THE PLASTEIN REACTION

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ABSTRACT

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By

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Since 1886 the plastein reaction has been investigated to determine if specific enzymes, under empirical conditions, catalyze protein synthesis. Chymotrypsin is an acyl transferase which normally transfers to water. When water becomes limiting, chmotrypsin may transfer to free N-terminal amino acids thus initiating protein synthesis through a condensation-type reaction. A decrease in TCA-solubility of plastein over that of the hydrolyzate, the insolubility of plastein in solvents, the inability of plastein to pass through dialysis membrane, and dramatic changes in color and viscosity of the plastein over the hydrolyzate have been cited by previous investigators as evidence of peptide bond formation.

The ninhydrin reaction was used to monitor changes in the number of N-terminals exposed. Column chromatography and disc gel electrophoresis were used to monitor changes in molecular weight profiles. These methods along with others gave no indication of any increase in molecular

weight of the plastein over the hydrolyzate. The changes in physical properties of the hydrolyzates, such as insolubility and molecular weight increases, were explained on the basis of hydrophobic aggregation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

The problem that concerned us was the nature of the plastein reaction. There is abundant literature on this topic with considerable conflicting conclusions.

A Japanese research team at the University of Tokyo have devoted considerable effort since 1970 to the investigation of the plastein reaction. They reported that soy protein peptic hydrolyzate produced a white, tasteless plastein. They reported that amino acids could be incorporated into plastein when corresponding ethyl esters were added to the reaction mixture. Mixtures of complementary protein hydrolyzates resulted in a plastein with a higher P.E.R. than either of the original proteins.

Vegetable proteins have played an increasing role in human nutrition and there is no indication that their incorporation into food products will decrease. In an effort to provide high quality, low cost vegetable proteins with improved functional properties, the plastein reaction was investigated.

The primary objective of this study was the investigation of the claim that the plastein reaction is

a peptide bond forming reaction synthesizing polymeric proteins from monomer peptides. The incorporation of amino acids using their corresponding ethyl esters was also investigated. From data obtained in these investigations a suggested mechanism for plastein formation was proposed.

LITERATURE REVIEW

History

In 1886 Danilewski observed the formation of a precipitate when stomach extracts were added to a concentrated peptic hydrolyzate. Danilewski believed the precipitate to be produced by the stomach enzymes since no precipitate resulted if the stomach extracts were first boiled. In 1895 Oknew confirmed these observations. In 1901 Sawjalow investigated the enzymatic phenomenon and designated the resulting precipitate, "plastein." Wasteney and Borsook (1930) published an informative review article covering the early work on plastein.

Preparation of Plastein

The historical studies with plastein established three conditions for plastein formation: (1) a peptic hydrolyzate, (2) a sufficient concentration of the hydrolyzate, and (3) a plastein-forming enzyme at the correct pH and temperature, Fig. 1.

Traditionally, egg albumin was used as the substrate for the plastein reaction (Horowitz and Haurowitz, 1959). Fujimaki *et al*. (1970) performed the plastein

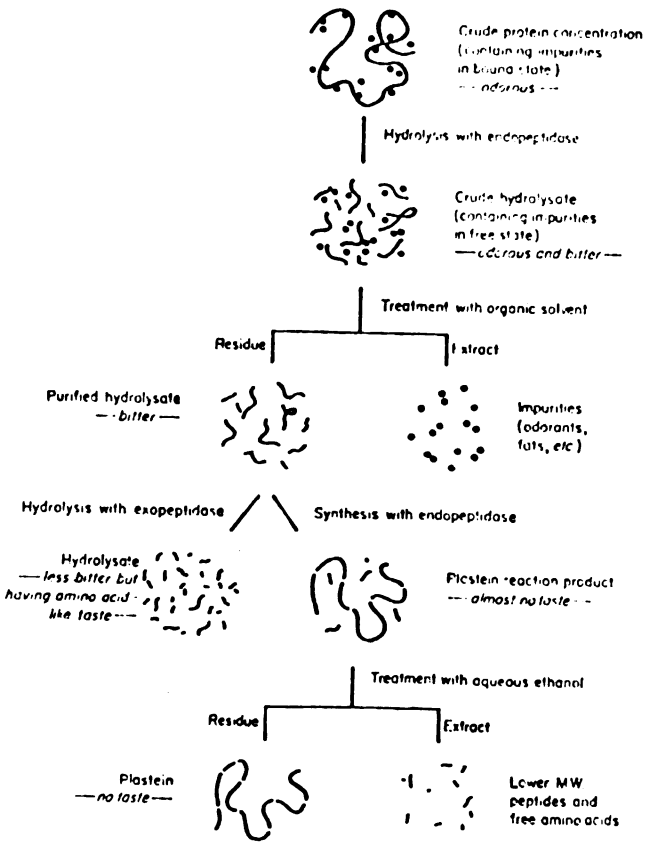

FIGURE 1. PROCESS FOR DEODORIZING AND DEBITTERING, AND FOR SYNTHESIZING PLASTEIN (FROM ARAI ET AL., 1975).

reaction on a wide variety of plant and animal proteins. Yeast, soy, and gluten protein hydrolyzates consistantly gave the highest plastein yields. They described plastein as a bland, white, protein-like substance possessing a relatively high degree of insolubility.

Hydrolysis

The hydrolysis of egg albumin was usually achieved by the addition of pepsin (Horowitz and Hoaurowitz, 1959). Yamashita et al. (1970) reported enzyme/substrate ratios of 1-3/100, employing a solution containing approximately 1% soy isolate at pH 1.8 for 24 h. This treatment resulted in about 80% hydrolysis of the protein which Yamashita considers optimum for plastein formation. Yamashita et al. (1970) reported a molecular weight profile for the peptic hydrolyzates of soy protein and the resulting plasteins by means of gel filtration over Sephadex G-75. A significant shift to higher molecular weight substances in the plastein was noted. Tasi et al. (1974) observed that hydrolysis fractions with average molecular weights between 685 and 1043 were more "plastein productive" than fractions above or below this molecular weight range.

Concentration

Relatively high concentration of the peptic hydrolyzate is essential for plastein formation. The concentration of the peptic hydrolyzate is reportly responsible for the shift of enzyme "cleavage" properties to "peptide

bond forming" characteristics. Horowitz and Haurowitz (1959) reported that 40% egg protein hydrolyzate was a necessary condition for the plastein reaction. Tasi et al. (1972) reported that 6-8% soy peptic hydrolyzate was necessary for plastein formation, but maximum plastein yields occurred between 30-40% soy hydrolyzate. Concentration of the peptic hydrolyzate was usually accomplished under vacuo at temperatures below 40 C. Tauber (1951b) reported that extremely high peptide concentrations were unnecessary if a 26% sodium chloride solution was used to dilute the protein to 4% before subjection to the plastein reaction.

Plastein Synthesis

Traditionally, plastein synthesis was catalyzed with pepsin. Subsequently, selected plant extracts were found to be "plastein active." Tauber (1951a) introduced α-chymotrypsin as a plastein-forming enzyme. Pepsin induces the formation of a precipitate as the plastein reaction proceeds, whereas chymotrypsin produces highly viscious solutions. Fujimaki et al. (1970) reported excellent yields of plastein from soy isolate by employing chymotrypsin as the catalytic agent. None of a wide variety of endopeptidases, exopeptidases, and various microbial proteases exceeded chymotrypsin in plastein productivity. Taminato et al. (1972) prepared an insoluble preparation of α-chymotrypsin bound to filterpaper. The

plastein reaction was then performed to dispell any possibility that the plastein reaction is a product of peptide-enzyme aggregation. Yamashita et al. (1971a) reported plastein yields were a function of pH, concluding that the pH optimum for chymotrypsin shifts from around 7.0 for hydrolysis, to between pH 4 and 6 for optimum plastein formation.

Proposed Mechanisms for Plastein Formation

Cyclization

Virtanem and Kerkkomem (1948) observed no increase in molecular weight of plastein when compared to the peptic hydrolyzate. They attributed the change in physical properties of the hydrolyzate to cyclization of the peptides.

Condensation

The condensation reaction catalyzes peptide bond formation by the direct condensation of α-amino and α-carboxyl groups. Determann et al. (1963) reported that in high concentrations oligopeptides are converted to polymeric forms. The monomers must be at least a tetrapeptide with L-leucine, L-phenylalanine, or L-typosine as the C-terminal residues. For example, when a pentapeptide with L-tyrosine at the C-terminal and L-phenylalanine at the N-terminal, a pentadecapeptide would be produced. Boyer (1971) discussed the thermodynamics of the condensation reaction.

Transpeptidation

Transpeptidation reactions occur when enzymes catalyze the transfer of acyl groups to water and other acceptor molecules. Lehninger (1971) describes chymotrypsin as a hydrophobic acyl group transferase. Transpeptidations are classified into two categories: (1) acyl transfer (carboxyl transfer) and (2) imino transfer (amine transfer). These reactions are catalyzed by esterases and the serine proteases. Horowitz and Haurowitz (1959) believed that amino acyl residues were transferred from donor peptides to acceptor peptides or amino acids. Otherwise there was no way to explain the constant amount of α-amino nitrogen before and after the plastein reaction. Also, acceptor peptides must be poor in threonine, asparatic, and glutamic acids and rich in leucyl, isoleucyl residues to participate in the plastein reaction. Thus, the insolubility of plastein could be explained on the basis of the nonpolar side chains of leucine, isoleucine, and phenylalanine.

Yamashita et al. (1973), using isotopic oxygen (0^{18}) techniques, reports that transpeptidations occur via acyl and imino transfer but that condensation reactions seem to be the major mechanism involved in the plastein reaction. Arai et al. (1975b) concluded that Ser-195 of chymotrypsin serves as the target of acyl-enzyme intermediate formation. In the reverse reaction, His-57 serves as the acid-base catalytic site for acyl enzyme formation. The proposed mechanism is presented in Figure 2.

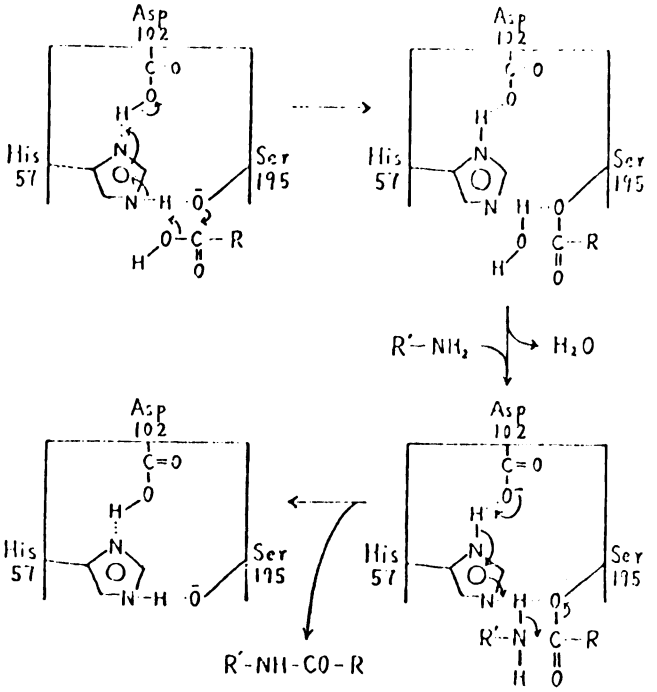

FIGURE 2. PROPOSED PROCESS FOR THE REVERSE REACTION OF α-CHYMATRYPSIN (FROM ARAI ET AL., 1975).

Contemporary Investigations

Model Systems

Determann and Wieland (1961) synthesized the plastein active pentapeptide L-tyrosyl-L-isoleucyl-glycyl-L-glutamyl-L-phenylalanine. A plastein was produced from this pentapeptide using pepsin as the catalyst. Utilizing UV spectrophotometry of the DNP-derivative, a molecular weight of 2250 was determined for the plastein. Subsequently, Determann et al. (1962) synthesized nine plastein-active pentapeptides. The substrate plastein mixture contained 100 mg of peptide in 0.1-0.2 ml of water, adjusted to pH 4 and incubated at 37 C for 15-24 h with less than 1 mg of pepsin. The enzyme was destroyed by heating to 80 C and plasteins were isolated by centrifugation. Plasteins formed by the use of pepsin possessed low molecular weights and an average degree of polymerization of only 2.5.

Determann et al. (1963) conducted experiments designed to study the effect of chain length on the plastein reaction. They found tetra-, penta-, and hexapeptides to be plastein-active while di- and tri-peptides did not polymerize in the presence of pepsin. Aromatic residues at the carboxyl terminal of the peptide appears to be essential for the formation of a plastein. The substitution of alanine for tyrosine at the N-terminal position in the pentapeptide did not effect its ability to

form plasteins, but similar substitution at the carboxyl-terminal caused inactivity of the peptide. Fujimaki (1971) showed that peptic hydrolyzates of soy protein effectively liberated bound odorants and lipids from the native protein, however, the hydrolyzates possessed extremely bitter flavor. Following the plastein reaction the peptide mixture was completely void of bitterness. Insolubility in aqueous systems is a characteristic of plasteins. Aso et al. (1973) obtained good solubility with 0.3 M sodium dodceyl sulfate (SDS) or 2N sodium hydroxide (NaOH). They concluded that hydrophobic bonding was largely responsible for the water insolubility of plastein.

Gel Filtration

Yamashita et al. (1970) estimated the molecular weight of soy hydrolyzate and soy plastein by gel filtration over Sephadex, and observed that the average molecular weight of the hydrolyzate increased by approximately three fold following the plastein reaction.

Gel Electrophoresis

Tasi et al. (1974) published the results of polyacrylamide gel electrophoresis in a phenol-acetic acid-water (1:1:1) buffer system, indicating that the molecular weight increased from 600-1000 to 11-27,000 as a consequence of the plastein reaction. Ultracentrifugal sedimentation analysis revealted an average molecular weight of 20,000.

Amino Acid Analysis

Yamashita et al. (1970) reported amino acid analyses for soy protein and soy plastein. Aso et al. (1974) showed that plasteins could be enriched with the ethyl esters of specific amino acids. Yamashita et al. (1974), using a diethyl ester preparation of glutamic acid, reported a 17% increase in glutamic acid content of the resulting plastein. The plastein was extensively washed and dialyzed prior to assay. Amino acid incorporation is of practical importance when one considers that proteins of low nutritional quality can be improved by the addition of limiting amino acid residues. Yamashita et al. (1971) demonstrated that plasteins composed of hydrolyzates of complementary proteins yielded higher P.E.R. values than either of the original proteins.

EXPERIMENTAL PROCEDURES

Materials and Methods

Preparation of Proteins

Whole Casein

Fresh skim milk was adjusted to pH 4.6 with 6N HCl. The precipitated casein was then filtered through three layers of cheese cloth resuspended in water and adjusted to pH 7.0 with 6N NaOH. The casein was acid precipitated and resuspended in water two additional times before it was shell frozen in preparation for freeze drying.

Soy Protein Isolate

Soy Promine-D isolate was donated by Central Soya (Chicago, Ill.). Promine-D is prepared from isoelectric precipitated soybean proteins which were resuspended in water at pH 7.0 and sprayed dried using low heat for maximum solubility. All protein preparations were stored in air tight containers or plastic bags at 0 C.

Preparation of Plastein

Enzyme-Induced Plastein

A 1% solution of casein or soy protein was hydrolyzed at pH 1.6-2.0, using a pepsin-protein ratio of 1:100 (w/w) at 37 C for 24 H. The pepsin was 3X crystallized, a preparation from Nutritional Biochemical Corporation. The peptic protein hydrolyzate was neutralized to pH 7.0 using 6N sodium hydroxide (NaOH). Concentration of the peptic hydrolyzate was carried out using a rotary evaporator at 40 C. A vacuum of 28 inches was produced with a mechanical vacuum pump. The condensate was collected which enabled estimation of protein concentration to between 30 and 35% protein. After concentration, the pH was adjusted to 7.0 when necessary, and incubated with α-chymotrypsin at 37 C at an enzyme:protein ratio of 2:100 (w/w). α-Chymotrypsin was a salt-free, 3X crystallized preparation obtained from Nutritional Biochemical Company. The reaction was allowed to proceed for 24 h. The resulting plastein was washed with a 100-fold excess of 50% ethanol and centrifuged at 1000 x g for 20 min. The white plastein pellet was suspended in water, shell frozen, and lyophilized on the laboratory's freeze dryer (Arai et al., 1975b).

Plasteins were formed in dilute protein digest solutions containing only 4% total solids and 26% sodium chloride. The procedure of Tauber (1951b) was followed with the following modifications. Soy hydrolyzate was

used as the substrate in place of egg albumin, the plastein reaction was carried out at pH 7.0 instead of 7.3 and sodium fluoride was omitted from the salt solution.

Enzyme Activity

The activity of α-chymotrypsin was determined by the L-tyrosine ethyl ester method of Rick (1963). L-tyrosine was purchased from Sigma Chemical Company and L-tyrosine ethyl ester HCl (TEE) from Nutritional Biochemicals Corporation. The α-chymotrypsin used was the same as that used for the plastein reaction. All chemicals and enzyme preparations were stored at 0C. Spectrophotometric measurements were made with a Beckman DK-2A ratio-recording spectrophotometer at a wavelength of 234 nm using silica cuvettes with a light path of 1 cm. L-tyrosine was made up to one millimolar in tris buffer (Appendix). A 2 mM solution of tyrosine ethyl ester using the tris-buffer plus 4.44 ml of a 5% $CaCl_2$ was added per 100 ml of solution. The $CaCl_2$ was added to the L-tyrosine ethyl ester solution to help stabilize chymotrypsin. The enzyme solution contained 50 µg chymotrypsin/.1 ml in tris-HCl buffer. Three ml of a 1 mM L-tyrosine solution was used as a reference in the DK-2A spectrophotometer. One and a half milliliter of a 2 mM L-tyrosine ethyl ester solution was diluted to 3 ml with enzyme and buffer. The decrease in adsorbance was plotted against time. The activity of

α-chymotrypsin was determined as follows:

$$\frac{A\ E_{234}/min}{\frac{\text{adsorbance 1 } \mu \text{ mole}}{\text{assay volume}} \cdot \text{mg enzyme}} = \mu \text{ mole TEE hydrolyzed/min/mg chymotrypsin.}$$

Chymotrypsin activity was determined according to the method of Rich (1963) in sodium chloride solutions (26%) with the modification that all solutions contained 26% sodium chloride (w/w) except the enzyme solution.

Heat-Induced Plastein

A 35% solution of casein or soy protein hydrolyzate was prepared according to the method of Arai et al. (1975a) and adjusted to pH 7.0 after hydrolysis and concentration. One to two ml portions of this solution were placed in a test tube and then into a boiling water bath for two min. The heat-induced plastein was washed with a 100-fold excess of 50% ethanol, centrifuged at 1000 x g for 20 min and stored at 0 C for no more than 48 h.

Analytical Materials and Methods

Amino Acid Analysis

Amino acid analyses were performed on 22 h acid hydrolyzates of protein samples (soy and casein), utilizing the Beckman 120 C amino acid analyzer according to the methods of Moore and Stein (1954), Moore et al. (1958), and Spackman et al. (1958). Protein samples (2 mg/ml) were weighed into a 10 ml glass ampule and 5 ml of once-distilled 6 N HCl was added. A drop of octanol was added to reduce

foaming when a problem. Each ampule was frozen in a dry-ice ethanol bath under vacuum. The samples were slowly thawed under vacuum to remove the dissolved oxygen. The contents were again frozen and sealed under vacuum with an air-propane flame. The sealed ampules were placed in an oil bath at 110 C (± 0.1 C) for 22 h then removed and allowed to cool. The ampules were broken and one milliliter of a 2.5 μ mole/ml norleucine solution was added to each sample as an internal standard. The contents of the ampules were transferred to an evaporation flask connected to a rotatory evaporator and evaporated to dryness at 40 C. Samples were washed and dried until all traces of acid were removed. Acid-free hydrolyzates were dissolved in 0.067 M sodium citrate-HCl buffer (pH 2.2), containing a methionine antioxidant, thiodiglycol, BRIJ-35 (a detergent) and the preservative, pentachlorophenol. Each solution was transferred to a 5 ml volumetric flask and diluted to volume with citrate buffer pH 2.2. Two tenths milliliter sample volumes were removed for analyses.

Using a FORTRAN program developed at Michigan State University, samples were corrected for the internal standard and column difference amino acid. The computer program expressed the results in moles/1000 moles, gram residue/100 gram sample and gram residue/100 gram protein.

Incorporation of Tyrosine Ethyl Ester

Tyrosine ethyl ester-HCl was incorporated into soy and casein hydrolyzate according to the method of Aso et al. (1974) with the following modifications. Casein and soy hydrolyzates were concentrated to 33% protein and a 10 fold increase of tyrosine ethyl ester HCl was added. The hydrolyzate was incubated at 37 C for 24 h. One gram of the resulting plastein was washed with a 100-fold excess of 50% ethanol. Samples were centrifuged at 1000 x g for 20 min., shell frozen and lyophilized. A nitrogen determination and amino acid analysis were performed on the sample as previously described.

Column Chromatography

Casein and soy hydrolyzates were submitted to gel filtration over Sephadex G-75. The sephadex was equilibrated overnight in 0.1 M tris-HCl buffer containing 1 M NaCl (see Appendix). The slurry was poured into the column and after settling for 5 min the column outlet was opened to allow the gel bed to compress. The column was equilibrated with four volumes of the tris-HCl buffer. The void volume of the column was determined using Blue Dextran 2000 and the column volume with β-mercaptoethanol. The elutant was monitored with an Isco UV monitor at 280 nm.

Samples were dissolved in a SDS-phosphate buffer (see Appendix) and heated for five min in a boiling water bath. After cooling, 0.5 ml of a 0.5% protein solution was placed on the column by removing the running buffer above the column, layering the sample on top, and allowing the sample to run into the bed, followed with 2-3 ml of running buffer before continuing elution.

Polyacrylamide Disc Gel Electrophoresis

A stock solution of Cyanogum 41 was diluted and polymerized with 0.25 ml of a 1.5% ammonium persulfate solution and 50 µl of TEMED (see Appendix). Water was carefully layered on the gels cast in 8 cm long glass tubes with an outside diameter of 8 mm. The overlayer of water was removed after polymerization and the gels were placed in a Buchler 12-hole or in a laboratory constructed 6-hole, electrophoresis apparatus. The upper and lower buffer reservoir were filled with the appropriate running buffer and electrophoresed at 8-10 mA/tube using a Bio-Rad (model 400) or a Heathkit variable voltage power supply. All samples contained bromphenol blue as the marker dye. Electrophoresis in the presence of SDS was performed according to Weber and Osborn (1969). Fluorescent SDS electrophoresis and fluorescent photography was performed according to Talbot and Yphantis (1971).

15% SDS-Phosphate System

A 30% Cyanogum 41 stock solution was diluted 1:1 with 0.2 M phosphate gel buffer. The gel buffer was diluted 1:3 to yield a 0.05 M SDS running buffer (see Appendix). Electrophoresis was performed for 4-5 h at room temperature with 8-10 mA/tube.

15% SDS-Tris-Glycine System

A 15% Cyanogum 41 gel, containing tris was cast according to the method of Subbaih and Thompson (1974). The running buffer contained 25 mM tris-glycine (pH 8.3) and 0.1% SDS (see Appendix). Protein samples were dansylated according to the method of Talbot and Yphantis (1971), and electrophorsed for 4-5 h at room temperature. After electrophoresis, the position of the marker dye was marked on the glass tube with a fluorescent crayon and photographed with Polaroid type 57 high speed film.

Protein Identification

Protein (0.1-1.0%) solutions were prepared from freeze-dried preparations and dissolved in a 0.05 M sodium phosphate (Na_2HPO_4) buffer, pH 8.2. SDS was added to the protein solution in the ratio of 2:1 (SDS/protein). Twenty microliters of a 10% dansyl chloride in acetone solution was added per milliliter of protein solution with vigorous shaking. The mixture was placed in a boiling water bath for five minutes. After cooling, sucrose was added to

increase sample density. The dansylated protein was layered on a Bio-Gel P-6 column with an exclusion limit of 4,600 daltons to remove free dansyl. The Bio-Gel P-6 column had a bed volume of 46 ml, measured 26 cm in length, and was equilibrated with a 0.05 M phosphate buffer (pH 7.1) containing 1% SDS. The void volumes and column volumes were collected for electrophoresis.

Ultraviolet illumentation of dansyl (1-dimethyl-amino-5-naphthalene-sulfonyl chloride) was generated with a Black Ray UVL-21 with a peak output at 366 nm. The separation of free dansyl from proteins could be readily observed on the Bio-Gel P-6 column. Visual separation of proteins could be observed during electrophoresis by placing the UV source near the gels. Marker dye (bromphenol blue) was added to all protein samples before electrophoresis. Using a fluorescent crayon manufactured by Ultra-Violet Products (C-138 invisible chartreause), the position of the marker dye could be located on the gel tubes in the photograph.

Pictures of the fluorescent gels (15%) were taken with a 4" by 5" press-type camera equipped with a Polaroid single sheet adaptor. Polaroid type 57 (ASA 3200) film was used in combination with a Kodak Wratten gelatin filter No. 15 (yellow). The UVL-21 Black Ray ultra-violet light was suspended above the gels placed on a Plexiglass plate.

Exposure times were for one second at f4.6 to f8.0 depending on protein concentrations.

Molecular weight estimations were made from a plot of the relative mobilities vs. the log of molecular weights of the protein standards. The standards used were ovalbumin (45,000 daltons), chymotrypsin (25,000 daltons), and ribonuclease (13,700 daltons). Talbot and Yphantis (1971) reported that relative mobilities calculated from photographs of fluorescent gels gave accurate and reproducible molecular weight estimations. Relative mobilities were calculated from measurements of the protein migration zones and dye migration distance from the following relationship: R. M. (relative mobility) = distance protein migrated/distance dye migrated.

Ninhydrin

Changes in α-amino nitrogen were monitored with the ninhydrin assay. Protein solutions (0.1 ml of a 0.1% concentration) were adjusted to 0.5 ml by the addition of distilled water and 1.5 ml ninhydrin solution added (see Appendix). The mixture was heated in a boiling water bath for 20 min and cooled to room temperature. Eight milliliters of a 50% n-propanol solution was mixed with this solution and allowed to stand 10 min for color development. Absorbance was measured at 570 nm against a reagent blank. Casein and soy hydrolyzates were used to prepare a standard curve according to a procedure described by Clark (1964).

Nitrogen

Duplicate nitrogen analyses were determined by means of a micro-Kejldahl apparatus. Ten to twenty milligrams of dried sample were mixed with 4 ml of digestion mixture (see Appendix). Digestion was carried on for 1 h. The contents of the digestion flasks were allowed to cool for 30 min and 1 ml of 30% H_2O_2 was added. Digestion was continued for another hour and allowed to cool for 30 min. The flasks were rinsed with 10 ml of deionized water and allowed to cool for an additional 30 min. The mixture was neutralized with 25 ml of a 40% NaOH solution and the released ammonia steam distilled into 15 ml of 4% boric acid solution containing 5 drops of indicator solution (see Appendix). Distillation continued until a final volume of 75 ml was obtained. The ammonia-borate complex was titrated with a 0.020 N HCl solution standardized with tris-hydroxymethylaminomethane. Volumes from a blank were substracted from sample titration values.

Recovery Methods

The plastein reaction was performed with both soy and casein hydrolyzate concentrates at 33% concentration and washed with 100-fold quantities of 50% ethanol then shell frozen in a dry ice-ethanol bath and lyophilized. The hydrolyzates were weighed and multiplied by their nitrogen value. This value divided by the initial weight gave the plastein yield.

Thin Layer Chromatography

Thin layer chromatography (TLC) was used to determine the purity of L-tyrosine ethyl ester HCl (TEE) and L-tyrosine. TLC was performed according to a procedure described by Brenner and Niederwiser (1967).

In the presence of tyrosine or cystine, 0.1 M HCl was used to dissolve samples since these amino acids are only sparingly soluble in other solvents. Prepoured silica gel (Merck) plates, 20 by 20 cm and 250 μm thick, were activated in a forced air oven at 110 C for 10 min. Ten microliters of a 0.1% solution of TEE and tyrosine were spotted and dried with a hair dryer. After the samples were spotted, the plates were run unidimensionally in a chromatographic chamber equilibrated with n-propanol and water (70:30 v/v) at room temperature for 3 h. The silica plates were removed from the chromatographic chamber and air dried, then sprayed with a ninhydrin spray (see Appendix). After staining, the plates were air dried for 4 min at 110 C to achieve visible zones.

RESULTS AND DISCUSSION

Enzymatic Activities

Pepsin

The ninhydrin test was used to evaluate the extent of hydrolysis of casein by pepsin. Ninhydrin reacts stoichrometrically with free amino groups and the color produced is measured at 570 nm. Hydrolysis can be estimated by substracting initial values for the sample from values for the hydrolyzate (see Appendix). Data presented in Figure 3 illustrate that casein was approximately 70% hydrolyzed when exposed to pepsin at 37 C for 24 h. Most of the hydrolysis occurred in the first three hours of digestion. Using 25,000 as an average molecular weight for casein, 70% hydrolysis should produce peptides with a molecular weight of about 7,500. For a more accurate determination of the number of peptide bonds hydrolyzed, Whitaker (1972) recommends that the sample be subjected to reduced alkylation followed by the addition of trinitrobenzene sulfonic acid and observed at 570 nm.

α-Chymotrypsin

L-tyrosine ethyl ester (HCl) was used as a model substrate to calculate an activity of 0.4 μm/min/mg for

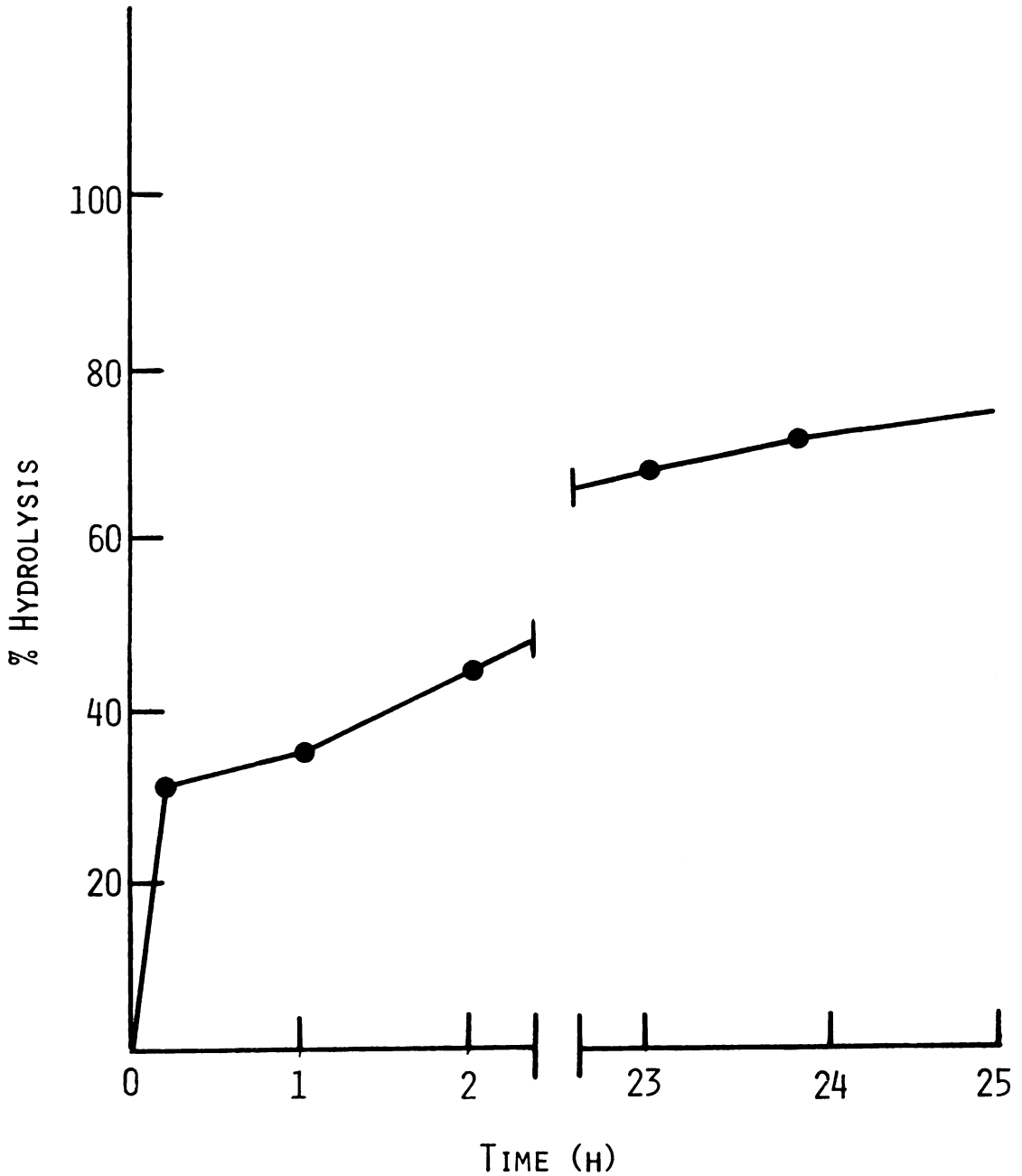

FIGURE 3. Hydrolysis of casein (1% solution) with pepsin (1:100 E/S) at 37 C (pH 7.0) as monitored by the ninhydrin reaction.

α-chymotrypsin at pH 7.0 and 25 C. Tauber (1951b) reported that substances similar to plasteins were formed when diluted protein digests (4%) in saturated NaCl (26%) solutions were reacted with α-chymotrypsin. In this study the activity of α-chymotrypsin in a saturated NaCl solution was 1.4 μM/min/mg at pH 7.0 and 25 C. It appears that in the presence of NaCl the activity of α-chymotrypsin increased about 3 fold. Turbidity can interfere with absorbance at low wavelengths (234 nm). Increase in turbidity was monitored at 600 nm. Thus, it was determined that the decrease in adsorbance was due to turbidity and that α-chymotrypsin was active in high salt solutions.

A 4% solution of L-tyrosine ethyl ester HCl in tris buffer, pH 7.0, containing 26% NaCl was monitored for changes in turbidity at 600 nm following the addition of α-chymotrypsin. After 3.26 h, absorbance increased by 0.36 units above that of the control and the solution was visibly turbid. Thus, as L-tyrosine ethyl ester is cleaved in high salt solutions, tyrosine associated and precipitated out of solution. Because tyrosine is a hydrophobic amino acid, aggregation occurs through hydrophobic forces. Brenner and Wiederwiser (1967) recognized the insolubility of tyrosine in buffer solutions and suggested that 0.1 N HCl be used as a solvent. Apparently the high salt concentration (26%) favors the aggregation of free tyrosine.

Tauber (1951b) reported that after 3 h at 37 C the peptic hydrolyzate solution (4%) in 26% NaCl became turbid following the addition of α-chymotrypsin, and after 24 h a precipitate formed. This observation was duplicated with soy hydrolyzate and the results correlated with those observed by Tauber. Additionally, it was observed that a heavy precipitate formed if the soy peptic hydrolyzate was heated for 5 min in a boiling water bath. Neurath (1963) reported that short polypeptide helices without side-chain interactions have only marginal stability in solution. Thus, if the salt solution depresses ionic side chain interactions, only hydrogen and hydrophobic side chain interactions remain to stabilize the system. The fact that heat above 60 C destabilizes hydrophobic interactions (Neurath 1963) and that α-chymotrypsin cleaves aromatic amino acids at the C-terminal residue suggests that aggregates are formed by hydrophobic interactions.

Plastein Recovery

Physical Characteristics

Soy hydrolyzate concentrate (30%) is turbid dark brown in appearance. Following the plastein reaction, the hydrolyzate changes color to a light tan. Casein hydrolyzate concentrate (30%) was opaque white with a pinkish cast. Following the plastein reaction, the protein concentrate changes in appearance to a chalk white (see

Figure 4). A dramatic increase in the viscosity of the plastein solutions was noted with soy and casein hydrolyzates. Soy hydrolyzate is extremely bitter, even in dilute solutions. Following the plastein reaction the taste is essentially bland. After washing the plasteins with 50% aqueous ethanol and centrifuging at 1000 x g for 20 min a snow white pellet was obtained.

Protein Recovery

A 50% ethanol wash was used to separate the plastein from lower molecular weight peptides and free amino acids. Both ninhydrin and Kjeldahl nitrogen assays were employed to estimate the percentage of protein in the ethanol wash and pellet (see Table 1). Plastein yields from soy and casein hydrolyzates did not exceed 30% and 7% respectively.

Fujimaki et al. (1970) expressed plastein productivity as the percentage of protein which is insoluble in 10% TCA. This TCA insoluble protein is collected by centrifugation and washed with 50% ethanol to remove bitter peptides. Whitaker (1972) reported that the change in TCA solubility of a protein-containing system is the method most widely used for monitoring hydrolysis of a protein. Because the amount of material soluble in TCA decreased instead of increasing during the plastein reaction, Fujimaki et al. (1971) assumed that synthesis had occurred. He reported that 95% of soy hydrolyzate and 48% of casein

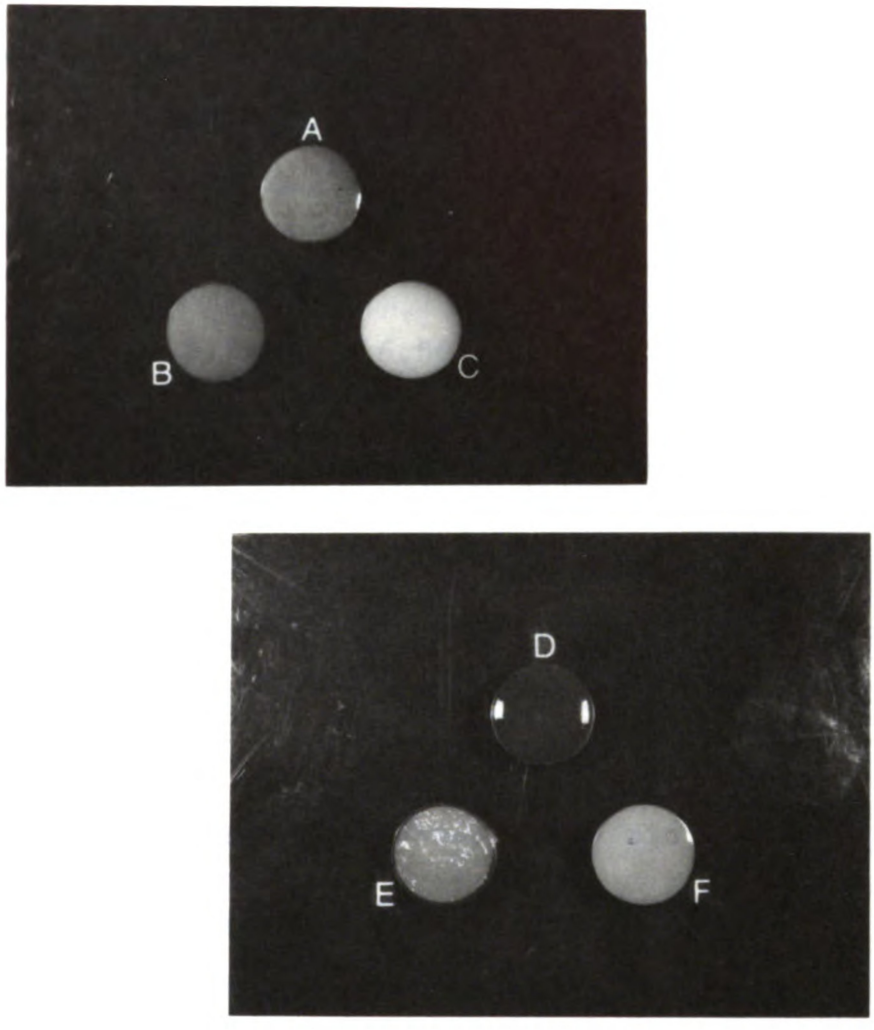

FIGURE 4. PICTURES OF CASEIN AND SOY PLASTEINS AND THEIR HYDROLYZATES (3C% PROTEIN): CASEIN PEPTIC HYDROLYZATE (A); HEATED CASEIN PEPTIC HYDROLYZATE (B); CASEIN PLASTEIN USING α-CHYMOTRYPSIN (C); SOY PEPTIC HYDROLYZATE (D); HEATED SOY PEPTIC HYDROLYZATE (E); AND SOY PLASTEIN USING α-CHYMOTRYPSIN (F).

Table 1.--Percent recovery of casein and soy plasteins from a 50% ethanol wash of the reactants.

Sample	% Protein in pellet		% Protein in wash	
	Ninhydrin	Kjeldahl	Ninhydrin	Kjeldahl
Casein hydrolyzate	0.0	--	100	--
Casein plastein	5.88	6.50	94.12	93.5
Heated Casein hydrolyzate[a]	0.0	0.0	100	100
Soy hydrolyzate	5.8	--	94.2	--
Soy plastein	27.0	27.0	73.0	73.0
Heated Soy hydrolyzate[a]	14.3	17.0	85.7	83.0

[a]Heated casein and soy hydrolyzates for 5 min at 100 C.

hydrolyzate were converted to plastein. Compared with results obtained in the present study, these values represent a difference of 72% and 86% yield for soy and casein plastein, respectively. A feasible explanation might be that much of the protein which is insoluble in TCA becomes soluble in aqueous ethanol. Thus, the yield of plastein from these sources need further study.

Molecular Weights

Ninhydrin

Whitaker (1972) reported that ninhydrin reacts stoichiometrically with free amino groups which can be

related to the number of peptide bonds hydrolyzed. As enzymatic hydrolysis proceeds, more color is generated because more amino groups are exposed per unit of protein (see Table 2). If the plastein reaction were a simple condensation or cyclization reaction, a decrease in the number of free amino groups would be expected, and a corresponding increase in units of protein per absorbance unit. The results of this study indicate that the reverse situation is the case. An increase in the number of free amino groups is concluded because of a decrease in units of protein per absorbance unit. This indicates additional hydrolysis of the protein system when α-chymotrypsin is added.

Table 2.--Units (mg) of protein required per unit absorbance change in excess of a reagent blank as determined by the ninhydrin assay.

Sample	mg/Abs
Casein protein	1.05
Casein peptic hydrolyzate	0.44
Heated casein peptic hydrolyzate[a]	0.40
Casein plastein	0.33
Soy protein	1.06
Soy peptic hydrolyzate	0.42
Heated soy peptic hydrolyzate[a]	0.40
Soy plastein	0.30

[a]Heated casein and soy hydrolyzates for 5 min at 100 C.

Gel Filtration

Sephadex G-75 produced elution patterns for casein and soy hydrolyzates and their corresponding plasteins (see Figures 5 and 6). These elution patterns demonstrated no evidence that there was significant increases in the molecular size of the plasteins. The disappearance of the void volume elution peaks and the increases in volumes for the slower eluting peaks suggest that hydrolysis continued during the plastein reaction. This observation contradicts those reported by Yamashita et al. (1970) who demonstrated, using Sephadex-gel filtration, a significant shift to higher molecular weight species after the plastein reaction. They used a standard buffer proposed by Wolf and Briggs (1956) which does not contain the high salt concentration nor was the protein sample treated with SDS before being applied to the column. Thus, the increase in molecular weight of plasteins observed by Yamashita was probably due to hydrophobic effects.

Polyacrylamide Gel Electrophoresis

Casein and soy hydrolyzates were dansylated and electrophoresed in 15% Cyanogum-41 polyacrylamide gels using either a tris-glycine or phosphate buffer system. Figure 7 illustrates that soy hydrolyzate consists of two major zones. A zone of approximately 90,000 daltons or greater and a wide zone with an average molecular weight of about 10,000. Casein digest contains only a single

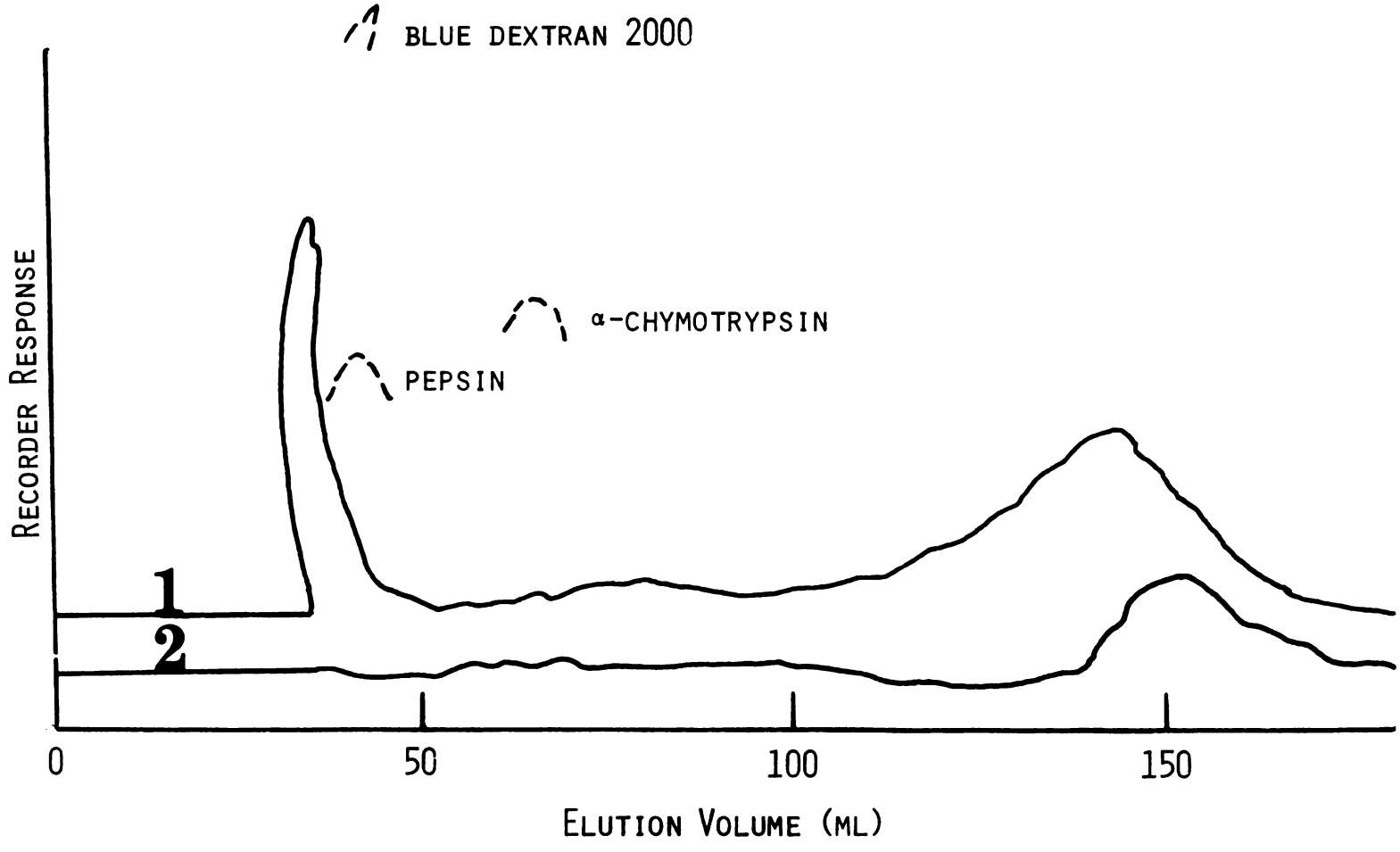

FIGURE 5. GEL FILTRATION (SEPHADEX G-75) CHROMATOGRAM OF CASEIN HYDROLYZATE (1) AND CASEIN PLASTEIN (2) IN TRIS-HCL BUFFER (1M NACL).

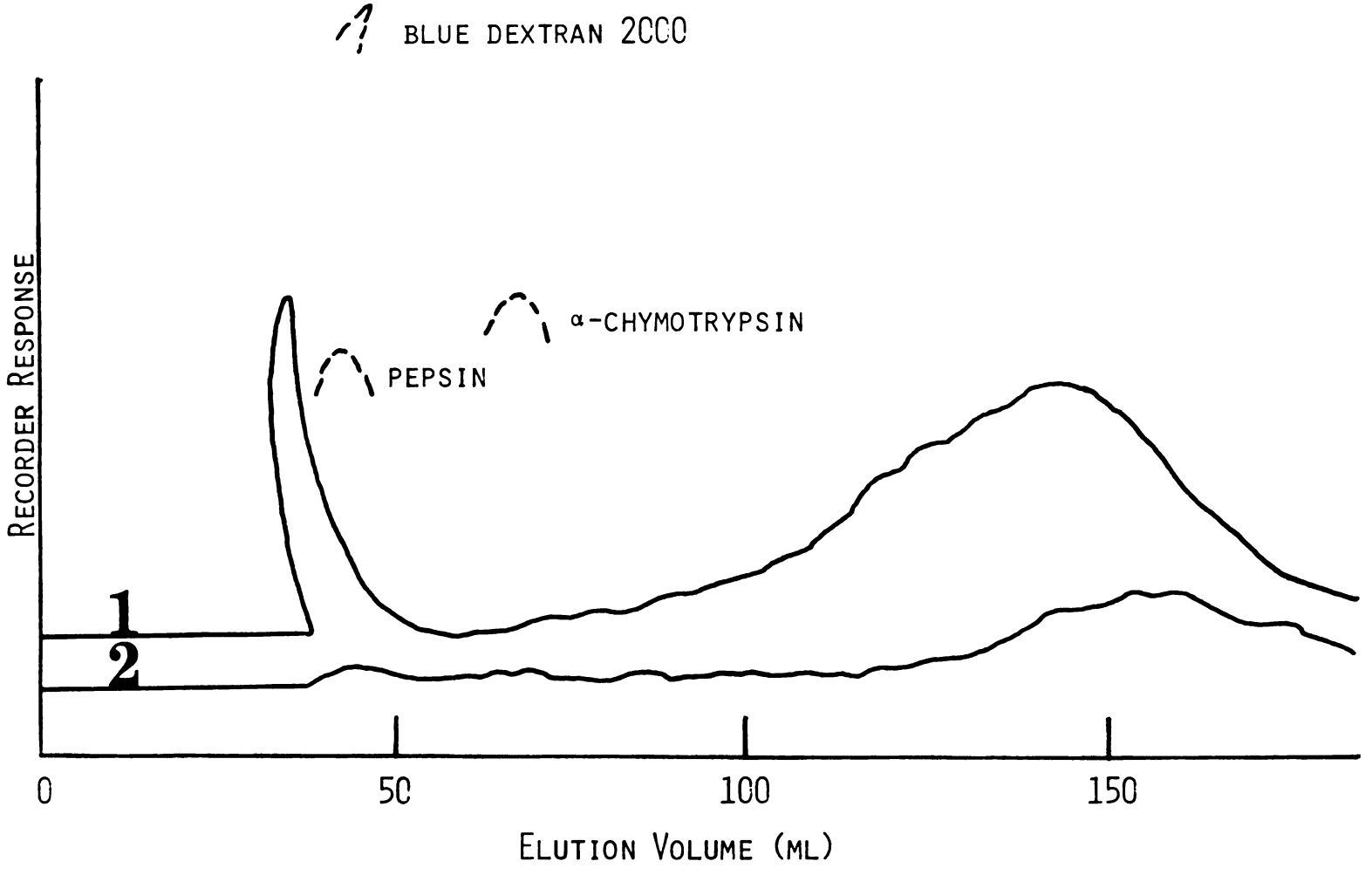

FIGURE 6. GEL FILTRATION (SEPHADEX G-75) CHROMATOGRAM OF SOY HYDROLYZATE (1) AND SOY PLASTEIN (2) IN TRIS-HCL BUFFER (1M NACL).

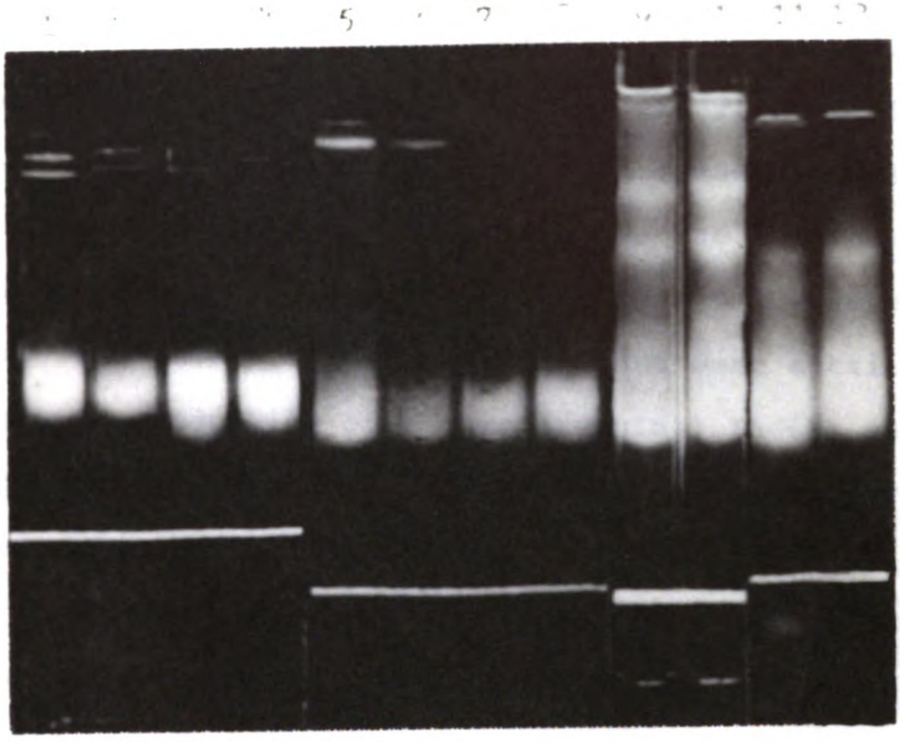

FIGURE 7. ELECTROPHEROGRAMS OF DANSYLATED CASEIN AND SOY WITH THEIR PLASTEINS IN A 15% CYANOGUM-41 TRIS-GLYCINE GEL SYSTEM: TUBE 1 & 2, HEATED SOY PEPTIC HYDROLYZATE; TUBES 3 & 4, HEATED CASEIN PEPTIC HYDROLYZATE; TUBES 5 & 6, PEPTIC SOY HYDROLYZATE; TUBES 7 & 8, PEPTIC CASEIN HYDROLYZATE; TUBES 9 & 10, CASEIN PLASTEIN; AND TUBES 11 & 12, SOY PLASTEIN.

band with an average molecular weight of about 9,000. Heating the soy and the casein hydrolyzates had no effect on the electrophoretic patterns (see Figure 7). Similarly, the casein and soy plasteins showed no increase in molecular weight. The two zones located above the low molecular weight in the casein plastein (patterns 9 and 10) have molecular weights of 25,000 and 12,500 and were attributed to hydrolytic production of α-chymotrypsin. In the case of soy plastein only the 12,500 band was observed above the lower molecular weight zone.

The concentration of lower molecular weight proteins in the 50% aqueous ethanol wash of heated soy hydrolyzate was obvious when heated soy hydrolyzate was compared to the pellet and the decanted supernatant (see Figure 8).

These results demonstrate that there was no increase in molecular weight of the plastein over the hydrolyzate. The 50% ethanol wash extracts the larger polypeptides which were not hydrolyzed by pepsin. These results are contrary to those of Tasi et al. (1974) and Arai et al. (1975) who used 7.5% polyacrylamide gel and a phenol-acetic acid-water system and demonstrated an increase in molecular weight of the plastein over the peptic hydrolyzate. Mangino (1972) concluded that a phenol-acetic acid-water system is not sufficient to dissociate hydrophobic bonds of membrane proteins, thus Tasi

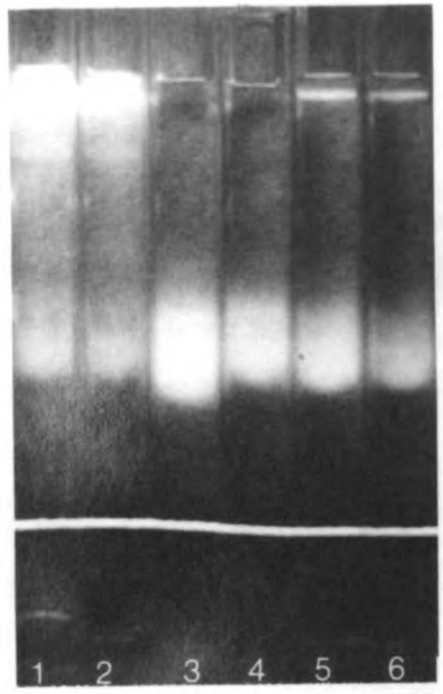

FIGURE 8. ELECTROPHEROGRAMS OF DANSYLATED HEATED SOY PLASTEIN USING A 15% CYANOGUM-41 GEL IN AN SDS PHOSPHATE SYSTEM: 50% ETHANOL HEATED SOY PLASTEIN PELLET (1 & 2); 50% ETHANOL HEATED SOY PLASTEIN WASH (3 & 4); AND HEATED SOY HYDROLYZATE BEFORE THE WASH (5 & 6).

and Arai may well have observed the hydrophobic aggregation of polypeptides.

Castimpoolas et al. (1968) followed the natural degradation of soy globulins during seed germination with 7% polyacrylamide gel using a tris-glycine discontinuous buffer system. They observed a high molecular weight protein zone that failed to show indications of proteolysis after 16 days of germination. Thus certain fractions of the soy globulin appear resistant to enzymatic hydrolysis.

In this current study, protein hydrolyzates were dansylated because of difficulty in staining the electrophoretically resolved zones. Tasi et al. (1974) and Arai et al. (1975) also experienced difficulty in staining hydrolyzed proteins. Talbot and Yphantis (1969) reported that as molecular weight of the enzymatic substrate decreased, the binding capacity of napthal blue black for the proteinaceous products also decreased. Swank and Munkres (1971) theorized that low molecular weight peptides were leached from the gel by the staining solution. Dansylation overcomes these problems since the gels are never removed from the tubes in which they were cast and electrophoresed. The identification of protein zones was increased 100-fold over napthal blue black (Talbot & Yphantis 1969).

Swank and Munkres (1971) proposed a disc electrophoretic method for analysis of oligopeptides in the range

of 8,500-1,800 daltons. Their procedure coupled with an improved dansylation procedure, i.e., eliminating the formation of dansylhydroxide, would perhaps allow for the location of the 2250 dalton plastein monomer reported by Determann and Wieland (1961). The value of sodium dodecyl sulfate (SDS) as an effective dissociating agent for apolar associated species of such low molecular weight is questionable. Results obtained in this study indicate that a low molecular weight compound such as dansylated L-tyrosine ethyl ester not only appeared in the void volume of a P-10 column but demonstrated lower electrophoretic mobility than expected on the basis of size.

Amino Acid Analyses

Hydrophobic Analyses

Amino acid analyses were performed on 22 h HCl hydrolyzates of casein and soy protein and their corresponding plasteins (see Tables 3 and 4). Bigelow (1967) proposed a method for calculating the average hydrophobicity of a protein from its amino acid composition. Tanford (1962) defined the hydrophobicity of an amino acid based on the free energy of transferring one mole from an aqueous solution at a fixed concentration to an ethanolic solution at the same concentration. Transfer free energies (HØ) for side chains were calculated in calories/residue by substracting the free energy change of glycine from each

Table 3.--Amino acid analyses of casein hydrolyzates and its plastein.

Amino acid (acid hydrolyzed)	Reference values[a]	Casein samples[b]		
		Hydrolyzate	Plastein	Heated[c]
Ala	2.39	2.73	3.68	2.71
Arg	3.67	3.90	3.40	3.32
Asp	6.13	7.14	6.54	6.98
Cys/2	0.28	0.00	1.82	0.00
Glu	19.63	22.53	9.92	22.17
Gly	2.05	1.70	3.29	1.67
His	2.74	3.00	2.39	2.64
Ile	5.26	5.21	4.83	5.19
Leu	7.93	5.58	13.30	8.86
Lys	7.18	8.26	5.81	7.64
Met	2.46	0.80	1.19	1.78
Phe	4.45	5.29	7.56	5.04
Pro	9.53	11.79	9.24	10.73
Ser	5.22	5.54	6.01	5.49
Thr	4.15	4.10	5.20	4.12
Trp[e]	1.70	----	----	----
Tyr	5.67	5.79	7.27	5.45
Val	6.09	6.64	8.47	6.20

[a]Literature values: Gordon et al. (1949).

[b]Grams amino acid residues/100 grams protein.

[c]Heated casein hydrolyzate for 5 min at 100 C.

[e]Not determined.

Table 4.--Amino acid analyses of soy hydrolyzates and its plastein.[e]

Amino acid (acid hydrolysis)	Reference values a	Reference values b	Soy samples[c] Hydrolyzate	Soy samples[c] Plastein	Soy samples[c] Heated[d]
Ala	4.08	3.60	3.80	3.93	3.76
Arg	7.45	7.55	7.57	7.98	9.58
Asp	11.51	10.38	12.16	10.30	11.80
Cys/2	1.78	1.34	0.50	0.73	0.23
Glu	16.94	18.42	20.65	13.76	15.15
Gly	4.88	3.44	3.71	3.38	3.00
His	2.66	2.25	2.43	2.13	2.09
Ile	5.20	4.40	4.80	7.07	5.73
Leu	6.73	6.66	8.30	11.02	10.31
Lys	5.81	6.01	6.19	6.54	7.10
Met	1.25	1.37	0.62	0.33	0.21
Phe	4.29	4.46	5.75	8.11	7.79
Pro	6.27	5.30	5.89	4.48	4.88
Ser	5.45	4.61	5.25	5.29	5.78
Thr	3.58	3.66	3.78	3.88	3.35
Trp	1.34	1.17	----	----	----
Tyr	3.34	3.51	3.76	4.77	4.12
Val	4.97	4.55	4.84	6.30	5.14

[a]Literature values: Yamashita et al. (1971b) g A.A./100 g protein.
[b]Literature values: Rackis et al. (1961) g A.A./100 g protein.
[c]Grams amino acid residues/100 grams protein.
[d]Heated soy hydrolyzate for 5 min at 100 C.
[e]Not determined.

amino acid (see Appendix). Average hydrophobicities calculated for soy and casein hydrolyzates and their plasteins are presented in Table 5. These data indicate an increase of 220 calories/residue for the plasteins over their respective hydrolyzates. This increase in average hydrophobicity for the protein can only arise from a relative increase in hydrophobic amino acids. The increase in free energy of transfer was larger for heated soy hydrolyzate than for heated casein hydrolyzate, indicating that hydrophobic amino acid residues participate to a greater extent in stabilizing soy.

As protein becomes denatured, an increase in turbidity is frequently observed. SDS is an excellent anionic detergent which disassociates hydrophobic bonds

Table 5.--Hydrophobicities of casein and soy hydrolyzates and their derived plasteins.

Sample	HØ cal/residue	Lit.[a]
casein hydrolyzate	1,215	1,215
casein plastein	1,435	---
heated casein hydrolyzate	1,236	---
soy hydrolyzate	1,044	---
soy plastein	1,264	---
heated soy hydrolyzate	1,181	---

[a]Value of α-casein reported by Bigelow (1967).

(Reynolds & Tanford, 1970). Smith and Circle (1972) report that secondary and tertiary structures of the major globular components of soy proteins appear to be composed of both random and compactly folded regions stabilized by hydrophobic bonds. Casein proteins assume a random-like structure in solution. Figure 9 illustrates that the solubility of casein is only slightly affected by SDS and that its hydrolysis by pepsin does not increase its solubility. Heating the casein hydrolyzate produced a slight amount of insoluble material which was quickly resolubilized by the addition of SDS. Notice the significant increase in protein insolubility which results from the plastein reaction. This high level of insolubility was reduced by the addition of SDS, indicating the strong contribution of hydrophobic effects.

Soy proteins reflect their increased sensitivity to SDS (Figure 10) which was expected because the protein structure is substantially stabilized by hydrophobic bonds. Because SDS is capable of solubilizing heat- and enzyme-induced destabilization effects, it appears that these aggregates are essentially hydrophobic in nature.

Incorporation of L-Tyrosine Ethyl Ester

The purities of L-tyrosine ethyl ester and L-tyrosine were determined by thin layer chromatography (Figure 11). L-tyrosine ethyl ester contained traces of free tyrosine. L-tyrosine contained significant impurities

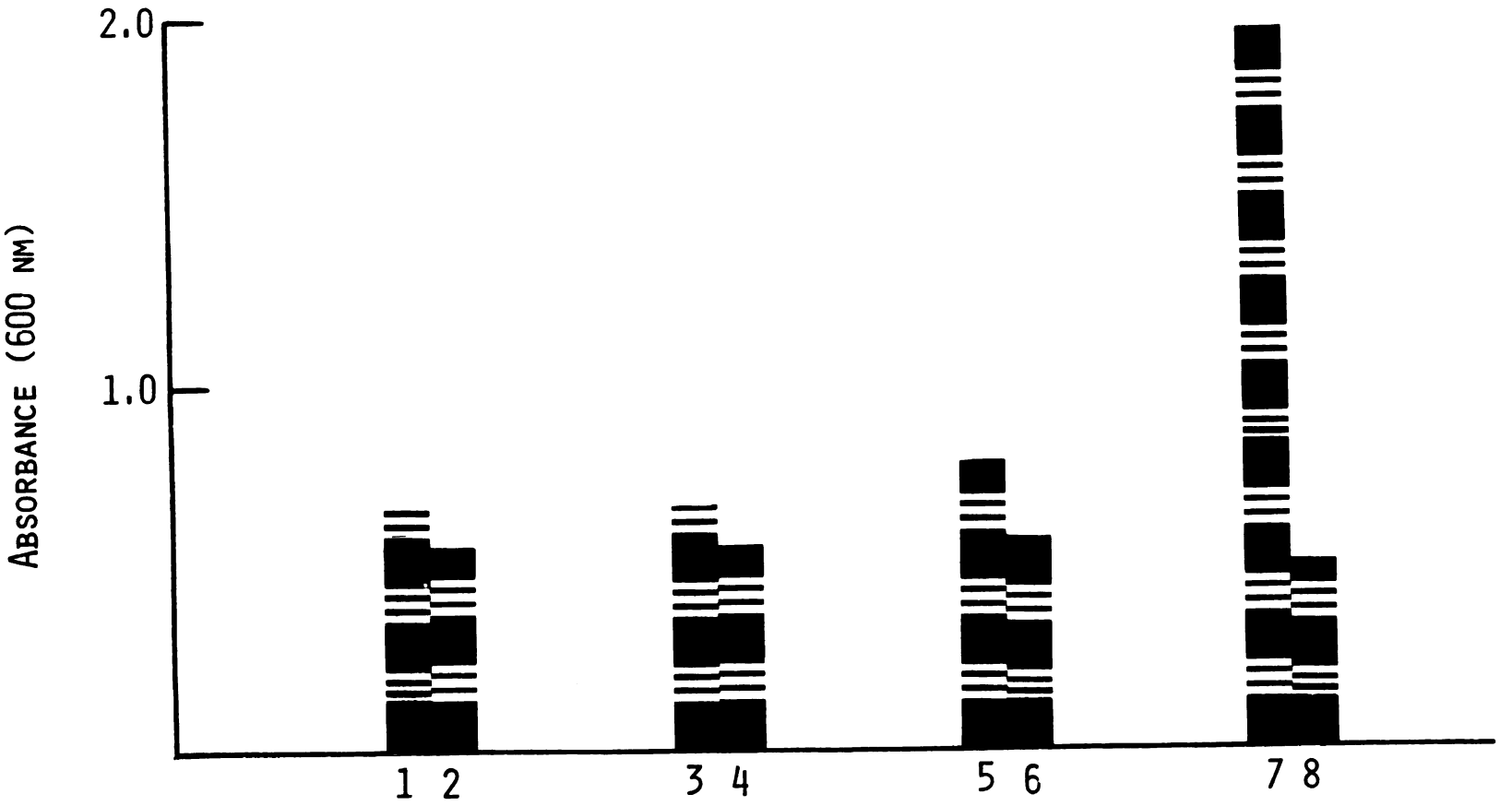

FIGURE 9. EFFECTS OF SDS ON CASEIN PROTEINS AND ITS PLASTEINS: WHOLE CASEIN IN WATER (1) AND SDS (2); CASEIN HYDROLYZATE IN WATER (3) AND SDS (4); HEATED CASEIN HYDROLYZATE IN WATER (5) AND SDS (6); AND CASEIN PLASTEIN IN WATER (7) AND SDS (8).

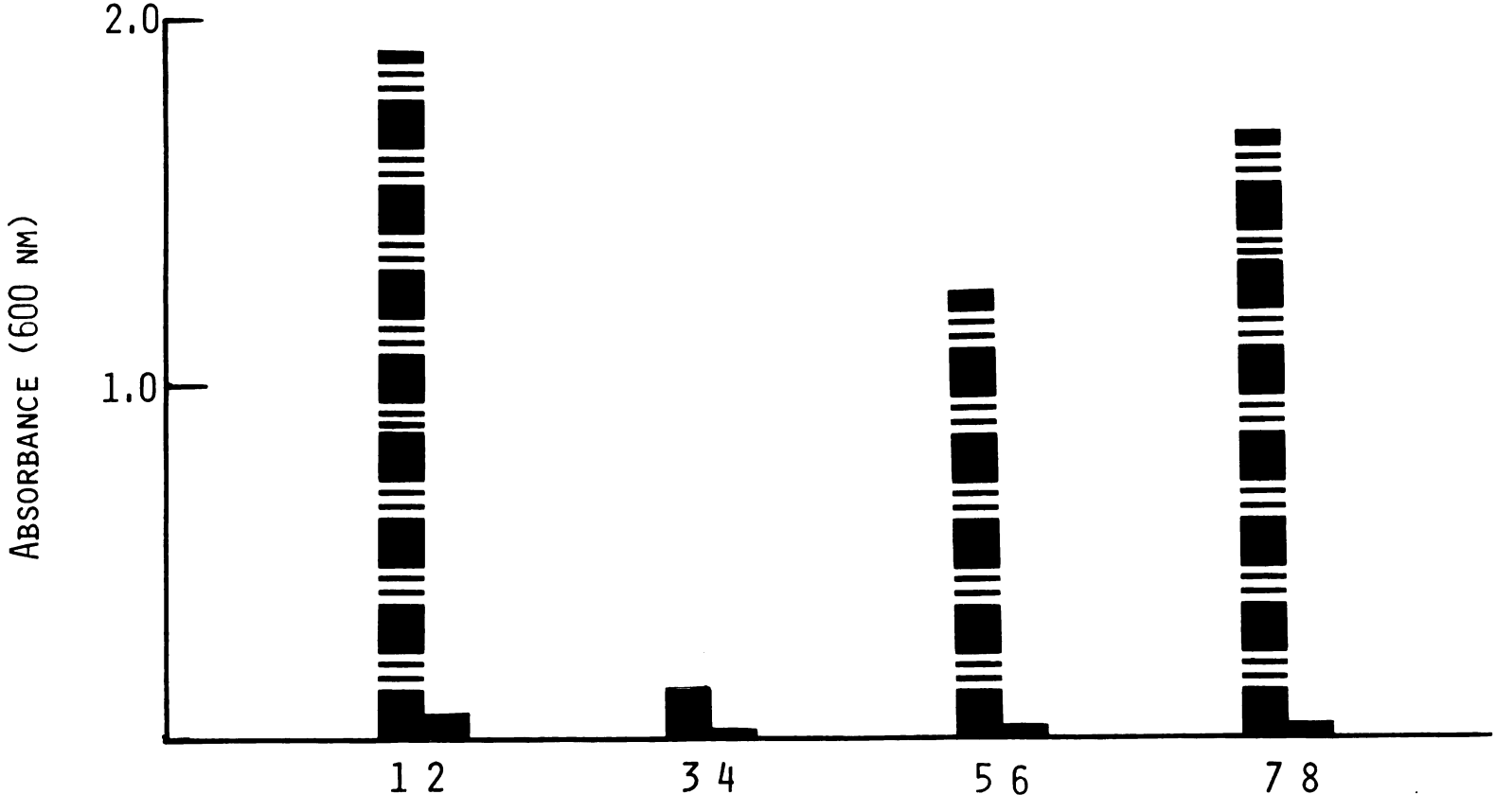

FIGURE 10. EFFECTS OF SDS ON SOY PROTEINS AND ITS PLASTEINS: WHOLE SOY IN WATER (1) AND SDS (2); SOY PEPTIC HYDROLYZATE IN WATER (3) AND SDS (4); HEATED SOY HYDROLYZATE IN WATER (5) AND SDS (6); AND SOY PLASTEIN IN WATER (7) AND SDS (8).

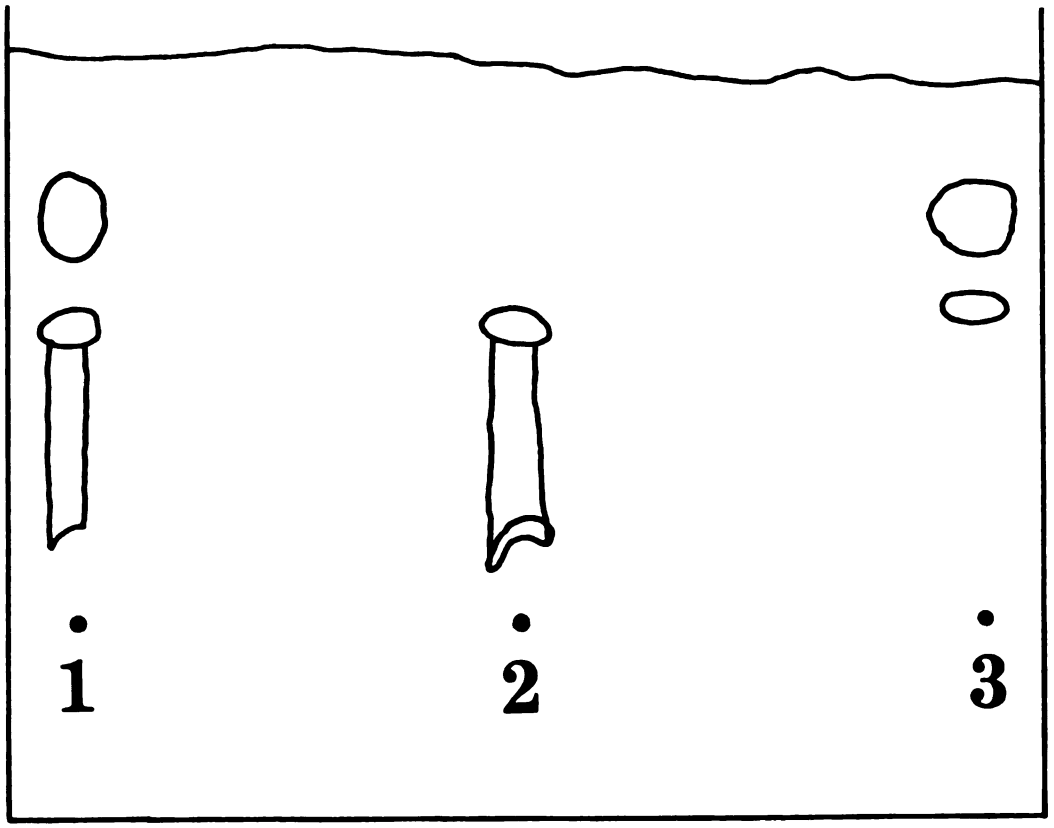

FIGURE 11. THIN LAYER CHROMATOGRAM OF TYROSINE AND ITS ETHYL ESTER ON SILICA GEL PLATES RUN WITH N-PROPANOL AND WATER (70:30 V/V) FOR THREE HOURS: POSITION 1, AN EQUAL MIXTURE OF 2 & 3; POSITION 2, L-TYROSINE; AND POSITION 3, L-TYROSINE ETHYL ESTER (HCL).

as evident from the trailing zone. An acid hydrolyzate of L-tyrosine ethyl ester was compared to an unhydrolyzed sample by amino acid analysis. Approximately 10% of the tyrosine ethyl ester was free tyrosine.

A ten-fold excess of tyrosine ethyl ester was added to the casein hydrolyzate (600 mg TEE/g protein). The plastein reaction was initiated and after an ethanol wash (1:100 v/v) the plastein pellet was analyzed for its amino acid composition. Almost complete incorporation of the tyrosine into the casein plastein was observed (Table 6). Casein plastein was heated in the presence of tyrosine and the incorporation of tyrosine appears roughly equal to that obtained with the ethyl ester preparation. Free tyrosine was added to a casein peptic hydrolyzate concentrate and heated for 5 min at 100 C. The product was washed with ethanol as above and amino acid analysis was performed on the pellet. The free tyrosine was insoluble in the ethanol and was present in the pellet fraction, amounting to 90% tyrosine.

The incorporation of L-tyrosine into soy hydrolyzates gave similar results for both heated and enzymatically activated plasteins. Soy plastein exhibited a 9.5-fold increase in L-tyrosine content whereas the heated soy hydrolyzate contained less than a 2-fold increase in tyrosine. Again free tyrosine showed a 7-fold increase over the native soy protein (Table 7).

Table 6.--L-tyrosine ethyl ester incorporation into casein plastein.*

Amino acid (acid hydrolysis)	TEE**	Samples			
		Casein plastein	Casein Plastein + TEE	Casein Hydroly-zate + Heat + TEE	Casein Hydroly-zate + Heat + Tyrosine
Ala	--	2.73	1.35	2.78	0.20
Arg	--	3.90	1.11	1.92	0.27
Asp	--	7.14	4.25	9.32	0.66
Cys/2	--	0.00	0.69	1.20	0.00
Glu	--	22.50	5.97	10.95	1.40
Gly	--	1.70	1.55	3.75	0.16
His	--	3.00	0.70	1.39	0.21
Ile	--	5.21	3.13	6.06	0.49
Leu	--	5.58	3.80	8.00	0.74
Lys	--	8.26	2.19	4.01	0.76
Met	--	0.80	0.56	1.08	0.00
Phe	--	5.29	2.96	5.20	2.15
Pro	--	11.79	4.23	7.45	0.60
Ser	--	5.54	3.75	7.41	0.51
Thr	--	4.10	2.51	5.36	0.52
Trp***	--	--	--	--	--
Tyr	10.0	5.79	58.36	18.69	90.94
Val	--	6.64	2.90	5.61	0.38

*Gram residues/100 grams protein.
**Gram free tyrosine/100 gram L-tyrosine ethyl ester.
***Not determined.

Table 7.--L-tyrosine ethyl ester incorporation into soy plastein.*

Amino acid (acid hydrolysis)	TEE**	Samples			
		Soy plastein	Soy plastein + TEE	Soy Hydrolyzate + Heat + TEE	Soy Hydrolyzate + Heat + Tyrosine
Ala	--	3.93	2.05	3.38	2.91
Arg	--	7.98	2.94	6.05	4.40
Asp	--	10.30	6.39	11.78	6.23
Cys/2	--	0.73	0.63	0.53	0.48
Glu	--	13.76	8.13	14.17	7.64
Gly	--	3.38	2.17	3.67	2.74
His	--	2.13	0.93	1.56	1.52
Ile	--	7.07	3.25	5.73	4.70
Leu	--	11.02	4.97	8.64	7.56
Lys	--	6.54	2.50	4.11	3.36
Met	--	0.33	0.26	0.61	0.35
Phe	--	8.11	4.43	6.38	5.51
Pro	--	4.48	3.05	5.11	6.14
Ser	--	5.29	3.82	6.91	3.95
Thr	--	3.88	2.65	4.35	3.21
Trp***	--	--	--	--	--
Tyr	10.0	4.77	49.21	12.09	35.17
Val	--	6.30	2.90	4.93	4.13

*Gram residues/100 grams protein.
**Gram free tyrosine/100 gram L-tyrosine ethyl ester.
***Not determined.

Horowitz and Haurowitz (1959) established that ethyl ester of C^{14}-labeled phenylalanine, tyrosine, threonine, asparatic acid, glutamin acid, leucine, isoleucine, and histidine were incorporated into plastein. They subjected their test specimens to extensive dialysis at different pH values. The amino acid-incorporated plasteins were extracted with boiling water, acetone, and dinitrofluorobenzene without significant loss of the incorporated residues. Paper chromatography of these plasteins showed no evidence of free phenylalanine or its ethyl ester. Aso et al. (1974) reported that L-lysine could be incorporated by the same mechanism contradicting the observations of Horowitz and Haurowitz (1959). The lysine enriched plastein was washed with 10 volumes of 50% ethanol made to 0.1 N with NaOH followed by 90 volumes of diluted HCl and collected by centrifugation. Similarly Arai et al. (1975a) reported that the methionine content of protein could be increased through the incorporation of the methionine ethyl ester.

CONCLUSIONS

The objective of this study was to investigate various aspects of the plastein reaction. Chymotrypsin appeared to be enzymatically active during the plastein reaction and was responsible for the changes in physical properties observed. There was no evidence that cyclization, transamination, or transpeptidation reactions occurred during the plastein reaction. Incorporation of amino acids from their ethyl esters does not appear to be the result of peptide bond formation. Instead, as the apolar amino acids are cleaved from their ethyl esters by chymotrypsin, they aggregate and precipitate.

Hydrophobic interactions in proteins is complex and seems to result from apolar amino acid side chains in contact with a polar solvent (water). The water seeks to maintain its structural integrity despite the interruption of the polar amino acids. Thus, water becomes highly ordered near aliphatic and aromatic apolar amino acid residues. This entropy difference is the basis for the hydrophobic effect.

The experimental evidence to support hydrophobic interaction in plastein formation is considerable. Column

chromatography and disc gel electrophoresis results indicated that there was no increase in the molecular weight profile of the hydrolyzate attributed to the plastein reaction. The heat-induced insolubility of peptic digests was completely reversed by SDS. The specific activity of pepsin exposes N-terminal phenylalanyl groups. Chymotrypsin exposes C-terminal phenylalanyl, tyrosyl, and tryptophanyl groups. These three amino acid residues represent three of the four most hydrophobic amino acids (Tanford, 1962).

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LITERATURE CITED

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APPENDIX

APPENDIX

Enzyme Activity

Tris-Buffer

A 0.05 M tris buffer was prepared by dissolving 6.057 g tris-hydroxymethyl aminomethane in 900 ml of distilled water and adding 23 ml of 2N HCl. This solution was diluted to 1 lt after the pH was adjusted to 7.0.

Column Chromatography

Column Buffer

A 0.1 M tris-HCl buffer was prepared by adding 12.114 g tris-hydroxymethyl aminomethane to 900 ml distilled water. 58.5 g sodium chloride was added and sufficient 2N HCl was added to reach pH 8.0. This solution was diluted to 1 lt.

Sample Buffer

0.5 ml of a 1/2% protein solution was added to 1/2 ml of a 0.01 M phosphate solution containing 1% SDS and 0.02% sodium azide.

Polyacrylamide Gel Electrophoresis

TEMED

N,N,N',N',-Tetramethylethylenediamine

Staining Solution

To 454 ml of 50% aqueous methanol, combine 1.25 g Comassie Brilliant Blue, and 46 ml glacial acetic acid.

Destaining Solution

75 ml of glacial acetic acid is combined with 250 ml methanol, and 675 ml of distilled water.

Dansyl Chloride

1-dimethylamino-5-naphthalenesulfonyl chloride was made 10% (100 mg/ml) in acetone and packed under nitrogen. Dansyl chloride was purchased from the Pierce Chemical Company.

SDS Phosphate Gel Buffer

7.8 g of $NaH_2PO_4 \cdot H_2O$ was combined with 38.6 g of $Na_2HPO_4 \cdot 7H_2O$ in 900 ml of distilled water. 2 g of SDS was added and 0.02% sodium azide was added to prevent microbiological growth. The solution was then diluted to 1 lt.

SDS Phosphate Running Buffer

SDS phosphate gel buffer was diluted 1:3 with distilled water.

Tris Glycine Running Buffer

0.046 M tris glycine buffer was prepared by adding 5.6 g tris to 28.8 g glycine and adjusting the pH to 8.3 with glycine. 2 g SDS was added and using distilled water the solution was diluted to 1 lt.

Tris Glycine Spacer Buffer

0.062 M tris buffer was prepared by adding 7.5 g tris 50 800 ml distilled water and adjusting the pH to 6.7 with 2 N HCl. This solution was made 2% in SDS and diluted to one liter.

Tris Glycine Gel Buffer

0.76 M tris was made 0.1% with SDS and adjusted to pH 8.9 with glycine.

Ninhydrin

Ninhydrin Solution

400 mg of stannous chloride dihydrate was dissolved in 250 ml of 0.2 M acetate buffer at pH 5.0. This solution was mixed with 250 ml of methyl cellosolve (ethylene glycol monomethyl ether) containing 10 g of dissolved ninhydrin and stored in a glass bottle at 0 C.

Citrate Buffer

4.3 g citric acid was combined with 8.7 g Na_3 Citrate $\cdot$ $2H_2O$ in 250 ml of solution. This solution was adjusted to pH 5.0 with NaOH or HCl.

Kjeldahl

Digestion Mixture

5.0 g of $CuSO_4 \cdot 5H_2O$ and 5.0 g of SeO was made up to 500 ml with concentrated H_2SO_4.

Indicator Solution

400 mg bromocresol green and 40 mg ethyl red were dissolved in 100 ml of 95% ethanol.

Thin Layer Chromatography

Ninhydrin Spray

0.039 g of ninhydrin was dissolved in 350 ml absolute ethanol, 14 ml colodine, and 135 ml glacial acetic acid. This solution was stored at 0 C.

% Hydrolysis Calculation

$$\frac{\text{Pepsin hydrolyzed sample - protein}}{\text{Acid hydrolyzed sample - protein}} \cdot 100 = \% \text{ Hydrolysis}$$

Hydrophobic Calculations

Amino acid	HØ (kcal/residue)
Try	3.00
Ile	2.95
Tyr	2.85
Phe	2.65
Pro	2.60
Leu	2.40
Val	1.70
Lys	1.50
Met	1.30
Cys/2	1.00
Ala	0.75
Arg	0.75
Thr	0.45
Gly	0.00

Each amino acid is multiplied by the HØ value. This product is totaled for all the amino acids and divided by the number of amino acids in the protein. This is the average hydrophobicity of a protein and is expressed in cal/residue.