 minutes and 121°C - 5 seconds, the higher stability of 7S/milk protein systems compared to 11S/milk protein mixtures were also noticed, although to a lesser degree. Sodium caseinate combined with soy protein revealed the greatest stability at 74°C - 15 minutes and 121°C - 5 seconds when combined with both 7S and 11S proteins. The higher overall solubility of 7S/milk proteins compared to 11S/milk protein mixtures probably resulted from the 11S protein sensitivity to calcium.

Gel electrophoresis was used to ascertain if milk proteins altered the overall characteristics of soy proteins. The 7S molecule, when combined with sodium caseinate (Figure 16) and β-lactoglobulin (Figure 17) showed the independent effect of calcium on the gel patterns. When combined with sodium caseinate, the subunit which represented the 7S protein faded slightly with increased calcium levels. The 7S, combined with β-lactoglobulin, and treated with calcium, showed similar transitions in the absence of calcium and the milk protein. In the non-urea

system the 7S was represented by three bands at 63°C for 5 and 10 minutes, yet faded with increased holding time of 30 minutes, and vanished at 74 and 121°C. Also, at the two higher temperatures, soluble aggregates were noticed once again, as noticed in model systems lacking calcium. It was, therefore, concluded that the calcium effect was not as influential as temperature.

Some 7S/milk protein mixtures showed greater susceptibility to calcium precipitation than others. The sodium caseinate mixture was the most stable, particularly at higher temperatures to treatment with calcium, while the 7S/β-lactoglobulin mixture appeared to be most susceptible. Although the 7S protein appeared in electrophoretic patterns at all calcium treatments and time temperature relations, the solubilities were reduced by β-lactoglobulin sensitivity to calcium and subsequent precipitation.

The electrophoretic patterns, which contained the 11S protein showed the extreme sensitivity of this protein to calcium. In Figure 18 when combined with sodium caseinate, the two subunit bands which represented the 11S protein decreased with increased calcium concentration. A more pronounced demonstration of calcium sensitivity was in a non-urea gel system combined with β-lactoglobulin. The 11S band diminished substantially when 1 mM calcium was present and disappeared completely at 3 and 8 mM calcium levels. It was concluded that the influence of divalent calcium on 11S stability was more important than temperature treatment. Milk proteins failed to alter the 11S susceptibility to various calcium levels, although the sodium caseinate mixture resulted in the highest solubility compared to the other milk proteins. This increased solubility was attributed to the reduction of the effective calcium level by the sodium caseinate.

Formation of Lysinoalanine (LAL) in Soya Protein

The initial procedure for determination of lysinoalanine (LAL) was by ascending chromatography on cellulose TLC plates, with a mobile phase of 1-butanol-formic acid-water (23/4/5:v/v/v) (Sternberg et al., 1975b). The procedure resulted in ample resolution and demonstrated the purity of the LAL standard obtained from Dr. J. Finley. However, the tedious procedure lacked the sensitivity to yield reproducible, quantitative values. For this reason, an alternative method was selected. The basic column of an amino acid analyzer, when eluted with citrate buffer, pH 5.28, showed the LAL retention time similar to lysine. This problem was resolved when the column was increased from 6 to 9 cm. A standard run, which included glycosamine, galactosamine, the acid, basic and neutral amino acids, showed suitable resolution (Figure 26).

The question of LAL formation in soybeans was confirmed in this study as by others (Sternberg et al., 1975a; DeGroot and Slump, 1969). The question raised then: Is lysinoalanine formed under normal processing conditions and/or present naturally?

The examination of the native bean would involve the direct hydrolysis of a ground sample. Problems caused by the lipid fraction on the analyzer necessitated fat extraction with pentane followed by 30% ethanol in diethylether, $0^{o}C$, prior to analysis. Peptane was selected since the low boiling point of $36^{o}C$ failed to cause any soy protein alterations. The LAL detected was 305 µg/g defatted soy protein. Since the defatted soy was exposed to minimal heat treatment and not alkali treated (both of which increase LAL formation), it may be suggested that LAL was present naturally.

The possibility of lysinoalanine formation in the native bean was suggested. Bohak (1964) and Patchornik and Sololovsky (1964) demonstrated LAL was formed by condensation of two adjacent or spatially arranged cystine and lysine amino acids. Also, Dieckert and Dieckert (1976) suggested the soy proteins are "liberal" proteins, which the authors define as proteins that remained functional while sustaining numerous alterations in the primary amino acid structure.

Heat treatment of a 1% solution of soy acid-precipitated protein at pH 6.8 and 10.0 was examined (Table 2). The selection of pH 6.8 approximated the pH of water extractable soy protein while pH 10 approximated the level used for spinning soy protein fibers. Heat treatment of 50^{o}C represented the temperature of the pH 10 dope solution (Smith and Circle, 1972).

The sample treated at pH 6.8 ambient temperature for 30 minutes showed a LAL value 4.7 times higher compared to the fat extracted sample. The large LAL concentration was unexplainable since the maximum pH during extraction of soy APP fraction was pH 7.6. Sternberg et al. (1975a) showed the formation of LAL was possible when exposed to neutral and acid conditions, though in much less amounts. When the 1% APP, adjusted to pH 6.8, was heated at 50^{o}C for 30 minutes, the LAL detected was 5% larger than ambient temperatures. A 1% APP solution increased to pH 10.0 resulted in an increased LAL formation of 20-22% for samples treated at ambient temperature and 50^{o}C - 30 minutes compared to 1% APP-no heat-30 minutes. Increased alkaline condition was found to favor an increase in LAL concentration.

Soy APP/sodium caseinate mixture was examined for possible depression in LAL formation by a non-soy protein. A combination of soy APP and

sodium caseinate (57:43) resulted in 706 μg LAL/g protein mixture. Since the APP was the only contributor of LAL, the adjusted value was 1,239 μg LAL/g soy APP. This was a 19% reduction compared to 1% solution of soy APP treated at pH 6.8 - 50^{o}C for 30 minutes. This reduction was unaccountable unless the sodium caseinate had diluted the APP sufficiently to reduce the spatial arrangement necessary for LAL formation.

Sternberg et al. (1975a) commented after examination of a wide range of protein foods: "The formation of LAL in proteins by heating at pH values considerably lower than those obtained during alkali treatment, . . .suggest the ubiquity of LAL in cooked foods and that humans have long been exposed to protein containing LAL". Thus, Sternberg et al. (1975a) suggested LAL presence has been with us as long as protein foods have been known. Since no detrimental aspects have been observed to date (except in rats fed free LAL) (DeGroot, 1976), little concern over the presence of LAL appears justified at this time.

CONCLUSIONS

Soy protein interactions on a molecular level were conducted in a neutral pH, low ionic strength buffer (pH 7.0, μ=0.1). Various chemical and physical procedures revealed soy proteins were affected by heat treatment encountered in normal processing operations of fluid milk. The interactions, being characterized by the particular proteins involved, were similar whether the soy proteins were present alone or in combination with various milk proteins.

The acid-precipitated proteins (APP) were shown to be composed of four boundaries by analytical ultracentrifugation. The 7S species was a major protein of this fraction. When dialyzed against phosphate buffer (pH 7.0, μ=0.1), it existed as a dimer. Therefore, all work which used phosphate and cacodylate buffers (pH 7.0, μ=0.1) resulted in the 9S conformation.

Soy protein was investigated with a fluorescent probe, ANS, to determine the effect of hydrophobic interaction on aggregation. The 7S demonstrated a marked increase of RF at 70°C, which correlated with the hydrophobic formation of a soluble aggregate as determined by gel electrophoresis. The 11S molecule was found to undergo an increase in RF at 80°C, followed by a marked decrease at 100°C. This correlated with an increase in hydrophobic aggregation of the 11S molecule, followed by disassociation into subunits, as detected by gel electrophoresis.

Various reagents were used to ascertain the effect of hydrogen bonding and thiol-disulfide interactions on soy 7S and 11S. For both proteins, urea caused the greatest effect at temperatures high enough to cause protein denaturation. Confirmation of hydrophobic interaction was achieved by reducing intra- and inter-molecular hydrogen bonding by use of increased salt concentration. This resulted in a linear increase in the hydrophobicity of both soy protein fractions.

The possible involvement of thiol-disulfide groups in the soy 7S and 11S were ruled out. When the 7S fraction was heated at 63, 74 and 121°C, little change in total -SH content was apparent, the 11S decreased immediately to zero upon heating. Soy protein heated in combination with β-lactoglobulin showed no marked decrease in total sulfhydryl content and thus suggested little or no thiol group interaction.

Soy and milk proteins combined in the absence of calcium suggested the independent nature of reactivity for soy 7S and 11S. The 7S when heated at 74 and 121°C, resulted in the formation of soluble aggregates, while the 11S heated at 121°C disassociated into subunits. These characteristics were noticed even in the presence of various milk fractions. One common characteristic of soy 7S and 11S proteins was the stability when heat treated in the presence of sodium caseinate compared to combinations with other milk proteins.

The addition of calcium to 7S and 11S-milk protein mixtures revealed the extreme sensitivity of the 11S fraction to the divalent cation, even in the presence of milk proteins. Immediate aggregation was demonstrated when the 11S was not detected by gel electrophoresis, either as a native protein at 63° and 74°C or 121°C as a faster moving band. The sensitivity of the 11S to calcium predominated over that of temperature effects. When

calcium was incorporated into the sodium caseinate-11S mixture and heated at 74 and 121°C, the most soluble combination resulted.

The 7S-milk protein mixtures were more stable to calcium than the 11S mixtures. The 7S formed soluble aggregates at 74 and 121°C in the presence or absence of calcium. Proteins which comprised the 7S-milk protein mixture acted independently of each other as determined by gel electrophoresis. The 7S/sodium caseinate was found to have the highest solubility at the higher levels of calcium when heated at 74 and 121°C.

Lysinoalanine (LAL) was found in defatted soybeans and extracted APP fractions. LAL occurred naturally as demonstrated when 305 µg/g protein was found in fat extracted samples. One percent APP solutions, treated at two pH values and temperatures, also showed formation of LAL. LAL formation was favored by high pH, with a 20-22% increase of LAL in soy APP treated at pH 10.0 compared to pH 6.8.

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APPENDIX

Kjeldahl Method

A 15 mg protein sample was digested with 4 ml of digestion mixture (5.0 g $CuSO_4.5H_2O$, 5.0 g SeO_2 in 500 ml of concentrated H_2SO_4) in a micro-Kjeldahl flask for 90 minutes. The sample was cooled and 2 ml of 30% H_2O_2 added. Digestion was continued for 90 additional minutes. The digestion flask was removed, allowed to cool, rinsed down with 10 ml of water, and attached to a steam distillation apparatus. The digestion mixture was made alkaline by addition of ca 25 ml of 40% NaOH (w/v). The released ammonia was steam distilled into 4% boric acid solution containing five drops of indicator (400 mg bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol). The ammoniumborate complex was back titrated with 0.02 N HCl to an olive endpoint. A factor of 6.25 was used to convert percent nitrogen to crude protein.

Biuret Method

The procedure followed that by Layne (1957) with slight modifications. The Biuret reagent was composed of a 1:1:1 mixture of A, B, C; where A was 1.125 g of $CuSO_4.5H_2O$ per 250 ml of water, B was 4.5 g of sodium tartrate per 250 ml of water, and C was 22.5 g NaOH pellets per 250 ml of water.

Protein was determined by taking 1.0 ml of sample (1 to 10 mg of protein) and adding 4.0 ml of Biuret reagent. Color development was allowed to continue for 30 minutes. The absorbance was measured at 540 nm and compared to a standard curve constructed with 3x crystallized bovine serum albumin.

Lowry Method

The procedure followed that of Lowry et al. (1951) with one revision--the mixing of reagents on the day of the analysis. Reagent A was a 1:1 mixture of 2% sodium tartrate and 1% $CuSO_4.5H_2O$. Reagent B consisted of 2% $NaCO_3$ in 0.1 N NaOH. On the day of use 50 parts of Reagent B was mixed with 1 part of Reagent A (designated as Reagent AB).

Protein concentrations, which ranged from 50 to 500 µg in 1.0 ml of solution, had 5.0 ml of Reagent AB added. Following 10 minutes incubation, 0.5 ml of phenol reagent (Folin-Ciocalteau reagent-Fischer) was added in less than 2 seconds with a blow out pipet. The blue color was allowed to develop for 20 minutes and measured at 540 nm. A standard curve was constructed with 3x crystallized bovine serum albumin.

Sulfhydryl Determination

The determination of cysteine was adapted and modified from the procedures of Ellman (1959) and Beveridge et al. (1974). Free sulfhydryl (exposed to surface of the proteins) was determined by taking 0.5 ml of sample and adding 2.0 ml of 0.1 M phosphate-0.001 M disodium EDTA buffer, pH 8.0. To this was added 0.03 ml of Ellman's reagent (40 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) from Aldrich Chemical Company was

dissolved in 10 ml of 0.05 M phosphate buffer, pH 7.0). Color development was allowed to proceed for 15 minutes (color intensity was constant up to 30 minutes) and absorbance measured at 412 nm using ε of 12,000.

Total -SH was carried out by taking 0.25 ml of sample, adding 3.0 ml of a phosphate-urea buffer, pH 8.0 (0.1 M phosphate, 0.001 M disodium EDTA, 8 M urea (Sigma) made to pH 8.0). This was incubated for a minimum of 1 hour at room temperature, followed by addition of 0.05 ml of Ellman's reagent. After 15 minutes for color development, absorbance of the yellow solution was measured at 412 nm using ε of 12,000. Purified β-lactoglobulin was used to check recovery values.

Lysinoalanine-Hydrolysis Procedures

Nine to twelve mg of protein was accurately weighed into 10 ml ampoules. To this was added 1-4 ml of 6N HCl (redistilled), the volume depending on the carbohydrated content. The ampoules were frozen in a dry ice-ethanol bath, evacuated under high vacuum while frozen, melted, and refrozen, followed by sealing with a butane flame. Hydrolysis for APP samples at pH 6.8 and 10.0 were for 22 hours and the remaining samples at 48 hours at 110°C.

Following hydrolysis the ampoules were opened and 1.0 ml of S-B-(4-pyridylethyl)-DL-Penicillamine added as an internal standard for recovery calculations. The ampoule contents were quantitatively transferred to a 50 ml round bottom flask and attached to a rotary evaporator. The HCl was evaporated off under vacuum and the hydrolyzed residue was washed three times with water. The sample was picked up in citrate buffer, pH 2.22, and made to 5.0 ml. A sample of 0.2 ml was applied to a 9 cm column

(Beckman Resin Type PA 35) and eluted with citrate buffer, pH 5.28.

The lysinoalanine standard was supplied by Dr. John Finley, Western Regional Laboratory, USDA.