MODIFICATION OF SOYBEAN PROTEINS BY IMMOBILIZED

PROTEASES/

by

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"I can write another dissertation on the basis of what I owe."

- Anonymous -

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1.0   INTRODUCTION

Coupled with ever increasing world population, pressure from
the limited energy, food, land, water and other resources is signi-
ficant today. Animal protein production requires very large inputs
of energy, land, and labor. In 1977, 91 percent of the estimated
27.1 million metric tons of cereal, legume and other plant protein
suitable for human consumption was fed to livestock in the United
States (Pimentel and Pimentel, 1977). In turn, these livestock
produced only 5.3 million metric tons of proteins that could be
consumed by humans. The energy inputs as feed for animal protein
production are 19 - 164 times more than those of plant protein
(Hamdy, 1977). This shows that the biological process by which plant
protein is converted into animal protein for human consumption is
expensive and inefficient.

On the other hand, the amount of plant protein per hectare
is eight times in general the yield of animal protein per unit
area (Pimentel et al., 1975). Of the edible plant protein sources,
soybean yields one of the largest amount of protein, 510 - 785 kg/
hectare (Nagy et al., 1978). Furthermore, soybean storage proteins
comprise 55% of the final seed weight, the highest percentage proteins
of any commercially grown seed crop (Beachy, 1982). When crops are
compared on the basis of kcal fossil energy input to kcal protein
output, soybean is highly efficient. Therefore, considering grams of

protein and energy used, plant protein production is much more efficient than animal protein. Moreover, the nitrogen fixing legume requires less energy per unit protein production than cereal grains (Rickland and Radke, 1981).

As for the protein price, soybean protein costs $ 1.03/lb., whereas beef costs $ 12.40/lb. protein basis in 1981 (Langsdorf, 1981). Evidently, soybean proteins are and will remain the cheapest source of protein for human use (Hamdy, 1977).

It has been reported that in 1976 79% of the protein produced in the world was plant protein (Hardin, 1979). The amount of soybean protein production in the world is about the same as that of animal protein (Roozen and Pilnik, 1973). In the U.S., soybean has been the second major cash crop (Wolf and Cowan, 1971). In addition, soybean is the world's main international protein source (Rackis, 1977).

Soybeans justifies usage in prudent-diet foods in terms of health, nutritional, convenience, stability and economic attributes (Hamdy, 1977). Soybeans are a good source of calcium, magnesium, phosphorous, copper and zinc (Liener, 1977). The incorporation of methionine and valine in the soybean protein can improve the biological value to approach that of milk or meat proteins. On the other hand, Young et al. (1979) clearly indicated that for young children and adults consuming well balanced diets methionine supplementation was unnecessary or possibly undesirable. Usually, the caloric energy of soybean protein products do not exceed 4.0 calories/g. Soybean

isolates and defatted soy flours allow maximum flexibility to develop products with the required fatty acid ratio of polyunsaturated to mono-unsaturated to saturated fatty acids (1:1:1) with their ratio of 3:1:1 (Orthoefer, 1978). Soybean contains no cholesterol and low levels of sucrose and dextrose (Carroll et al., 1979). Thus, the supplementation of soy protein in a diet may offer means of lowering serum cholesterol levels and decreasing the high incidence of coronary heart disease (Kritchevsky, 1979; Carroll, 1981). Sodium levels on the basis of protein range from 0.4 mg/g - 1.5 mg/g protein which is one fourth of animal source protein and an acceptable range to work with and meet the maximum salt intake requirement (Haytowitz, 1981). Moreover, legumes are good sources of complex carbohydrates (O'Dell, 1979). Soy-based formulas can be utilized to eliminate symptoms of cow milk allergy (Thomson, 1979).

Soybean proteins have the following shelf-life stabilizing factors (Hamdy, 1977): high water and fat holding capacity, no prooxidants like heme pigment which accelerate the oxidation of polyunsaturated fatty acids in meat, eliminated enzymatic activity during processing of soybean proteins and binding capacity to some added flavoring components. Also, phenolic antioxidants of soybean protein hydrolysate were found (Yee et al., 1980; Pratt et al., 1982). Consequently, stable shelf-life products could be manufactured if the proteolytic processes were carefully controlled.

The consumption of this crop, as a major protein source in

the Orient especially, has been tremendous. However, in the U.S.,
soybean derived protein products have been only recently marketed,
because the flavor, texture, and other functional properties are
comparatively strange to American people. Moreover, soybean products
have problems related to legal aspects of protein addition, anti-
nutritional factors, flatulence and amino acid content in addition to
undesirable functional properties (Wolf and Cowan, 1975; Rackis,
1977). Fortunately, anti-nutritional factors such as trypsin
inhibitors, hemagglutinins, goitrogens, antivitamins, and phytate can
be inactivated by heat treatment (Jaffè, 1973; Liener, 1974; Liener,
1979).

Since 1960, the production of soybean conentrate and isolate
have been increasing as a result of major changes in the U.S. food·
industry (Wolf and Cowan, 1975). Use must be made of desirable
functional properties of soybean proteins in order to develop
derivatives to be applied in the areas of conventional use of animal
proteins and in new food products. To achieve the best results as
food ingredients, the functional properties of soybean proteins must
be modified.

Modification of functional properties of vegetable proteins can
be achieved by physical, chemical, and biological methods and by
combination of these. Methods of extraction of protein or other
components, annealing effect of temperature, modification of porosity
and particle size during drying, all effect significantly the

functional properties of the soybean proteins (Pour-El, 1981).
Ultrasonic treatment may serve as an alternative mean to achieve
desired properties through aggregation in soybean food products
(Wang, 1981).

Alkaline hydrolysis resulted in racemization of amino acids
through removal of hydrogen from the alpha-carbon of amino acids,
splitting of disulfide bond, loss of cystine, serine and threonine
via beta-elimination reactions (Feeney, 1977). It also resulted in
the formation of potentially toxic compounds such as lysinoalanine
from lysine, ornithinoalanine from ornithine, lanthionine from
cysteine, and beta-amino alanine from dehydroalanine (Franzen and
Kinsella, 1976a). Unidentified compounds resulted from histidine
and crosslinking of the proteins make them more resistant to digestion
(Sikorsk and Naczk, 1981; Robbins and Ballew, 1982). This indicates
the need for precaution regarding chemical changes involved in food
protein treated with alkali (Hayashi and Kameda, 1980). Acid
hydrolysates are salty from the neutralization step and frequently
bitter due to the formation of hydrophobic peptides (Franzen and
Kinsella, 1976b).

Amino acid side chains can be chemically modified by commonly
used reagents by alkylation, acylation, esterification, amide
formation, reduction, oxidation, and electrophilic substitution
(Feeney, 1977). However, chemical modification of food proteins
has not been studied as extensively as enzymes and other proteins

of biochemical interest because of the following drawbacks (Feeney, 1977). For instance, the soapy flavor of succinylated casein prevents its use, because any chemically modified protein must be acceptable organoleptically in order to be of any value (Kinsella, 1976). Loss in nutritional value would be unavoidable. By chemical modification in the digestive tract, toxic products are formed. Possible, toxic compound results from the digestion of modified proteins.

The mechanisms of microbiological methods for soybean modification are similar to enzymatic modification and require large quantities of materials and energy needed for growth and maintenance of microorganisms, in addition to the modification process.

Enzymatic modification has advantages: specificity, effectiveness at low concentrations, requirements of mild conditions and of low energy input, and general safety. Moreover, enzymatic hydrolysis of food proteins generally results in profound changes in terms of variety of functional properties of the protein (Alder-Nissen and Olsen, 1979) with the specificity of the enzymes (Waxdal, 1971). Soluble proteolytic enzymes have been widely used to modify food proteins. Moreover, nutritional value of a soy hydrolysate was higher than that of the control (Rham et al., 1978). However, commonly occurring problems associated with soluble enzymatic modification of proteins are excessive hydrolysis which deteriorates functional properties such as bitter tasting hydrolysate; decreased emulsifying capacity and stability; decreased foaming capacity and

stability; and liquid retention. In addition, the elimination or inactivation of enzymes used to treat proteins is a critical problem once the desired modification of functional properties is achieved (Phillips and Beuchat, 1981). If heat inactivation is used, the proteins may be denatured and revert to insoluble forms. Obviously, washing out the enzyme at its isoelectric point would also remove a portion of the protein which is solubilized by the enzyme. Inactivation of enzyme by chemical means may cause significant changes in the protein.

The use of immobilized proteases will solve these inherent problems associated with soluble enzymatic modification. The advantages of the use of the immobilized enzymes over the soluble enzymes are conservation of enzyme, easy separation from the substrate, greater control of the extent of enzymatic action, continuous flow operation, enhanced stability, long half-lives, predictable decay rate, variety of design, rapid termination, no contamination of enzyme, and changes in physical and chemical properties of enzyme (Ferrier et al., 1972; Stanley and Olson, 1974; Zaborsky, 1974a; Kilara and Shahani, 1979). With covalent bonding methods, additional advantages are less steric hindrance, high activity, strong binding force, good general applicability and changeable substrate specificity (Chibata, 1978).

The characteristics of multienzyme systems are: enhanced substrate transfer efficiencies, stabilization of intermediates

sharing cofactors, unidirectional reactions or controls, establish-
ment of a hydrogen ion gradient or a redox potential, and provision
of hydrophobic areas of reactions (Kilara and Shahani, 1979).
Therefore, the use of multi-enzyme systems in many processing
operations may be necessary.

With the enzymatic treatments of proteins, changes in protein
properties can be expected such as: changes in molecular properties
like molecular weights, primary, secondary, tertiary, and quaternary
structures, together with reactive side chains and ionization.
Physical properties can also be affected. Hydration can occur, as
well as changes in rheological, thermal and surface properties and
in other functional properties. Peptide bond breakage by enzyme(s)
alters functional properties by a complex series of changes such as
dissociation of subunits or by opening a compact globular structure to
expose the hydrophobic interior to an aqueous phase. Since functional
properties of proteins are influenced by the molecular properties of
ingredient proteins, systematic elucidation of the physical properties
of component protein is important to understand the mechanism of
functional properties.

The main objectives of this research were to develop immobilized
protease(s) and to modify molecular size and molecular charge of
soybean proteins. This research emphasized the relationships
between molecular and functional properties of modified soybean
proteins.

The first phase of this study was designed to find the optimum conditions of immobilization of proteases. The second phase was concerned with physical and operational parameters of immobilized protease(s). The third phase involved the functional properties of modified soybean proteins by immobilized protease(s). The fourth phase dealt with modification of soybean proteins by molecular size and with molecular charge manipulations. The fifth phase was conducted to measure molecular size and molecular charge of various modified soybean proteins. The sixth phase was undertaken to find the relationships between functional and molecular properties of soybean proteins with the average molecular weights and average molecular charges.

The data obtained in this research further knowledge aimed at utilizing immobilized protease(s) to change functional properties of proteins as well as molecular weights and molecular charges to explain various functional properties of proteins.

2.0   REVIEW OF LITERATURE

The modification of plant proteins has received much attention
recently since the conversion of high quantities of plant protein
into suitable form for direct consumption has increased (Terrell
et al., 1979; Rizvi et al., 1980; Peng et al., 1982). Soybean
protein shows much potential as a food protein for future human
consumption because of its abundance, low cost, and high yield.

Soybean protein has been characterized extensively with
emphasis on its subunit structure (Catsimpoolas et al., 1968;
Catsimpoolas, 1969; Catsimpoolas and Ekenstam, 1969; Catsimpoolas
et al., 1971; Badley et al., 1975; Kitamura and Shibasaki, 1975;
Kitamura et al., 1976; Draper and Catsimpoolas, 1977; Moreira et al.,
1979; Iyengar and Ravestein, 1981; Koshiyama et al., 1981a). Like-
wise, literature concerning the functional properties of soybean
protein is abundant (Circle et al., 1964; Paulsen and Horan, 1965;
Hang and Jackson, 1967; Yasumatsu et al., 1972a; Yasumatsu et al.,
1972b; Yasumatsu et al., 1973; Wolf, 1974; Shen, 1976; Anonymous,
1979; Volkert and Klein, 1979; Morris, 1980; Patel et al., 1980;
Stone and Campbell, 1980; Tornberg, 1980). However, studies on the
molecular properties such as molecular weight and molecular charge
of protein insofar as attempts to elucidate the mechanism of several
functional properties are limited.

The successful supplementation of foods and the replacement

or simulation of traditional proteins depend on the availability of new proteins with acceptable functional properties (Wang and Kinsella, 1976). To get desirable functional properties of soybean protein, enzymatic modification can be considered as an attractive method to modify the functional characteristics (Adler-Nissen and Olsen, 1979). Although immobilized enzyme techniques offer significant advantages over soluble enzymes for protein modification, little information has been reported on their use.

2.1   Immobilized enzymes

Enzymes attached to insoluble supports offer particularly attractive advantages for many purposes including their use in the food processing industry. Moreover, immobilized enzymes will solve the problems associated with soluble enzymes: excessive hydrolysis (Roozen and Pilnik, 1973; Adler-Nissen, 1976; Beuchat, 1977b; Fujimaki et al., 1977), destabilizing of emulsions and foams (Beuchat et al., 1975; Groninger and Miller, 1975; Puski, 1975; Beuchat, 1977b; Kabirullah and Wills, 1981; Phillips and Beuchat, 1981), decreased liquid retention (Beuchat et al., 1975), and residual enzymatic activity (Rahm et al., 1978; Phillips and Beuchat, 1981).

Since the first immobilized enzyme was prepared in 1916 by Nelson and Griffin who adsorbed invertase on charcoal and aluminum hydroxide gel, a tremendous number of papers on immobilized enzymes have been published (Richardson, 1974). Gutcho (1974) and Johnson

(1979) extensively reviewed patents regarding preparations and engineering of immobilized enzymes. There are numerous methods for achieving the immobilization of enzymes (Falb, 1974; Zaborsky, 1974a; Chibata, 1978; Kilara and Shahani, 1979). The general methods are adsorption, entrapment, microencapsulation, ion exchange, cross-linking, and covalent attachment.

Stanley and Olson (1974), and Zaborsky (1974a) classified the covalent methods for immobilization into the following groups according to the mode of linkage: diazo method, peptide bonding method, alkylation method and carrier binding method. The functional groups that take part in the covalent binding of enzyme to carrier are alpha- or epsilon-amino groups, alpha-, beta- or gamma-carboxyl groups, sulfhydryl group, hydroxyl group, imidazole group, and phenolic group (Chibata, 1978). The acid azide reaction involves the epsilon-amino groups of lysine to form an amide (Weetall, 1975a).

Glutaraldehyde was used to insolubilize enzymes and proteins without supporting materials by Avrameas (1969), Jansen and Olson (1969), Hynes and Walsh (1969), Avrameas and Guibert (1971), and Jansen et al. (1971). Since the reaction is simple, gentle and inexpensive, many reports for immobilization with glutaraldehyde have been published (Zaborsky, 1974a; Bouin et al., 1976; Chibata, 1978; Iyengar and Rao, 1979; Puvanakrishnan and Bose, 1980). An additional advantage of using the glutaraldehyde method is that there are no toxic substances produced. Glutaraldehyde cross-linked

collagen used in the manufacture of sausage casing has been applied
for GRAS (Generally Recognized As Safe) status as direct human food
ingredient (Anonymous, 1982).

Ueki et al. (1974) reported that the formation of intermolecular
crosslinkages between the enzyme molecules through glutaraldehyde
is dependent on the pH. During the formation of crosslinkages, phos-
phate protected the catalytically essential residues of ribonuclease
(Zaborsky, 1974b).

Several theories have been developed to explain the crosslinking
reaction of glutaraldehyde with proteins. The glutaraldehyde reaction
involves a Schiff's base formation between an amine carrier and
usually an available amine in the protein (Weetall, 1975a). The
simple Schiff's base formation was found to be valid as the initial
step in the crosslinking reaction (Branner-Jorgensen, 1978). However,
Quiocho (1974) disagreed with formation of a Schiff's base as the
basis of the reaction of glutaraldehyde with proteins.

For the selection of a carrier, Messing (1975), Chibata (1978),
and Kilara and Shahani (1979) reviewed the following aspects:
nature of enzyme, particle size, surface area, molar ratio of
hydrophilic to hydrophobic groups, mechanical strength, microbial
resistance, thermal stability, chemical durability and functionality,
cost, and regeneration.

Porous glass has been used in many investigations (Weetall,
1969; Weetall and Hersh, 1969; Weetall, 1970; Line et al., 1971;

Ferrier et al., 1972; Shipe et al., 1972; Zaborsky, 1974a; Weetall, 1975a; Pitcher et al., 1976; Weetall et al., 1976; Bliss and Hultin, 1977; Chibata, 1978). However, on many accounts, it is attractive to consider the use of nylon as a support matrix. Nylon is readily available in a wide variety of physical forms such as films, membranes, powders, hollow fibers, and tubes. Nylon structures are mechanically strong and non-biodegradable. Nylon 6 and Nylon 66 are relatively hydrophilic, suggesting that they can support an environment on which to immobilize enzymes that is conducive to the stability of the protein (Hornby and Goldstein, 1976).

Hornby and Filippusson (1970) reported the immobilization of trypsin on the inside surface of a nylon tube through the diazonium salt formation after hydrolysis with 1N hydrochloric acid. Evidently, hydrolysis increased the number of free carboxyl and amino groups on the surface of polyamide polymers like nylon by a factor of 2 - 5 (Johnson, 1979). Amino groups of enzymes and the inside surface of a nylon tube were cross-linked with glutaraldehyde (Sundaram and Hornby, 1970; Jansen et al., 1971; Hornby et al., 1972; Smiley et al., 1974). Goldstein et al. (1974) immobilized trypsin on polyiso-nitrile nylon through either its amino or acrboxyl groups depending on buffers. Alpha-galactosidase immobilized on nylon microfibrils was utilized to hydrolyze the oligosaccharides (Reynolds, 1974).

Recently, Torchilin and Martinek (1979) reviewed new methods for insolubilization of enzymes without using conventional carriers.

The main methods considered were: addition of low molecular weight compounds; addition of organic solvents; chemical modification of key functional groups on enzymes; and intramolecular cross-linking with bifunctional reagents. Phenolase was encapsulated in liquid surfactant membranes by May and Li (1974). Moreover, Reiner and Doring (1978) discussed the covalent bonding of proteins to soluble polymers such as polyethylene glycol, and polysaccharides.

Ionizing radiations were utilized to polymerize soluble monomers and polymers (Kawashima and Umeda, 1975). Kawashima (1978) reported the use of ionizing radiation to entrap enzymes by polymerizing a water soluble synthetic monomer and a polymer in the presence of an enzyme.

Kobayashi et al. (1975) reported that in order to increase the activity of beta-galactosidase some protective agents were added to the enzyme acrylamide-bis acrylamide solution before polymerization. The optimum pH for immobilization was between 8.0 and 8.5, which is the median value of the isoelectric points of lysozyme (10.5 - 11.0) and collagen (6.8) (Bernath and Vieth, 1974). This implies that at this pH, where there is a maximum amount of net charge difference on the two proteins, the maximum formation of complexes.

Increases in concentrations of soluble enzyme and coupling time led to enzyme derivative on microcrystalline cellulose by ethyl chloroformate with higher bound protein and activity (Kennedy, 1974). Bernath and Vieth (1974) demonstrated that the amount of lactase

impregnated by a collagen membrane increased with increasing enzyme concentrations, approaching a saturation value asymptotically at higher enzyme concentrations.

Hultin (1974) reviewed naturally and artificially immobilized multienzymatic systems. Matrix-bound multi-enzyme systems were first developed by Mosbach and Mattiasson (1970). Furthermore, Mattiasson and Mosbach (1971) immobilized three enzymes whose efficiency was higher prior to reaching steady state than the efficiency of the system by corresponding soluble enzymes. Enzymes bound to the same particles are advantageous in the initial phase of the reactions, thus giving better economy, especially when involving reactions catalyzed by several enzymes (Mosbach et al., 1974). Binding of the enzymes to different particles offers better control of immobilization and activity of individual enzymes. The efficiency of the immobilized system was improved with an increasing number of sequential enzymes (Mattiasson and Mosbach, 1971; Brodelius, 1978). Co-immobilization allows a higher flow rate in a packed bed reactor. If the first enzymatic reaction is thermodynamically unfavorable, the second enzyme will shift the equilibrium, so a higher reaction rate is obtained (Srere et al., 1973). When the enzymes were working intermittently on the same macromolecular substrate, the proximity of the enzymes allowed much faster reaction rate (Martensson, 1974). For the evaluation of the functioning of the dual enzyme system, Bouin et al. (1976) immobilized glucose oxidase and catalase

to a support of nickel-impregnated silica alumina through 2.5%
glutaraldehyde. Sundaram (1978) immobilized rabbit muscle enzymes
after treating nylon-poly-(L)-lysine and nylon polyethyleneimine by
glutaraldehyde in pH 9.4 bicarbonate buffer. However, there is no
report on the multi-immobilized systems to modify food proteins.

Information on changes in enzymatic properties caused by
immobilization is necessary for the application of immobilized
systems. Changes in enzymatic properties are caused by changes in
the enzyme itself and in the physical and chemical properties of the
carrier used for immobilization (Chibata, 1978). Marconi et al.
(1974) indicated that the efficiency, defined as a percentage ratio
between the activity in enzymes entrapped in the organic fibers and
the activity in the solution of the same amount of enzyme, depended
on the concentration of the enzyme in the fiber. The theoretical
activities of immobilized enzymes relative to soluble enzymes were
17% for trypsin on silica immobilized by glutaraldehyde (Haynes and
Walsh, 1969), 24 - 60% for carboxypeptidase on AE-cellulose by
glutaraldehyde (Ueki et al., 1974), 65% for galactosidase on
polyacrylamide gel (Thananunkel et al., 1976), 98% for B_6 enzyme
on Sepharose by diazotization (Fukui and Ikeda, 1975), and 160% for
ribonuclease A with dimethyl adipimidate (Hartman and Wold, 1967).
When enzyme coupling was performed with a smaller volume of enzyme
solution, a higher proportion of the enzyme was coupled, thus giving
a more economic use of the soluble enzyme (Kennedy, 1974). Bouin

et. al. (1976) concluded that the functioning of dual enzyme systems would depend on the ratio among the amounts of active enzymes and on the absolute activity of enzymes that were obtained in the insoluble support. It was apparent that the grade of purification affected little the enzyme activity and the ratio of enzyme activity (Yokote et al., 1975).

As structural conformation changes of the enzyme protein may occur on immobilization, the degree of affinity between enzyme and substrate may be affected. According to review by Chibata (1978), little or no change occurred to the Michaelis constant on immobilization in some cases, although significant changes of up to two orders of magnitude could be found in a few cases. Enzyme activity on substrates of high molecular weight is markedly reduced by steric hindrance, which obstructs the approach of the substrate to the enzyme molecule. Experimentally, enzymes covalently bound to a water-insoluble support of enzymes which were insolubilized by crosslinking with multi-functional reagents possessed steric and diffusional limitations as well as decreased binding of substrates to the active sites (Olson and Richardson, 1974; Woychik et al., 1974).

Michaelis constants for immobilized enzymes are highly dependent on the external and internal diffusion effect (Bernath and Vieth, 1974; Lee and Tsao, 1974; Vieth and Venkatasubramanian, 1976). The Km values of immobilized enzymes were higher than those of the native enzymes (Marconi et al., 1974; Richardson and Olson, 1974; Zaborsky,

1974a; Zuzuki et al., 1975). However, Sundaram and Hornby (1970),
Fukui and Ikeda (1975), Okada and Urabe (1975), and Pitcher et al.
(1976) reported that Km values of immobilized urease, tryptophanase,
alpha-amylase, and lactase were similar to those of soluble enzymes,
respectively. The scarcely altered affinities for enzyme and
substrate suggest that the steric environment around the active
center of each enzyme is not influenced by immobilization. An
opposite phenomenon was observed because of attractive forces between
the substrate and carrier. As an example, trypsin immobilized on
CNBr-activated Sepharose was reported (Chibata, 1978). The long
length of the spacer from support materials enhanced binding properties
toward proteins and enzymes (Zemek et al., 1982).

The apparent Km values of immobilized glucose isomerase
increased in accordance with the increase of flow rate (Yokote et al.,
1975). Geenfield and Laurence (1978) critically evaluated the
deviation of Km by introducing the Damkohler number which is a
measure of the severity of film diffusional resistance. The
Lineweaver-Burk plot was sensitive to points near the extremes of
the line rather than in the center (Kennedy, 1974). Therefore,
Km values actually have little value other than providing some gross
indication of the existence of a microenvironmental effect (Bernath
and Vieth, 1974).

The changes in the pH-stability curve depend on the change of
the enzyme protein and/or the water insoluble carrier. The pH-

activity profile of the entrapped enzyme is quite similar to that of the free enzyme when substrate and product are not electrically charged as long as they are free from diffusional effects (Kobayashi et al., 1975). Similarities in the pH-activity profiles of soluble and immobilized enzymes were observed; urease on nylon by glutaraldehyde (Sundaram and Hornby, 1979), 1-asparaginase on collagen (Bernath and Vieth, 1974), glucoamylase on N-vinylpyrolidone by radiation (Suzuki et al., 1975), and beta-galactosidase on polyacrylamide (Kobayashi et al., 1975; Thananunkel et al., 1976).

When an enzyme is bound to a polycationic carrier like DEAE-cellulose, the positive charge on the enzyme protein increases, and the pH of the immobilized enzyme region becomes more alkaline than that of the external solution. Accordingly, the enzyme reaction effectively proceeds on the alkaline side of the external buffer pH, and the optimum pH apparently shifts to the acid side (Chibata, 1978). On the other hand, the shifts in the pH optima could be attributed to either microenvironmental effects or to changes in the enzyme caused by the reaction with covalent reagents (Woychik et al., 1974; Puvanakrishnan and Bose, 1980). Immobilized enzymes showed a shift in pH optimum toward the acidic side as compared to soluble enzymes. Examples are urease immobilized on glass through diazotization (Weetall and Hersh, 1969), beta-galactosidase on glass through glutaraldehyde (Woychick et al., 1974), glucoamylase on acrylamide by radiation (Kawashima and Umeda, 1975), glucose

isomerase on phenol aldehyde resin by triazinyl chloride (Yokote
et al., 1975), and lactase on silica through glutaraldehyde (Ford
and Pitcher, 1975; Pitcher et al., 1976). A highly negatively charged
membrane would attract hydrogen ions, maintain a lower microenviron-
ment pH than the bulk solution, and thus stabilize the enzyme in a
highly alkaline substrate stream (Bernath and Vieth, 1974).
Immobilized beta-galactosidase to polymethylene polyphenylisocyanate
shifted the pH optimum to alkaline side, indicating that the matrix
is negatively charged (Richardson and Olson, 1974). The charge of
pH-activity profile of tryptophanase by immobilization was caused
by the localized proton which was eliminated from the alpha-hydrogen
of the amino acid substrate prior to the subsequent rate-determining
beta-elimination step (Fukui and Ikeda, 1975). On the other hand,
Goldstein et al. (1974) indicated that the pH-activity profiles of
succinyl trypsin on nylon were broader and displaced toward more
alkaline pH values. The optimal activity was observed at pH 9.5.

With fiber entrapping a large amount of enzyme, flattening of
the pH-activity curve around the optimal pH occurred (Marconi et al.,
1974). The degree of broadening of the pH-activity curve increased
with the increase in acylation number and acyl carbon chain length
of alpha-amylase (Okada and Urabe, 1975). At pH extremes, the
activity of the entrapped enzymes is higher than that of the free
enzyme because the denaturation effect of pH is more dramatic for
the enzyme in solution. Broad pH optima of immobilized trypsin

on aminofunctional glass were reported by Line et al. (1971). The
glucose oxidase and catalase immobilized systems exhibited optimal
activities at pH 5.5, which is the optimal pH for glucose oxidase
(Bouin et al., 1976).

The catalytic activity of enzymes is dependent on temperature,
but the activity is lost at temperature above a certain limit due
to the denaturation of enzyme protein. Chibata (1978) reviewed
varied activation energy after immobilization as compared to soluble
enzymes. Low activation energies of immobilized enzymes were observed;
carboxypeptidase on AE-cellulose by glutaraldehyde (Ueki et al.,
1974), penicillin acylase on fibers (Marconi et al., 1974), and
lactase on Titania porous particles through glutaraldehyde (Pitcher
et al., 1976). The activation energy of immobilized lactase by the
silane-glutaraldehyde method was calculated to be 12.0 kcal/g-mole
(Ford and Pitcher, 1975). Therefore, the reaction rate in this case
was entirely diffusion controlled. The activation energy of immo-
bilized glucose isomerase decreased in accordance with the increase
of flow rate (Yokote et al., 1975).

It is often reported that immobilized enzymes are more heat
stable than soluble enzymes. Enzymes immobilized by bifunctional
reagents like dimethyl adipimidate and glutaraldehyde had enhanced
thermal stability compared to the native enzymes (Zaborsky, 1974c).
The immobilized enzymes having high thermal stability were trypsin
on glass immobilized by isothiocyanate (Weetall, 1969), trypsin on

oxidized cellulose by glutaraldehyde (Van Leemputten and Horis-
berger, 1974), alpha-chymotrypsin on polyacrylonitrile by imido-
ester (Zaborsky, 1974b), beta-tyrosinase on Sepharose by diazoti-
zation (Fukui and Ikeda, 1975) and glucose isomerase on phenol-
formaldehyde resin by triazinyl chloride (Yokote et al., 1975).
It is generally believed that thermostability of an enzyme is decided
principally by its amino acid sequence and the specific conformation
derived from it (Okada and Urabe, 1975). Experimentally, Kawashima
and Umeda (1975) indicated that the optimum reaction temperature of
immobilized glucoamylase was slightly higher than that of native
glucoamylase. The heat resistance of bacterial alpha-amylase at
$75^{o}C$ was enhanced by acylation (Okada and Urabe, 1975).

In many papers optimum temperature and thermostability of
enzymes immobilized by the entrapping method were found to be almost
similar to those of free enzymes (Kobayashi et al., 1975). However,
the heat stability of glucoamylase gel immobilized by irradiating
N-vinylpyrrolidone was slightly inferior to that of the native
enzyme (Suzuki et al., 1975). Lactase immobilized on collagen had
decreased temperature stability at low temperatures (Bernath and
Vieth, 1974).

Two of the most important factors affecting the industriali-
zation success of immobilized enzymes are: (a) the enzyme activating
stability under various operational variables; (b) the enzyme
activating stability under various storage conditions. The

stability of immobilized enzymes is expressed in terms of the number of days required for the activity to fall to half its initial value. It is affected by feed composition, operating temperature, and flow rate (Ford and Pitcher, 1975; Yokote et al., 1975). Glucose isomerase adsorbed on the internal surface of controlled-pore alumina showed 40 days of column half-life in sulfite buffer at 60°C (Messing, 1975). The half-life of glucose isomerase-DEAE-cellulose complex in a column was 10 - 11 days (Park and Toma, 1975). However, Weetall et al. (1976) reported that long half-life of glucoamylase immobilized on porous ceramic support was 113 days at 50°C.

Weetall and Hersh (1969) showed that urease on glass support through diazotization had been employed continuously in a column over long periods without detectable losses in enzymatic activity. Pepsin covalently attached to aminofunctional glass retained activity for over 30 days under wet cake condition (Line et al., 1971). Smiley et al. (1974) indicated that alpha-amylase immobilized on nylon tube through the glutaraldehyde reaction did not lose any activity for more than 10 days of continuous operation on 1% dextrin at 40°C. Neither the glass-bound nor collagen-bound galactosidase showed any loss of activity after 5 days of continual hydrolysis of 5% lactose at pH 7.0 (Woychik et al., 1974). Following the initial decrease in activity, stable-limit activity was maintained over 80 hr of continuous operation at 37°C (Lin et al., 1976). Weetall (1970) showed that enzymes covalently coupled to porous glass were

found to have greater stability than enzymes covalently attached

cellulose wehen stored for several weeks at a 4°C and 23°C, dry or in

distilled water. Johansson et al. (1973) reported that when

immobilized trypsin was used in the flow microcalorimeter during a

4-week period at 25°C, enzyme activity decreased about 15%. Microbial

contamination on immobilized glucose isomerase was not observed when

the substrate was high (40%) and operational temperature was high

(60°C) (Yokote et al., 1975).

The storage stability is an important factor to be considered in

application of immobilized enzyme for later use. The immobilized

trypsin could be stored for several months without loss of activity

at 2°C in 0.025N HCl (Chibata, 1978). This stabilization was caused

by a reduction of autolysis, such as blocking of the catalytic

activity of trypsin toward substrate lysine residue, since the

epsilon-amino group of lysine in the enzyme protein was protected by

immobilization. Messing (1975) found that when an enzyme was buried

in a pore having a diameter of less than 1,000 $\overset{\circ}{A}$, it would be

impossible for microorganisms which generally have dimensions greater

than 1,000 $\overset{\circ}{A}$ to gain access to this enzyme. As compared with soluble

urease, Sundaram and Hornby (1970) showed that the storage stability

of immobilized urease on nylon tube in phosphate buffer at 4°C was

higher than that of soluble urease. For many months, immobilized

enzymes were stable under storage conditions; alpha-galactosidase

(Reynolds, 1974), carboxypeptidase (Ueki et al., 1974), glucose

oxidase (Messing, 1974), and beta-galactosidase (Kobayashi et al., 1975). Stability of immobilized beta-galactosidase showed 86 - 100% of original enzymatic activity for 29 - 97 day periods (Richardson and Olson, 1974). Smiley et al. (1974) found that for more than 3 months at room temperature, the alpha-amylase-nylon tube lost enzymatic activity by 25%. For 15 days of storage period, alcohol oxidase on photo-cross-linkable resin lost 25% of its enzymatic activity (Fukui et al., 1978).

Originally most of the immobilized enzyme techniques have been used in outside of food areas such as in the investigation of enzymatic properties, studies on protein structure, analytical systems, experimental medical therapy, and preparation of pharmaceuticals. Currently enzymes utilized in the food industry have been reviewed by Kilara and Shahani (1979), Reilly (1980), Roland (1980), and Weetall (1980). Potential applications of immobilized enzymes to food industry can be realized by two processes: degradative process and replacement type process (Weetall, 1975b). Unfortunately, few applications of immobilized proteolytic enzymes have been made to improve functional properties of plant proteins. However, the use of some immobilized proteolytic enzymes has been reported. The application of immobilized papain to the production of chillproofed beer was reviewed by Brodelius (1978). Immobilized pepsin and rennin have been used to coagulate milk (Richardson and Olson, 1974). Alpha-galactosidase entrapped in polyacrylamide gel has been reported

for the degradation of raffinose and stachyose in soybean milk
(Thananunkul et al., 1976; Rackis, 1977). Bliss and Hultin (1977)
immobilized protease from _Streptomyces_ _griseus_ to porous glass which
was effective for the removal of fungal glucose oxidase and
partially effective to remove soluble protease. To retard milk
oxidation, Shipe et al. (1972) solved the problems regarding soluble
trypsin by replacing it with immobilized trypsin. As an another
approach to prevent the cooked flavor of heated milk, Swaisgood
(1977) developed a process which could convert sulfhydryl groups to
disulfides by immobilized sulfhydryl oxidase originated from raw
milk. Pronase immobilized on porous glass liberated different
quantities of five amino acids from casein than did soluble pronase
(Detar et al., 1975). Kinsella (1976) discussed the potential uses
of immobilized enzymes with ultrafiltration reactors for the production
of protein hydrolysates. Yun et al. (1981) reported that curd
tension of milk coagulum increased linearly with the extent of
proteolysis, which was controlled by employing proteases in an
immobilized form. Recently, Adu-Amankwa et al. (1981) immobilized
Penicillium _duponti_ enzyme on reconstituted collagen by macromolecular
complexation, impregnation and covalent crosslinking techniques for
the hydrolysis of soybean proteins. Without presenting data or an
explanation, they mentioned that the enzyme-modified proteins had
desirable whipping, foaming ability and spreading properties.

Glass (1981) and Semenov et al. (1981) discussed the use of

enzymes to catalize peptide bond formation and to manipulate blocking
groups during peptide synthesis. When the hydrolyzed protein is
incubated with certain proteolytic enzymes under appropriate condi-
tions, the hydrolysis is reversed and a high molecular weight
protein-like substance is formed (Yamashita et al., 1971; Arai et
al., 1974; Arai et al., 1975; Eriksen and Fagerson, 1976; Yamashita
et al., 1974; Aso et al., 1977; Rackis, 1977; Monti and Jost, 1979).
The properties are different from the original protein. Therefore,
the creation of some new functional properties might be possible.
Although the acyl-enzyme intermediate is known to be transferred to
other nucleophiles, such as the amino groups of amino acids and
peptides (Fujimaki et al., 1977), the plastein reaction is not throughly
understood and there is a controversy relating to the mechanism
(Satterlee, 1981).

2.2 Electrophoresis

Electrophoretic techniques offer very high resolution in the
separation of proteins and provide physicochemical parameters
pertaining to their characterization in terms of molecular size and
charge.

The differential migration of proteins in an electric field
depends on the magnitude of their surface charge density at a
particular pH (Catsimpoolas, 1977). The ionic groups that are
involved are alpha, beta, and gamma-carboxylic, alpha and epsilon-

amino, imidazole, and sulfhydryl, phenoxy, and guanidinium (Whitaker, 1972).

The mobility of a protein subjected to electrophoresis is governed by its particular pH. At any pH value above or below its isoelectric point (pI), the protein has a negative or positive net charge and will migrate toward the positive or negative electrode (Lehninger, 1970). Since electrophoresis is carried out in a supporting medium such as porous gel, the size differences among various proteins can be utilized for separating them either synergistically or independently of charge (Catsimpoolas, 1977).

If the average pore size approaches the range of dimensions of proteins, the various protein fractions will be differentially retarded to degrees proportional to their dimensions (Davis, 1964). When $\log_{10}$ of relative mobility for a single protein is plotted versus gel concentration, a linear relationship is found which follows the equation: $\log_{10} R_f = \log_{10} Y_0 + K_R T$, as first described by Ferguson on starch gel (1964), where R_f is the relative mobility at gel concentration T, Y_0 is the extrapolated relative mobility at zero gel concentration, and the coefficient K_R is defined as the retardation coefficient. Later, this relationship was independently found on polyacrylamide gel by Hedrick and Smith (1968). The retardation coefficient is related to molecular size and Y_0 is related to molecular net charge (Rodbard and Chrambach, 1971; Frank and Rodbard, 1975). A Ferguson plot can be constructed by three to seven points

and then K_R and Y_O can be calculated directly by weighted linear regression (Rodbard, 1976).

Ionic detergent molecules such as sodium dodecyl sulfate (SDS) equalize the charge density on the protein surface (Weber and Osborn, 1969; Rodbard, 1976; Catsimpoolas, 1977). Under these conditions the electrophoretic velocity is totally dependent on the retardation coefficient of the protein which is related only to its size and shape (Chrambach and Rodbard, 1971). Ideally, in the presence of SDS, Y_O should be a a constant, irrespective of K_R (Rodbard, 1976).

Rodbard and Chrambach (1970) developed a unfied theory for gel electrophoresis and gel filtration. The number of average molecular weight by gel filtration was characterized by its principal statistical moments (Catsimpoolas, 1974; Hsieh et al., 1979). Rodbard et al. (1971) proposed gel gradient electrophoresis for improving resolution and measuring molecular size. Rodbard and Chrambach (1971) concluded that the knowledge of free mobility and protein radius made possible calculation of net charge on a molecule.

Recently, Anderson (1980) examined water-extractable, acid-precipitable, and whey proteins from soybeans by disc gel electro-phoresis in pH 8.9 tris-glycine buffer, at gel concentrations ranging from 4 to 13%. Retardation coefficients and relative free mobilities of the soy proteins were grouped and used for the identification. Frank and Rodbard (1975) mentioned that the relation-ship between K_R and Y_O for 12 standard proteins showed 0.68 of the

correlation coefficient. The components from soybean protein did not show a clear relationship between the two constants (Anderson, 1980).

Chrambach et al. (1976) articulated the choice of buffers and solubilizing agents. Hjelmeland et al. (1978) classified solubilizing agents on the basis of charge for the protein separation. At present, SDS is the most widely used ionic addition to polyacrylamide gel electrophoresis (PAGE) buffers and samples. SDS virtually eliminates conformational and charge density differences among proteins and reduces the effect of variability on partial specific volume, hydration and axial ratio (Chrambach and Rodbard, 1971). At detergent concentrations near or below the critical micelle concentration, SDS binding to protein may undergo marked changes (Frank and Rodbard, 1975). Above this level, the binding increases rapidly. Hjelmeland et al. (1978) showed that the critical micelle concentrations of SDS and Triton X-100 were 8.2 and 0.24 mM in H_2O, respectively. Ionic detergents have higher critical micelle concentrations than non-ionic detergents. Weber et al. (1972) recommended that the weight ratio of SDS to protein be at least 3:1.

Electrophoresis at high SDS concentration was preferred by Fairbanks et al. (1971). However, excessive amounts of SDS produced an inordinately wide starting zone in the stacking gel, leading to impaired resolution (Wyckoff et al., 1977). Danno and Hoseney (1982) observed that SDS is a good solvent for protein separation because

it disrupts both hydrogen and hydrophobic bonds. SDS-PAGE provided
the only effective separation of the meat analog protein soluble in
SDS (Rizvi et al., 1980). Moreover, SDS-containing buffers were
most effective than other solvents such as, 2 - 8M urea, 6M guanidine-
HCl, and phenol-acetic acid-urea-cetyltrimethylammonium bromide, in
terms of both their ability to solubilize wheat proteins and of their
suitability as buffers for column chromatography (Bottomly et al.,
1982). According to Schofield and Baianu (1982), approximately 95%
of flour and gluten protein are extracted by SDS. Because of a high
level of binding of SDS, the charge contribution of the protein would
be not more than 10% of the charge introduced by the anionic detergent
binding (Weber et al., 1972). However, Hearing et al. (1976) reported
that SDS did not provide an equalized charge density on the molecular
surface due to folding of the protein or forms of steric hindrance.

For the charge evaluation, the detergents must have minimal
effects on the surface net charge in order to give protein-detergent
complexes which retain their native electrophoretic properties
(Hjelmeland et al., 1978). Nonionic solutes such as glycerol,
sucrose, urea and nonionic detergent (Triton X-100) can be added to
the gel and buffer solvent if free of ionic contaminants (Chrambach
et al., 1976).

In the presence of 4M urea in the gel, two forms of gamma-casein
were separated by Groves and Kiddy (1968). Melachouris (1969)
detected twenty well-defined zones from milk casein without using

2-mercaptoethanol on a 9% gel containing 7M urea. Hood et al. (1981)

used 10 mM 2-mercaptoethanol and 5M urea for the sample preparation

as well as 5M urea for the gel preparation to dissociate alfalfa

protein. To minimize the possibility of formation of artifact bands

due to carbamylation reaction in a boiling water bath, urea had to

be prepared freshly from pure reagent or deionized just prior to

use (Cole and Mecham, 1966).

Hearing et al. (1976) indicated that Triton X-100 had many

advantages relative to urea; the nonionic detergent was a parti-

cularly advantageous choice of solvent, due to its excellent protein

solubilizing properties, the preservation of protein conformation

and function without denaturation of proteins. They also demonstrated

that Triton X-100 did not dissociate proteins into their subunits;

on the contrary, it provided a favorable environment for normal

protein aggregation. Therefore, the molecular size and charge of

protein can be determined by using this detergent.

Hill and Breidenbach (1974) utilized beta-alanine plus 8M urea

as a dissociating system to characterize 2S, 7S, 11S and total fract-

ion of soybean storage proteins. Puski and Melnychyn (1968) utilized

tris-glycine or acetate buffer containing 6M urea and 0.1M 2-mercapto-

ethanol to dissolve soy protein. Ethylene glycol (45%) was included

in the buffer during chromatographic separations of zein to disrupt

protein-protein association through hydrophobic interactions (Esen,

1982). Kawasaki and Ashwell (1975) reported that the tendency

towards self-association of hepatic membrane protein through hydrophobic regions was completely reversed by addition of Triton X-100.

Laemmli (1970) mentioned that in gels of higher acrylamide concentration an additional three low molecular weight structural proteins were separated. For the low molecular weight proteins, the high concentration of acrylamide (12.5%) was recommended (Weber and Osborn, 1975).

Although the heavy binding of SDS to denatured protein swamps out even relatively small differences in intrinsic net charge, molecular mass determinations with SDS are difficult, especially when the amino acid composition deviates significantly from the average composition for proteins used as a standard (Iyengar and Ravestein, 1981). The accuracy of the quantification of subunit distributions from SDS-PAGE depends on the basic amino acids, particularly lysine, contained in each polypeptide type bound to anionic dye, Commassie Brillant Blue R-250 (Hu and Esen, 1981; Schofield and Baianu, 1982). Because the content of such basic amino acid residues in different types of subunits varies and because of the deviation of contents from standards, the values given by these authors can not be regarded as absolute, but they give useful information for different fractions. When gel preparation methods, voltage, and temperature were kept constant, the reagent purity and concentration, the electrophoresis time and type of conditions in the apparatus all

affected electropherograms (Lookhart et al., 1982).

2.3   Soybean proteins

Soybean proteins have been reviewed extensively by Wolf (1972, 1974, 1977b) and Kinsella (1979). Traditionally, the major components are classified according to their sedimentation properties (Wolf and Cowan, 1971; Wolf, 1972; Hill and Breidenbach, 1974; Kinsella, 1979). The investigations in which an ultracentrifuge was used have shown that soybean globulins consist of four components with sedimentation constants equal to about 2, 7, 11, and 15S. Among them, approximately 90% of the proteins in soybeans, mostly globulins exist as dehydrated storage proteins (Wolf and Cowan, 1971). The remaining proteins are composed of intercellular enzymes (lipoxygenase, urease, amylase), hemagglutinins, enzyme inhibitors and membrane lipoproteins (Kinsella, 1979). Recently, extensive studies have shown that soy proteins were tremendously heterogeneous (Hu and Esen, 1981). Hu and Esen (1982) found that soybean seeds contained at least 1,000 different polypeptides from the three solubility fractions.

The trypsin inhibitors in soybeans are important in relation to the utilization of soy proteins from both a nutritional standpoint and functional properties. The smallest protein, the Bowman-Birk inhibitor with a molecular weight of 7861, can inhibit trypsin well and chymotrypsin slightly (Wolf, 1977b; Baintner, 1981). It is

remarkably stable to heat, acid and proteolytic digestion, resulting in the residual trypsin inhibitor activity remaining in heated soybean protein products because the seven disulfide crosslinks make the molecule fairly rigid (Wolf, 1972; Johnson et al., 1980). Nevertheless, the microwave heating for 1.5 min completely inactivates trypsin inhibitor in soybean milk (Armstrong et al., 1979). Lei et al. (1981) found that the attractive conditions for inactivating soybean trypsin inhibitors were 2.5 mM cysteine, pH 9.0 and 80°C for 10 min without affecting solubility. Likewise, Friedman et al. (1982) reported the complementary cooperative effect of heat and thiols in activating soybean trypsin inhibitors in a commercial soy flour. Kunitz trypsin inhibitor with 181 amino acid residues and two disulfide bonds is effective on trypsin and chymotrypsin (Wolf, 1977b). This inhibitor is relatively heat labile (Liener and Tomlinson, 1981). Koshiyama et al. (1981c) claimed that 2S globulins of soybean proteins contained some inhibitory activities against trypsin and/or alpha-chymotrypsin and Kunitz trypsin inhibitor, which were identical to α_3 protein. Moreover, the thermal stability was the highest in α_4 protein, but that of α_2 protein was lost entirely at 79°C (Koshiyama et al., 1981b). Furthermore, Ellenrieder et al. (1980) and Ellenrieder et al. (1981) reported that the high molecular weight substances separated from soybean extracts by gel filtration on Sephadex G-75 accelerated thermal inactivation of trypsin inhibitory activity of soybean extracts and purified inhibitors.

Four agglutinins (lectins), which cause blood cells to clump together or agglutinate, are glycoproteins containing 4.5% D-mannose and 1.2% N-acetyl-D-glucoseamine and have a molecular weight of 120,000 (Lis and Sharon, 1973). With dissociating solvents such as 0.1% SDS or 6M guanidine hydrochloride, the molecular weight was reduced to 30,000 as a result of dissociation into four subunits (Wolf, 1977b). The possible mechanism of the toxicity is the combination of the ingested agglutinin with glycoproteins of cell membrane lining of the intestinal wall, interfering with normal nutrient absorption (Filho et al., 1979). Therefore, agglutinins contribute to the deterious effects of unheated soybean products (Sharon and Lis, 1972; Liener, 1974). Fortunately, heat treatment enhanced the nutritive value of raw legumes (Jaffé, 1973; Liener, 1974). The biological function of agglutinin combines specifically with the soybean-noduling bacteria (Mort and Bauer, 1980).

The storage proteins, 7S (conglycinin), and 11S (glycinin) are the major components of soybean proteins (Wolf and Cowan, 1971). Because of different properties and physical behavior of these globulins, several methods have been used to fractionate and characterize these proteins (Eldridge and Wolf, 1967; Catsimpoolas et al., 1968; Catsimpoolas et al., 1971; Saio et al., 1973). To fractionate 7S and 11S globulins of soybean proteins, calcium salt was used (Saio et al., 1973). The cold insoluble fraction is rich in 11S protein (Eldridge and Wolf, 1967). Furthermore, calcium salt up to

10 mM can be used to fractionate 7S rich fraction soluble in the solution. The cold precipitation method by Wolf and Sly (1965) to separate 7S and 11S proteins may be time-consuming and low in yield for practical application (Saio et al., 1973). Catsimpoolas et al. (1968) reported that the 7S and 11S components were found to be dissociated into 14 and 12 subunits, respectively, in the solvent system of phenol-acetic acid-0.2M 2-mercaptoethanol (2:1:1, w/v/v) made in 5M urea.

The 7S fractions contain lipoxygenase, hemagglutinin and predominately the 7S globulins (Wolf and Cowan, 1971). Catsimpoolas and Ekenstam (1969) isolated alpha, beta, and gamma-conglycinins and glycinin by immunoelectrophoresis from 7S globulins which comprised about one-third of the total soy proteins. Catsimpoolas and Ekenstam (1969) demonstrated that Koshiyama's 7S preparation presented gamma-conglycinin. They found that the molecular weight of beta and gamma-conglycinins were 330,000 and 210,000, respectively. The monomer (7S form) having 5.9% carbohydrates was observed above 0.5 ionic strength, whereas the dimer (9S form) was favored below 0.1 ionic strength above the isoelectric point (Kinsella, 1979). Between ionic strength of 0.1 and 0.5, the mixture of 7S and 9S form existed (Koshiyama, 1968). Thanh and Shibasaki (1979) reported that the beta-conglycinin had a protomer (7S) at acidic pH (pH 4.8) and a dimer conformation (10S) at low ionic strength (I 0.5) in the pH region 4.8 - 11.0. The major 7S soybean protein, beta-conglycinin, was

found to consist of six isomers (β_1 - β_6 conglycinins) having different

sugars, different N-terminal residues, and different amino acid

composition (Thanh and Shibasaki, 1978a). Furthermore, Thanh and

Shibasaki (1978b) showed that the isolated α, α', and β subunits of

β-conglycinin were unfolded in phosphate buffer containing 6M urea

and upon removal of urea, self-association of the subunits occurred,

forming ten possible molecular species of β-conglycinin. According to

Thanh et al. (1975), the electrophoretic heterogeneity of the native

7S globulin was due to the association of its three major subunits

into six trimeric species.

A considerable amount of knowledge about glycinin (11S) has been

already available in the literature (Wolf, 1977b; Kinsella, 1979;

Iyengar and Ravestein, 1981). For the isolation of 11S soybean

protein, the Eldridge 11S fractions can be prepared from an aqueous

extract of soy flour by cooling to $4^{o}C$ (cold insoluble fraction) or

calcium precipitation (Eldridge and Wolf, 1967; Saio et al., 1973).

The cold insoluble fraction had all 11S components, and also contained

other fractions (Puski and Melnychyn, 1968). Generally, 11S components

moved faster than 7S components in 0.08M tris-chloride buffer, pH 8.7,

containing 5M urea and 0.1M 2-mercaptoethanol. According to Eldridge

and Wolf (1967), the cold insoluble fraction contained 65% of 11S

and 10% of 7S fractions with 100% yield of 11S fraction.

Glycinin was generally considered to be composed of 12 subunits

arranged in two similar half molecules of six subunits each

(Catsimpoolas et al., 1968; Catsimpoolas, 1969; Badley et al., 1975; Wolf, 1977b). Moreira et al. (1979) isolated seven poly-peptides with three acidic isoelectric points and with four basic isoelectric points from glycinin in alkaline and acid-6M urea, after reducing and alkylating the protein with 4-vinylpyridine in 6M guanidine hydrochloride. Moreover, Kitamura et al. (1976) found four acidic subunits (A_1, A_2, A_3, A_4) present in the approximate molecular ratio of 1:1:2:2 and four basic subunits designated as B_1, B_2, B_3 and B_4 based on the relative mobilities in the approximate molar ratio of 1:1:2:2 in the acidic gel in 7M urea. Kitamura and Shibasaki (1975) concluded that there were sequential homologies between A_1, A_2, and A_3 subunits, and that the primary structure of an A_4 subunit was relatively different from that of other three subunits. This conclusion was reached by using electrophoresis of CNBr-treated acidic subunits in acidic and in alkaline systems in the presence of urea as well as in double gel immunodiffusion. Furthermore, Mori et al. (1981b) classified glycinin from 18 cultivars into five groups according to the differences in molecular charges of subunits consisting of 6 - 7 acidic subunits and 3 - 8 basic subunits.

The monomer of 11S proteins was composed of three subunits with molecular weight of 22,300 and three subunits with molecular weight of 37,200 (Catsimpoolas et al., 1971). The dimer composed of 12 subunits showed a molecular weight of 357,000 (6 x 22,300 plus 6 x 37,200). Consequently, the subunits were recognized as two sizes and charges,

acidic (35,000 - 42,000) and basic (17,000 - 20,000) (Badley et al.,
1975; Wolf, 1977b; Moreira et al., 1979). Kitamura et al. (1976)
showed that the fourth acidic subunit had relative molecular mass
of 45,000. Iyengar and Ravestein (1981) stated that the previous
reported value for the acidic subunits seemed to have been over-
estimated because of the high percentage of acidic amino acids and
the conformation of the SDS-protein polypeptide complexes which
deviated from those of proteins usually employed as standards for
molecular weights. Studies by electron microscopy suggested that
the region between the two hexagonal rings was hydrophilic involving
electrostatic and/or hydrogen bonding (Wolf, 1977b). The arrangement
within a hexagonal ring was proposed to be alternating acidic (A) and
basic (B) subunits (Badley et al., 1975). The intricate structure of
the 11S molecules can be disrupted by various methods, including
heat. Basic subunits precipitate almost quantitatively on heating
11S protein in buffer at $100^{o}C$ for 30 min because basic subunits are
more hydrophobic (Peng et al., 1982).

Phenylalanine, leucine, or isoleucine as N-terminals for the
acidic subunits, and glycinin for the basic subunits were reported
(Catsimpoolas et al., 1967; Kitamura et al., 1976; Wolf, 1977b;
Moreira et al., 1979). Iyengar and Ravestein (1981) found the
fourth N-terminal acidic subunit as arginine. Amino acid sequence
of the first 20 - 30 N-terminal residues of the four acidic subunits
revealed the identical regions of each subunit (Moreira et al.,

1979; Iyengar and Ravestein, 1981). Wolf (1972) reported that 7S and 11S soybean proteins contained more the number of lysine and arginine residues that that of tyrosine, tryptophan and phenyl-alanine. Catsimpoolas et al. (1971) found that the acidic subunits had higher contents of glutamic acid and proline, whereas the basic subunits were higher in the hydrophobic amino acids, such as leucine, tyrosine, phenylalanine, valine and alanine.

Badley et al. (1975), Kitamura et al. (1976) and Iyengar and Ravestein (1981) showed that each acidic and basic subunit was linked by one or more disulfide bond(s) forming intermediary subunits. In the absence of 2-mercaptoethanol, Mori et al. (1981b) indicated the intermediate subunits when the glycinins were analyzed by sodium dodecyl sulfate electrophoresis. With 0.01M 2-mercaptoethanol at neutral pH and an ionic strength more than 0.35, the glycinin monomer was stable and whereas the disulfide bonds for polymer formation were reduced, the disulfide bonds holding the acidic and basic subunits to form intermediary subunits were intact (Iyengar and Ravestein, 1981). Draper and Catsimpoolas (1978) observed a progressive increase in disulfide bond scission of glycinin with increasing urea concentration, reaching the maximum of 20 disulfide bonds at 8M urea. They showed that most of the disulfide bridges were buried in the interior of the protein molecule. The physical behavior of 11S fractions was affected by several factors such as ionic strength, pH, temperature, and solvent as discussed by

Kinsella (1979).

2.4   Molecular size modification

Although little research has been conducted quantitatively,
there is little double that the functional properties of the protein
are decided to a large extent by its molecular size (Roozen and
Pilnik, 1973; Deeslie and Cheryan, 1981).

There are several groups of enzymes in terms of their proteolytic
specificity (Waxdal, 1971). Therefore, the type of enzyme used may
affect the final product even if the hydrolysis is carried out in
such fashion as to achieve the same number of free amino groups per
unit weight of protein (Puski, 1975).

Proteolytic enzymes have been widely used to modify food
proteins. The results of proteolysis on the functional properties
have been studied in soybean (Roozen and Pilnik, 1973; Puski, 1975;
Kinsella, 1976; Rham et al., 1978; Zakaria and McFeeters, 1978;
Pour-El and Swenson, 1976; Horiuch et al., 1978), cottonseed
(Arzú et al., 1972), rapeseed (Hermansson et al., 1974), and sunflower
(Kabirullah and Wills, 1981).

Native globular proteins are generally resistant to proteolysis
because of the compact tertiary structure of the protein which
protects most of the peptide bonds (Aglemier and Montgomery, 1976).
When the breakage of a few peptide bonds destabilizes the molecule,
an irreversible unfolding occurs by which more peptide bonds are

exposed and the polypeptide chains are extensively degraded to intermediate size peptides in accrodance with the kinetic character- istics of the enzyme-substrate system (Adler-Nissen, 1976). Proteases exert their influences by catalizing the cleavage of the large molecular weight components or, rarely, the synthesis of peptide bonds (plastein reaction). With the increase in the number of polar groups and an increase in hydrophilicity of the product as well as a decrease in molecular weight of the peptide chains, a possible alternation in molecular properties and functional characteristics can be altered (Phillips and Beuchat, 1981). At the same time, the tertiary structures are dissociated into subunits and a compact globular structure is opened to expose the hydrophobic interior to an aqueous phase.

Lynch et al. (1977a) concluded that glycinin can be hydrolyzed into large peptide fragments by soluble pepsin and trypsin within a few minutes. They found that the acidic subunits of the protein with the components of higher molecular weights were hydrolyzed faster than the basic subunits with lower molecular weights. This was probably due to the greater hydrophobic character and therefore compactness of the basic subunits (Catsimpoolas et al., 1971). Native, acid-denatured glycinin and the carboxyamidemethyl (CAM)- acidic subunits, alkali-denatured glycinin and CAM-glycinin were attacked at very fast rate by trypsin (Lynch et al., 1977b). Hsieh et al. (1979) measured the average molecular weight of soybean

globulin by the gel filtration technique. They concluded that the logarithm of the average molecular weight of the peptides and the hydrolysis time had an approximately linear relationship.

Limited acid and alkaline treatments have been employed to solubilize protein from soy, single cell, leaf, rapeseed and fish (Hermansson et al., 1974; Kinsella, 1976). Aoki et al. (1980) reported that with the partial hydrolysis of 7S, 11S, and acid precipitated protein a remarkable increase in the emulsifying capacity of the proteins was observed in comparison with that of the unhydrolyzed proteins.

The isolation method is used to fractionate the proteins. It takes advantage of the differences in protein properties (Wolf, 1977b). Roozen and Pilnik (1973) reported that enzymatic depolymerization of soy protein in an ultrafiltration cell continuously separated peptides of desirable molecular weights from the reaction mixture. For the application, the molecular weight has to be low enough to ensure solubility in acid fruit juice media, but not so low as to give a bitter taste. By using phosphoric acid as an acidulant, Shemer et al. (1978) isolated a soybean protein isolate exhibiting unusual coagulation, viscosity, and emulsification properties. Sundar and Rao (1978) indicated that the acid extracted peanut proteins exhibited poor solubility but better emulsifying capacity compared to those of the alkali extracted protein. Their gel electrograms showed the greater differences in mobilities of each component of two

fractions. Lah and Cheryan (1980) found that a full-fat soy protein product produced by ultrafiltration with a 50,000 molecular weight cutoff had a higher protein dispersibility index than that of the raw material in the acidic and neutral pH regions. Similarly, nitrogen solubilities, oil adsorption, viscosity, and emulsifying and foaming properties of membrane-produced soy isolate were superior to those of the commercial isolate because the presence of whey proteins in membrane-produced isolates markedly enhanced the functional properties (Manak et al., 1980). The storage protein and non-storage protein fractions of cottonseed protein had different molecular weight, solubility and other functional properties (Lawhon et al., 1980).

It had been recognized that the proportions of 11S to 7S in total protein of soybean seeds considerably differed among varieties and that the differences of the proportion might be related to the physical properties of tofu-gel (soybean curd) prepared (Saio et al., 1969). They found that the tofu-gel from crude 11S was remarkably harder than that from crude 7S. Saio et al. (1973) showed that the physical properties of calcium gel prepared from 7S and 11S proteins were remarkably different. Saio et al. (1975) and Wolf (1977b) indicated that gels made from 7S fraction were significantly expanded and dense in contrast to 11S gels that were greatly expanded, porous and elastic. The emulsifying capacity and emulsion stability of the 7S protein rich fraction (PRF) generally expressed higher values than

those of the 11S PRF in the pH range 2 - 10 (Aoki et al., 1980).
Thus, it seems likely that the subunit composition of glycinin and
other components is related to the physical properties of foods made
from soybeans or their isolated proteins (Mori et al., 1981b).

It is well known that gliadin in wheat proteins appears to
influence loaf-volume potential, while glutenin affects its physical
(molecular size and shape) and chemical (tendency to aggregation)
properties (Khan and Bushuk, 1979). Bottomley et al. (1982) explained
that the ratio of two molecular sizes of wheat flours from four wheat
varieties seemed to correlate well with breadmaking quality of the
flours.

The relative ratio of electrophoretic two bands with molecular
weights of 55,000 and 40,000 from bovine muscle were proposed to get
the effectiveness of heat processing in destroying the food-and-mouth
disease virus (Caldironi and Bazán, 1980). In addition, Mahoney et al.
(1982) reported that the increased meltability of cheese products was
related to decreased protein molecular size.

Generally, enzyme treatment improves solubility of proteins at
all pH levels. Solubility profiles of enzymatically hydrolyzed
proteins were markedly different from their respective control
(Arzú et al., 1972; Hermansson et al., 1974; Puski, 1975; Sekul et al.,
1978; Kabirullah and Wills, 1981). Arzú et al. (1972) achieved 40 -
60% cottonseed solubilization under optimal experimental conditions
by two microbial proteases and bromelain. Obviously, the pepsin

preparation had a higher solubilizing effect on rapeseed protein than the papain preparation with unknown purity (Hermansson et al., 1974). At pH 4.5 or in the presence of 0.03M calcium chloride, Puski (1975) reported that the solubility of soy proteins treated with enzyme from Aspergillus oryzae increased with the increased enzyme treatment, although heat treatment lowered the nitrogen solubility index. Likewise, pepsin hydrolysis of a heated soy protein isolate rapidly increased the number of free amino groups in the protein, followed by small increase (Zakaria and McFeeters, 1978). Beuchat et al. (1975) showed that enzymatic hydrolysis with pepsin, bromelain and trypsin solubilized peanut flour protein to the extent that it was highly soluble in 0.03M Ca^{++} at a pH range normally associated with liquid milk. Kabirullah and Wills (1981) found increased solubility of sunflower protein hydrolysates by pepsin and trypsin as the degree of hydrolysis increased. Their chromatography illustrated lower molecular weight components of the hydrolyzed sunflower protein as compared to those of the unhydrolyzed protein. If the temperature was raised during the treatment with alkali and acid, Hermansson et al. (1974) indicated that the increase in solubility of rapeseed protein was enormous.

Since the number of free amino and carboxyl groups increases as a result of hydrolysis and because moisture uptake by proteins is proportional to the number of ionic groups present, it is not surprising to note that moisture uptake is increased by enzyme

treatment (Phillips and Beuchat, 1981). Peptization and permanent conformational changes may occur during heat treatment or exposure to acidic and alkaline pH conditions, influencing the capacity of proteins to absorb water. Hermansson et al. (1974) emphasized swelling characteristics as a very important parameter for food application, since most foods are water-swollen systems. Even moisture uptake of soy protein at 84% relative humidity and room temperature was increased in proportion to the extent of enzyme treatment (Puski, 1975). Moreover, high water adsorbing properties contributed by deamination and conformational changes of proteins may be desirable for incorporation into products requiring elevated water content at water activities low enough to inhibit microbial growth (Beuchat, 1977b).

Emulsion capacity of soy proteins increased by treatment with a neutral protease from Aspergillus oryzae, whereas emulsion stability decreased (Puski, 1975). It was theorized that enzyme digestion of protein would increase the number of peptide molecules available at the oil/water interface, and thus a larger area may be covered, resulting in the emulsification of more oil. However, because these peptides were smaller and less globular, they would form a thinner protein layer around the oil droplets resulting in an emulsion with less stability. Enzymatic treatment may also uncover buried hydrophobic groups which may improve the hydrophobic-lipophilic balance (HLB) for better emulsification (Phillips and Beuchat, 1981).

Therefore, limited enzyme treatment may not be detrimental to emulsion properties of soybean protein. Treatment with pepsin for 30 min at $22^{\circ}C$ or $50^{\circ}C$ restored the emulsion capacity of peanut protein, approaching values approximately 40% greater than those of the control (Beuchat, 1977b). Zakaria and McFeeters (1978) showed that there was an increase in emulsification activity of soy protein when the pepsin hydrolysis period was short and then the emulsifying activity decreased as the incubation time increased. They suggested that either hydrolysis of a few key polypeptides offered relatively large changes in functional properties or that changes other than proteolysis occurred which affected functionality. However, enzymatic digestion of proteins completely destroyed the emulsifying capacity of the peanut flour (Beuchat et al., 1975). Sekul et al. (1978) found that hydrolysis by papain decreased the emulsifying capacity of peanut protein. Indeed, the emulsion activity and emulsion stability of all hydrolysates of sunflower protein was lost irrespective of the method of enzyme inactivation (Kabirullah and Wills, 1981).

In general, foaming agents originating from proteins are prepared by their partial hydrolysis through which the foaming power is increased. However, the foam stability is decreased by this treatment (Halling, 1981). This decrease of the foam stability might be ascribed to the change of molecular structure during hydrolysis (Horiuch et al., 1978). The best foaming properties of rapeseed

protein concentrate were obtained by the acid hydrolysate and the CMC precipitated and 24 hr digested pepsin hydrolysate (Hermansson et al., 1974). Similarly, the mild hydrolytic treatment of succinylated protein enhanced its capacity to form stable whipped foams (Groninger and Miller, 1975). Sekul et al. (1978) indicated that partial hydrolysis of peanut proteins by papain significantly increased both foaming capacity and foam volume, especially where the pH was adjusted in two steps prior to the formation of the foam. As would be expected, following hydrolysis of sunflower protein with pepsin for 1 hr and enzyme inactivation by change of pH, foam expansion was markedly increased at all pH levels and to values that were much higher than those of egg albumin (Kabirullah and Wills, 1981).

2.5   Molecular charge modification

Chemical modification of proteins is routinely practiced in protein chemistry as a technique to study structure, conformation, active site residues and enzymatic mechanisms.

Several physiochemical properties of proteins are influenced by their reactive chemical groups, such as charged anionic and cationic group, and hydroxy, amide and thiol residues. Modification of these groups can alter the properties of proteins (Kinsella, 1976). Eisele and Brekke (1981) modified beef heart myofibrillar proteins by tetra-hydrofuran-2,3,4,5-tetracarboxylic dianhydride and

1,2,4-benzenetricarboxylic acid. Before the extraction of protein,
Choi et al. (1982) acylated cottonseed flour by maleylation and
dimethylglutarylation to modify functional properties.

Succinic anhydride is the most frequently used derivative for
protein derivatization. Succinic anhydride can react with free amino
groups, tyrosyl hydroxy groups and sulfhydryl groups in proteins,
forming amide, ester, and thioester linkages, respectively (Gounaris
and Perlmann, 1967; Konings et al., 1969; Grant, 1973). The protein
acylation reaction presumably follows the carbonyl addition pathway,
according to Franzen and Kinsella (1976a). These authors indicated
that the rate of reaction depended upon the rate of nucleophilic
attack, and acylation rates for homologous nucleophiles were inversely
related to their pK values. Consequently, high reactivity of a
protein group is usually the result of low pK. The majority of the
side chains are chemically modified when they are good nucleophile
and in the non-protonated condition (Feeney, 1977). Most acylating
agents react more readily with amino groups (Uraki et al., 1957).
Principally, succinic anhydride reacts with the epsilon-amino group
of lysine in protein, converting it to a negatively charged residue
by N-acylation from positive charged amino groups under acidic
conditions (Feeney, 1977).

Succinylation has three major effects on the physical character
of proteins: it increases net negative charge, changes conformation,
and increases the propensity of proteins to dissociate into subunits

(Kinsella and Shetty, 1979). As a result of this reaction, unfold-
ing and expansion cause larger molecules to exhibit higher intrinsic
viscosity because of expanded molecular specific volumes (Habeeb
et al., 1958; Konings et al., 1969). Chu et al. (1969) showed the
increased Stokes radius of Staphylococcal enterotoxin B on acylation.
This was due to lower electrostatic attraction between neighboring
cationic amino and anionic carboxyl groups of the proteins. Moreover,
extensive succinylation may cause proteins to dissociate into
subunits (Grant, 1973; Beuchat, 1977a). The sedimentation velocity
distribution data of Barman et al. (1977) showed that acetylation at
98% completion broke down 11S fraction of soy isolate into 2S and
7S subunits. They indicated that the dissociation of the quaternary
structure of the 11S globulins suggested a role for lysine in
stabilizing the multisubunit structures, through intrasubunit
hydrogen bonds which were lost in acetylation as well as electrostatic
repulsive forces of newly charged groups.

These molecular changes from acylation can affect functional
characteristics of proteins. To improve functional properties of
proteins for specific food application, acylation has been reported
for wheat protein (Grant, 1973), fish protein (Groninger, 1973;
Chen et al., 1975), cottonseed flour protein (Childs and Park, 1976;
Choi et al., 1981), soybean protein (Franzen and Kinsella, 1976a;
Barman et al., 1977), peanut protein (Beuchat, 1977a), leaf protein
(Franzen and Kinsella, 1976b), beef heart myofibrillar protein

(Eisele and Brekke, 1981), and sunflower protein isolate (Canella et al., 1979; Kabirullah and Wills, 1982).

The loss of positively charged groups shifted the isoelectric point to lower pH values, thus increasing solubility around the pI (Chen et al., 1975; Barman et al., 1977; Kabirullah and Wills, 1982). Upon succinylation, electrostatic repulsion occurred between the added carboxyl groups and the neighboring native carboxyl groups producing fewer protein-protein interactions and more protein water interactions to enhance aqueous solubility (Franzen and Kinsella, 1976b; Sundar and Rao, 1978; Barber and Warthesen, 1982).

Because of the enhanced solubility of succinylated protein, the reaction increased the emulsifying activity and emulsion stability mostly (Childs and Park, 1976; Franzen and Kinsella, 1976b; Sundar and Rao, 1978; Kinsella and Shetty, 1979; Waniska et al., 1981). The positive correlation between solubility and the ability of a protein to emulsify has been well documented (Franzen and Kinsella, 1976a; Choi et al., 1981; Barber and Warthesen, 1982; Kabirullah and Wills, 1982). As the protein becomes more soluble, it forms layers around fat droplets to facilitate association with the aqueous phase (Franzen and Kinsella, 1976b). Granular, insoluble proteins, however, separate from the oil phase or just float on the oil surface where they remain inert and contribute little toward emulsification. At low pH such as pH below 4.6, emulsifying activity and solubility showed decreased values (Chen et al., 1975). When the pH was

decreased, the net charge on the succinylated protein also decreased,
thus reducing repulsive force. The level-off of solubility of
succinylated proteins may be due to the pK (4.6) of succinic acid
(Chen et al., 1975; Barman et al., 1977). Pearce and Kinsella (1978)
found high emulsifying activities of succinylated yeast protein.
They suggested that this might be due to the solubility of proteins
and their resistance to surface denaturation. Addition of calcium
ion caused increases of both emulsion activity and stability with
fish protein extracted at high pH, but decreased only stability with
the succinylated counterpart (Chen et al., 1975). The divalent
calcium ion may act as a crosslinking agent holding the protein mole-
cules around the surface of the oil droplets, thus increasing
the strength of the protecting film. Sodium chloride in low
concentration greatly decreased the viscosity of dispersions of
succinylated fish protein (Groninger, 1973).

Succinylation had no effect on the viscosity of diluted solutions
of leaf protein nor on amino acid composition (Franzen and Kinsella,
1976b). The relatively small increase in protein viscosity resulting
from the reaction with excess succinic anhydride was due to the
counteracted unfolding of the polypeptide chain by the dissociation
of the protein complex (Grant, 1973; Choi et al., 1981).

The loss of charged groups of amino residues resulted in a
decreased number of water molecules bound per protein molecule
(Barman et al., 1977) and also reduced ionic attraction between

neighboring molecules that were responsible in part for stabilization
of protein gel (Chen et al., 1975). However, Beuchat (1977a),
Barber and Warthesen (1982), as well as Kabirullah and Wills (1982)
demonstrated that the unfolding of the protein structure and
dissociation of the protein into subunits would enhanced the entrap-
ment of liquid resulting from the increase in interaction properties
of the altered protein structure. They theoretized that the increase
in functional properties following acylation would be mainly due to
the change in charge, since surface properties were depended on the
protein solubility.

Acylation accounted for the looser texture, higher specific
volume and ligher color (Franzen and Kinsella, 1976b; Barber and
Warthesen, 1982). The feathering of vegetable protein in hot
beverages can be minimized by the derivatization of more than 20 -
30 acyl groups/10^5 g protein (Kinsella, 1976).

Theoretically, acylation is reversible upon acid hydrolysis
and mammalian kidneys contain an epsilon-lysine acylase that is
capable of hydrolyzing N-formyl and acetyllysines (Barman et al.,
1977). Sui and Thompson (1982) reported that succinylated whey
concentrate with 37% succinylation was still a good quality protein
with the net protein ratio higher than that of casein. However,
they recommended metabolic and toxicity studies of the succinylated
amino acids. Also, concerns must be expressed for low digestibility,
low availability of lysine, serine and threonine of succinylated

protein which may come from the acylation (Chen et al., 1975; Franzen and Kinsella, 1976b; Eisele et al., 1982). Practically, an acid labile amide bond with lysine epsilon-amino group of proteins by citraconic and maleic anhydride could offer potential applications because of the relative ease in deacylating lysine under the mild acid condition, and because of the improved functional properties of proteins (Brinegar and Kinsella, 1980; Barber and Warthesen, 1982). On the other hand, acylation may afford protection of labile lysine residues in a food product, giving rise to a reduction in non-enzymatic browning and therefore to improved nutritional stability.

Acetylation changed functional properties of proteins to a much lesser extent than succinylation insofar as solubility, foaming capacity, and emulsifying capacity (Waniska et al., 1981; Kabirullah and Wills, 1982).

When bovine serum albumin was treated with small amount of succinic anhydride, a rapid migrating electrophoretic band was produced that disappeared with further treatment (Grant, 1973). It would seem that a derivative with small amounts of the reagent had enhanced its negative charge, which would account for the high mobility in the alkaline buffer system of electrophoresis (Habeeb et al., 1958; Chu et al., 1969; Meighen and Schachman, 1970). However, the unfolding of the protein from the further succinylation eventually slowed the migration rate because of the sieving effect of the gel. Reduction of the disulfide bonds in the succinylated

protein followed by further treatment with succinic anhydride
resulted in a homogeneous product with a single band in the gel
electrophoresis (Grant, 1973). Likewise, Beuchat (1977a) reported
the dissociation of the protein of peanut flours to yield subunits
with greater mobility at 100 percent of succinylation as compared to
that of the untreated control.

2.6   Protein structure and functionality

The knowledge of the molecular properties of the proteins is
essential for understanding the basis of functionality, for modifying
proteins in order to acquire desirable functional properties, and
for predicting potential applications of modified proteins.

The factors influencing the functional properties of protein
products are the innate physiochemical characteristics of protein,
the processing and chemical modification steps that alter them, as
well as their environment (Pour-El, 1981). Furthermore, the physical
behavior of protein is determined by its amino acid composition, its
molecular size, primary structure, the conformation of the protein,
the charge distribution on the protein, the extent of inter and
intra-molecular bonding (quaternary structure) (Kinsella, 1979).
The properties of bonds and forces are the mediators affecting the
changes in size and conformation. A literature survey reported by
Wu and Inglett (1974) showed that a change in protein structure was
usually associated with changes in physical, chemical and functional

properties.

To obtain optimum functionality in uses, a highly soluble protein is required because soluble protein preparations are also easier to incorporate into foods. Kinsella (1979) reviewed numerous factors affecting apparent solubility of proteins; protein source, processing history, solvent composition, conditions, pH, and ionic strength. In globular proteins, the more polar-charged amino acids are oriented toward the surface. The breaking of peptide bonds results in the following modifications (Phillips and Beuchat, 1981): an increase in the number of polar groups, an increase in the hydrophilicity of the product, a decrease in molecular weight of the peptide chains, and a possible alternation in molecular configuration.

The pH and ionic strength of the aqueous solvent have the most significant effect on the solubility of proteins. Native soybean proteins show the classical aqueous pH-solubility profile at zero ionic strength with an isoelectric point around pH 4.5 (Wolf and Cowan, 1975). Ionic effects involve electrostatic, solvation, and salting in and salting out phenomena. Particularly, chloride ion binds to the positively charged protein groups in the acidic pH range and reduces solubility by accentuating electrostatic attractions (Fennema, 1977). Soy proteins are known to associate depending on salt concentration. At pH 7.6, 11S and 7S proteins dissociate into subunits at ionic strength below 0.1M and 0.5M,

respectively (Wolf and Briggs, 1958). The conformation changes
generated at low ionic strength and alkaline pH are reversible
(Koshiyama, 1968).

Disulfide bond-reducing agents, thiols (cysteine, 2-mercapto-
ethanol, dithiothreitol), and sodium sulfite by cleaving inter-
molecular bonds cause disaggregation of protein enough to increase
solubility (Wolf, 1972; Saio et al., 1975). However, heat applied
during the preparation of proteins reduces solubility of proteins
(Wu and Inglett, 1974; Wolf and Cowan, 1975). Because of the
differences in these treatments (heat, time, pH), commercial soy
proteins demonstrate wide variations in solubility.

Hydrophobic interactions are primarily responsible for the
formation of soluble aggregates which subsequently irreversibly
insolubilize through intermolecular disulfide bond interchanges
(Catsimpoolas and Meyer, 1970). According to Wolf and Cowan (1975),
soluble aggregates of soybean proteins were formed prior to the
formation of insoluble precipitates. Shen (1981) indicated that
soy isolate behaved as if they were composed of a completely soluble
fraction (A) and a completely insoluble fraction (B). The distribu-
tion of A and B depended on the previous history of the sample, such
as manner of extraction, precipitation, solvent treatment, heat
treatment, and drying.

Water holding capacity indicates the entrapped water and is
the ability to physically hold water against gravity. Thus, water

holding capacity is a quantitative indication of the amount of water
retained within a protein matrix under certain defined conditions
(Chou and Morr, 1979).

Water holding capacity is related to viscosity, hydration,
swelling, and gelation of food system and is influenced by pH,
ionic strength, and temperature which affect protein conformation
(Hutton and Campbell, 1981). Therefore, the isolation method and a
number of treatments to which proteins might be subjected in their
use in food can change water binding properties through molecular
changes (Hansen, 1978b).

Structurally, non-ionized amino acids bond an intermediate
amount of water and hydrophobic groups bind little or no water
(Kuntz, 1971). Polar amino acids with ionized side chains bind the
greatest amount of water (Hutton and Campbell, 1981). As equally
important consideration is whether the protein conformation permits
these binding sites to be stereochemically available for interaction
with water. Protein conformation affects the nature and availability
of the hydration site and hence the thermodynamic characteristics of
their water-binding reactions (Eagland, 1975; Chou and Morr, 1979).
A denatured, unfolded conformation should permit the protein molecules
to bind more water than in its native globular conformation.
Consequently, heat treatment enhanced water holding capacity,
probably due to conformational changes (Kinsella, 1979). On the other
hand, processes such as heating, concentrating, drying, and texturi-

zation which alter the quaternary structure of the protein system
by aggregation of individual molecules, may reduce the availability of
polar amino acid groups for binding water (Johnson, 1970; Chou and
Morr, 1979). The aggregation of protein through hydrophobic
interactions may also effectively reduce the total surface area of
the protein or cause the collapse of the protein matrix network.
However, the altered structural characteristics of the aggregated
protein systems may in some case provide additional protein-water
interaction by means of imbibition of water within the newly formed
structural network from a changed conformation, such as in gelation
(Eagland, 1975).

Lowering pH values below 4.0 converts carboxyl groups toward
a nonionized form, thereby reducing the water binding properties
because ionized amino acid groups bind considerably more water than
non-ionized groups. Since amino acid composition and arrangement
vary with protein origin, and variation in pH affects the ionization
of amino acid groups, the responses to pH also varies (Hutton and
Campbell, 1981). Consequently, water binding capacity is increased
as pH increase from 4.5 to 7.0 for soy protein. Ionic strength and
species have a significant effect on solubility, viscosity, gelation,
swelling, and water-binding capacity of proteins (Hermansson, 1972;
Hermansson and Akesson, 1975). Obviously, repulsive forces permit
additional water imbition within the protein network. In a review
of Ehninger and Pratt (1974), anions were found to have a stronger

effect on water holding capacity than cations. Moreover, salts compete with water for the binding sites on amino acid side groups. Generally, as ionic strength increased up to 0.1M NaCl, water absorption increased for the soy isolate and caseinate (Hermansson, 1973).

It was suggested that water absorption and solubility might be related to a point, perhaps maximum hydration, at which solubility continued to increase and hydration did not (Hutton and Campbell, 1977). However, there was no apparent correlation between solubility and water holding capacity (Kinsella, 1979). A highly soluble protein exhibits poor water binding. Solubility and water retention were minimal at the isoelectric point (Johnson, 1970). It was also considered that water absorption and viscosity were not always correlated (Hutton and Campbell, 1981).

Viscosity is important in product formulation, processing, texture control, and mouthfeel properties and in elucidating protein-protein interactions and conditions affecting conformational and hydrodynamic properties.

Shen (1981) reviewed factors such as conformation, exposure of hydrophobic groups, and charge distribution contributing to inter-molecular interactions that resulted in increased viscosity. In the slurries, the intermolecular protein-protein interactions dominate and are primarily responsible for the observed viscosity behavior and the progel as well as gel formation are evidecnes of strong intermolecular interactions (Catsimpoolas and Meyer, 1970; Catsimpoolas and Meyer,

1971a).

Processing, alkaline-acid and heat treatments enhanced swelling and increased viscosity of soy dispersions (Fleming et al., 1974; Hermansson, 1975). For the soluble molecules with their larger exposed hydrophobic surface areas should experience greater protein-protein interactions.

Dispersions of soy proteins demonstrate thixotropic behavior (Hermansson, 1975; Rha, 1978). Thus, the viscosity increases slowly and reverts to a much higher value if the slurries are allowed to stand. Viscosity was not necessarily correlated to the solubility (Shen, 1981).

The oil absorption is attributed mainly to the physical entrapment of oil (Hutton and Campbell, 1981). A correlation of -0.95 was found between oil absorption and the bulk density of alfalfa protein (Wang and Kinsella, 1976). Similarly, Schachtel (1981) showed that for the protein from Candida utilis, a strong negative correlation was observed between the fat absorption and the bulk density of the powders.

Factors affecting the protein-lipid interaction include protein conformation, protein-protein interactions and spatial arrangement of the lipid phase resulting from the lipid-lipid interaction (Catsimpoolas and Meyer, 1971b; Kinsella, 1976). Deng et al. (1981) found that the protein-protein interaction resulted in molecular aggregation and when measured as an increase in light scattering absorbance at 320 nm by a protein solution, the reaction was shown to be reversible between 4°C and 30°C. These investigators also demonstrated that the reduced

protein-protein interaction lost fat binding capacity with the prolonged chopping. Apparently, the high viscosity of a system contributed to greater ease of physical entrapment of oil (Hutton and Campbell, 1981).

All sunflower products absorbed more oil than the soy counterparts because the sunflower proteins appeared to be more structurally lipophilic than the soy proteins (Lin et al., 1974). Alfalfa leaf protein extracted with water and NaOH absorbed more oil than those extracted with NaCl or tris-buffer (Wang and Kinsella, 1976). Kinsella (1979) mentioned that protein preparations with low nitrogen solubilities had high fat absorbing capacities, whereas for emulsion formaton and stabilization protein solubility was desired.

Waniska et al. (1981) defined the terms relating to emulsion. The ability of protein to aid in emulsion formation and stabilization is emulsifying ability. The emulsifying capacity is the volume of oil emulsified per unit weight of protein. The emulsion stability is the ability of the discrete emulsion droplets to remain dispersed without creaming. The mechanisms of protein adsorption and reduction of interfacial tension sequentially involve diffusion of the protein to the interface, unfolding and spreading of absorbed molecules accompanied by intermolecular associations (Kinsella, 1979). The surface-active compounds reduce the surface and interfacial tensions between two immiscible liquids. Three separate functions are involved in formation of a stable emulsion: reduction of interfacial tension, formation of a rigid interfacial film, and electrical charges (Lynch and Griffin, 1974;

Powrie and Tung, 1976).

Proteins constitute an important group of emulsifiers because they behave in a manner similar to that of surface active agents by forming strong monolayer films at the interface as well as having charged groups. However, their structure, and hence their behavior are more affected by such variables as salt concentration, pH, and temperature than would occur with the use of conventional emulsifiers (McWatters and Cherry, 1981). Kinsella (1976) discussed the importance of the structural characteristics of the protein for the emulsifying properties. In fluid emulsions, a protein of molecular size adequate for stabilizing emulsion but too small to form an extensive network for gel formation, is needed (Rham et al., 1978). Halling (1981) indicated that the stability of many protein-stabilized emulsions to heating was remarkable compared to that of most other surfactants.

Electrical charges on droplets in emulsions can rise by ionization, absorption, or frictional electricity produced through the large shearing forces required for emulsion formation (Powrie and Tung, 1976). Salts may reduce charge repulsion between the proteins and enhanced hydrophilic association at the interface. The effects of electrical charge or balance between attractive and repulsive forces of particles are keeping droplets separated and preventing coalescence (Mcwatters and Cherry, 1981). Franzen and Kinsella (1976a) found that soybean protein-stabilized emulsions upon heating were most unstable in the isoelectric pH range. The aggregation of protein at pI can be counter-

acted by increasing salt levels (McWatters and Holmes, 1979a).

However, it is generally accepted that at or near the isoelectric point proteins stabilize emulsions by a mechanism of adsorption and interfacial denaturation to produce a physical barrier against coalescence of dispersed droplets (Cante et al., 1979). The steric or entropic force of repulsion between the surface films would be minimum at the pI, where the protein molecules are most compact (Halling, 1981). Yamauchi et al. (1980) found that the creaming stability was minimal and the viscosity was maximum at pH 5.0 over the range of pH 3.0 to 9.0. Evidently, pH changes the conformation of protein which affect the effective hydrophobicity. A correlation between emulsifying capacity and protein surface area has been noted by Cante et al. (1979).

Reduction of the disulfide bonds of bovine serum albumin reduced the emulsifying ability of the protein in the pH range of 4 - 7 (Waniska et al., 1981). Graham and Phillips (1976) showed that beta-casein, a disordered, flexible protein of comparatively high hydro-phobicity was more surface active than lysozyme which has a globular, rigid tertiary structure and a low hydrophobicity. On the other hand, Phillips (1981) found that the highly cohesive films formed by the adsorption of rigid globular molecules such as lysozyme and bovine serum albumin, were much more resistant to mechanical deformation than films containing the flexible beta casein molecules. Using a formula based on turbidity, volume fraction of dispersed phase, and weight of protein, Pearce and Kinsella (1978) calculated an emulsifying activity

index which was related to the interfacial area of the emulsion. With
pH change, urea and succinylation, Waniska et al. (1981) indicated that
electrostatic, hydrophobic and disulfide bonds of bovine serum albumin
were important in emulsifying ability because a certain degree of
tertiary structure was required for optimal emulsion formation.
Watanabe et al. (1981) showed that the incorporation of C_{10} - C_{12}
alkylesters of leucine into gelatin by papain gave products having a
higher ability to stabilize an o/w type emulsion. Thus, this process
enhanced the hydrophilic and lipophilic balance of the protein. Indeed,
Aoki et al. (1981) reported that the lipophilization of soy protein
decreased the HLB (hydrophilic-lipophilic balance) number and increased
the emulsion stabilization. Because of the molecular size, the soy
globulins diffuse relatively slowly, but once at the interface, they
initially spread easily, although the subsequent penetration of newly
arriving molecules into the film might slow further spreading (Tornberg,
1980).

While emulsion capacity decreased with protein concentration,
emulsion stability significantly increased (Lin et al., 1974).
Evidently, a stable emulsin was obtained at whey protein concentrations
more than 2% (Yamauchi et al., 1980). The solubility in an aqueous
phase is closely correlated with the surface activity of a protein
(Kinsella, 1979; Halling, 1981). Nevertheless, some workers noted the
evidence that the emulsifying capacity and the solubility of a protein
might not always be in parallel (Halling, 1981).

Emulsion and foam have similarities because they deal with the hydrophilic and hydrophobic entities. Proteins lower interfacial tension between gas and water, facilitating deformation of the liquid and expansion against its surface tension. These abilities are facilitated by alternation of tertiary and quaternary protein structures, which are dependent upon the physical-chemical conditions (Richert, 1979). However, Kitabatake and Doi (1982) indicated that the absolute surface tensions of protein solutions were not correlated to foamability, while the rate constant of surface tension decay of protein solutions was significantly correlated to foamability of protein solutions.

The protein films must be stable and exhibit a balance between their ability to engage in intermolecular cohesion required to form a membrane and the tendency to self-associate excessively which would result in foam instability (Powrie and Tung, 1976). Adsorption continually occurs around the bubbles to replace protein in areas of the interface where coagulation or stretching of the film is occurring (Cherry and McWatters, 1981).

Horiuchi et al. (1978) related foam stability to surface hydrophobicity. Hydrophobic derivatives of casein made by acylation had a much enhanced ability to stabilize the lamellae in foams and retard collapse (Halling, 1981). Phillips (1981) argued that the rate of surface denaturation and surface pressure development could be related to the structural characteristics of proteins. Moreover, Watanabe et al. (1981) found that the incorporation of alkyl esters of leucine by

papain into gelatin showed greater whippability.

Because liquid and air are still moving through the foam, salt and hydrogen bonds form between the polypeptides of the proteins to interact with each other and together with interactions between monolayer portions of the proteins, resulting in small aggregates formed; that is, the proteins coagulate into aggregates and the bubble bursts (Cherry and McWatters, 1981). In the more stable forms, there was no perceptible breakdown of the bubble until after the great majority of the liquid had drained from the foam (Jacobi et al., 1956).

An appreciable degree of tertiary structure in the dissolved protein is needed for maximal foam stability. Partially hydrolyzed proteins gave lower stability than the unhydrolyzed material, though the foam may be formed more readily (Eldridge et al., 1963). Partial hydrolysis or heating (70°C - 80°C) increased the tendency of the polypeptides to unfold at the interface and facilitate hydrophobic association, thereby increasing film thickness and viscosity, reducing air leakage, and enhancing stability (Kinsella, 1979, Kinsella, 1981).

Investigators working in this area widely believe that foam stability is at a maximum near the pI of a protein. Phillips (1981) reported the maximal foam stability at the pI of bovine serum albumin, where the rheological properties of the adsorbed films of the protein were also maximized. Sato and Hayakawa (1979) found that foam volume of yeast protein without salt exhibited a maximum value at pH 4.5, which was the pI of the protein. Near the isoelectric point of gluten,

the rate of drainage became minimum as the surface coverage of the adsorbed gluten molecules on the surface became minimum (Mita et al., 1977). However, the effect of pH appears extremely complex, depending upon several other variables (Richert, 1979).

Correlations between foam formation or stability and the solubility of proteins involved have been observed in some cases (Wu and Inglett, 1974; Kinsella, 1976; Cherry et al., 1979; Phillips and Beuchat, 1981; Halling, 1981). Solubility of soy protein was closely correlated with foaming, and a strong relationship existed between foam expansion and foam stability (Schachtel, 1981).

3.0   EXPERIMENTAL

3.1   MATERIALS AND EQUIPMENT

3.11   Supporting materials

3.11   Porous glass

Controlled size pore glass particles, 500 Å average pore diameter,
37 - 74 µm particle size, were purchased from Pierce Chemical Co.,
Rockford, Ill.

3.112   Nylon pellets

The nylon pellets (2.8 ± 0.4 mm long, 2 mm internal diameter) made
from Capron[R] Nylon 8200 was supplied by Specialty Chemical Div.,
Allied Chemical Corp., Morristown, N.J.

3.12   Enzymes

The soluble enzymes studied were: alpha-chymotrypsin crystal-
lized, Pfaltz and Bauer Inc., Stamford, Conn.), ficin (Pfaltz and
Bauer), papain (Pfaltz and Bauer, trypsin (purified, Fisher Sci., Co.,
Fair Lawn, N.J.), and trypsin (crystallized, Worthington Biochem.
Corp., Freehold, N.J.).

3.13   Substrates

3.131 Synthetic substrates

N-alpha-benzoly-L-arginine ethyl ester hydrochloride (BAEE) and

N-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Aldrich

Chemical Co., Inc., Milwaukee, Wis.

3.132 Soybean protein isolate

Promine D (4520), a commercial neutral isolated soybean protein

in the sodium form, was a gift from Central Soya Chemurgy Div., Fort

Wayne, Ind. The soybean protein isolate was dispersed in 0.07M

Sorenson's phosphate buffer (pH 7.5, 2.5% suspension, w/v) otherwise

mentioned, and blended for 1 min on the magnetic stirrer operated at

low speed. In order to destroy protease inhibitors, the suspension

was heated in boiling water for 40 min (Liener, 1979; Collins and

Beaty, 1980).

3.14 Reagents

Chemicals for the immobilization of enzymes were gamma-amino-

propyltriethoxysilane, Aldrich; glutaraldehyde, Fisher; 2,4,6-trinitro-

benzenesulfonate, Fisher; hydrazine hydrate, Fisher; dicyclohexylcarbo-

diimide, Eastman Kodak Co., Rochester, N.Y.; thiophosgene, Aldrich.

From Eastman, 2,4,6-trinitrobenzenesulfonic acid (TNBS) was obtained.

Folin-Ciocalteau reagent was purchased from Fisher.

Sources of reagents for electrophoresis were acrylamide, Biorad,

Richmond, Cal.; N,N'-methylenebisacrylamide (BIS), Biorad; N,N,N',N'-

tetramethylethylenediamine (TEMED), Biorad; 2-mercaptoethanol, Biorad; dithiothreitol, Biorad; urea, Biorad; Triton X-100, Biorad; sodium dodecyl sulfate, Biorad; bromophenol blue, blue, Biorad; coomassie brillant blue R-250, Biorad; tris, Biorad; glycine, Biorad; ammonium persulfate, Fisher; sodium phosphate (mono and dibasic), Fisher. Kodak Panatomic-X film was obtained from Eastman. The standard proteins were purchased from Biorad: myosin (200,000 dalton), beta-galactosidase (130,000), phosphorylase B (100,000), bovine serum albumin (68,000), ovalbumin (43,000); from Sigma Chemical Co., St. Louis, Mo.: catalase (60,000), soybean trypsin inhibitor (21,000), beta-lactoglobulin (18,400); from Fisher: pepsin (35,000), trypsin (23,000), hemoglobin (15,500); from Pfaltz and Bauer: chymotrypsin (11,000).

All other chemicals were analytical grade reagents.

3.15 Reactors

3.151 Continuous packed bed reactor

For the kinetic studies of immobilized enzymes, a packed bed reactor was constructed by using a jacketed glass column with a bed diameter of 1 cm. A peristaltic pump was used to maintain fluid flow and temperature was controlled in the column jacketed at 33°C otherwise mentioned. Either two or five grams of porous glass (3.111) or nylon pellets (3.112) were used as support in the column. Samples

were removed at specific time intervals from the system for analysis.

3.152   Batch stirred tank reactor

A 250 ml Erlenmeyer flask containing substrate and soluble enzyme
or immobilized enzyme(s) was placed in a water bath shaker Model OSW21
(Fermentation Design Inc., Allentown, Penn.). The agitator was
rotated at 200 rpm for various incubation times at 33°C and 55°C for
synthetic substrates and soybean proteins, otherwise specified. The
control sample was prepared similarly, except that the enzyme was not
placed in the flask. After incubation, samples were removed from the
reactor with immobilized enzyme(s). For the soluble enzymes, the
medium was heated for 10 min at 95°C to inactivate the enzyme.

3.16   Instruments

The following instruments were used in this experiment: Spectro-
photometer Spectronic[R] 710, Bausch and Lomb, Rochester, N.Y.; Beckman
Zeromatic[R] pH meter, Beckman Instruments, Inc., Fullerton, Cal.;
Biorad electrophoretic apparatus Model 155, Biorad; Power supply Model
38520, Gelman Instrument Co., Ann Arbor, Mich.; Gilford spectrophotom-
eter 250, Gilford Instrument Lab., Inc., Ft. Washington, Pa.

3.2   ANALYTICAL PROCEDURES

3.21   Methods of immobilization

3.211 Immobilization of enzyme to glass support

Porous silica glass particles were silanized with gamma-amino-propyltriethoxysilane using the method of Weetall (1970). The porous glass was first refluxed overnight in a 10% solution of gamma-amino-propyltriethoxysilane in toluene, washed with toluene and dried in an oven at 80°C for 30 min.

The alkylamino silane-glass derivative was covalently coupled to trypsin by the Schiff's base formation according to the method of Shipe et al. (1972). Fifty grams of alkylamino-glass was added to 200 ml of a 1:5 mixture of 25% aqueous glutaraldehyde and 0.1M phosphate buffer, pH 7.0. The glass stood at room temperature for 3 hr, was washed with distilled water and added to 40 mg of enzyme previously dissolved in 20 ml of 0.1M phosphate buffer. The reaction was continued for 2 hr at 4°C. The product was alternately washed with distilled water and 2M NaCl until no enzymatic activity in the effluent was detected. The immobilized enzyme was stored as a damp cake at 4°C until used.

3.212 Immobilization of enzyme to nylon support

3.2121 Acid azide method

The enzyme chemically attached to nylon pellets through the acid azide was prepared by the method of Hornby and Filippusson (1970). The nylon pellets were partially hydrolyzed by the treatment with 3.0M

HCl for 30 min at 30°C, after which the hydrolysis was arrested by washing through the pellets with water. So as not to interfere in subsequent steps in the process, the amine groups were destroyed by treating the tube for 2 min with an ice-cold solution of 1% (w/v) $NaNO_2$ in 0.5M HCl and then warming the pellets to 40°C and continuing the treatment for a further 20 min. The destruction of the primary amine groups was confirmed by the inability of a small sample of the surface of the pellets to form a red colored trinitrophenyl derivative with 2,4,6-trinitrobenzenesulphonate. The free carboxyl groups of the pellets were then treated to produce their acid hydrazide derivative. For this, the pellets were treated at 10°C with a mixture of 5.0% (w/v) hydrazine hydrate, 1.0% (w/v) dicyclohexylcarbodiimide in dioxane for 2 hr. The pellets were then washed by dioxane and then water. For the chemical attachment of the enzyme to the surface of the nylon pellets, the acid hydrazide derivatives were treated as follows. The pellets were treated with a mixture of 1.0% (w/v) $NaNO_2$ in 0.5M HCl for 20 min. The pellets were then perfused with ice-cold 1.0 mM HCl until their effluent failed to give a positive reaction for nitrous acid by the starch-iodide test. The process was always affected immediately prior to the coupling of the enzyme. A 0.2% (w/v) solution of trypsin in phosphate buffer, pH 8.0 ± 0.1 at 0°C was then treated for 2 hr at 4°C. After this, the pellets were washed free of any physically absorbed protein by perfusion with solutions of 1.0M NaCl, 0.5M $NaHCO_3$, 1.0 mM HCl and finally water. The pellets were stored at

4°C in the dry state until required.

3.2122   Glutaraldehyde method

Enzymes were bounded through the Schiff's base to nylon pellets by a modification of techniques described by Smiley et al. (1974). Nylon pellets were perfused at 45°C for 1 hr with a methanolic solution of $CaCl_2$ to remove amorphous nylon. The solution was composed of 18.5g $CaCl_2$, 18.5 ml water and 63.0 ml methanol. After rinsing until no Cl^- could be detected, the pellets were treated with 3.65N HCl at 50°C for 2 hr, otherwise specified. This provided considerably more free amino groups without adversely affecting the nylon structure. The pellets were thoroughly washed with water until free of Cl^- and then treated overnight at 25°C with 8.0% glutaraldehyde in 0.5M sodium phosphate buffer, pH 7.0. Excess glutaraldehyde was rinsed out with water and a solution containing 2.0 mg/ml of trypsin in 0.1M phosphate buffer, pH 7.0 was reacted at 5°C for 40 hr. Alternate washing with water and 0.1M NaCl removed excess enzyme. Final washing was done by recycling the substrate.

3.2123   Isothiocyanate method

The isothiocyanate derivative was prepared by refluxing the hydrolyzed nylon pellets overnight in a 10% solution of thiophosgene in chloroform (Weetall, 1970). The pellets were then washed with chloroform, air-dried and immediately coupled to a 0.2% solution of

enzyme at pH 9.0 in 0.2M carbonate buffer (Delory and King's) at room temperature.

3.22   Enzyme activity

3.221   Trypsin

The rate of hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) was measured by the increase in absorbancy at 255 nm, based on the method of Schwert and Takenaka (1955). The spectrophotometer was adjusted to give an optical density reading of zero with 0.5 mM BAEE in 0.05M tris-hydroxymethylaminomethane in the path of light. The tryptic activity of soluble enzyme and immobilized enzyme was determined by following the hydrolysis of BAEE when solutions of the substrate in Tris buffer, I 0.1 containing 0.25% (w/v) $CaCl_2$ were shaken with the enzyme (Hornby and Filippusson, 1970).

3.222   Alpha-chymotrypsin

The rate of benzoyl-L-tyrosine ethyl ester (BTEE) was determined from the change in absorbancy at 256 nm according to Hummel (1959). In the assay reactor, 5 ml of substrate (0.001M BTEE in 50% aqueous methanol) and 5 ml of Sorensen's phosphate buffer (0.07M, pH 6 - 8) or Clark and Lubs' borate buffer (0.1M, pH 8.5 - 10.0) were placed. At zero time, 100 µl of the diluted enzyme solution in 0.00M HCl or various amounts of the immobilized enzyme to the reactor were added

and stirred by a magnetic bar with a medium speed. The reaction
vessel was maintained at a specific temperature by a tubular heat ex-
changer. The rate of change of absorbance at 256 nm was measured for a
period of time. One unit was equal to the hydrolysis of one micromole
of substrate per minute. The value of change in molar extinction co-
efficient for the complete hydrolysis of BTEE was 944 (Walsh and Wilcox,
1970).

3.223   Papain

The method was based on the titrimetric determination of the acid
produced during the hydrolysis of benzoyl arginine ethyl ester
(Decker, 1977). The tritration was carried out with a grounded
Beckman Zeromatic^R pH meter with an external electrode. A 50 ml
reservoir type burette was used with its tip beneath the surface of
the magnetically stirred reaction system.

One unit of enzyme specific activity was defined as the activity
which gave rise to an increase of 0.01M NaOH per min per mg protein
required to maintain pH 6.2.

3.23   Modification of soybean proteins

3.231   Molecular size modification

3.2311   Controlled hydrolysis by immobilized protease(s)

Fifty ml of 2.5% soybean protein isolate in 0.07M Sorenson's phosphate buffer (pH 7.5) contained 0.02% sodium azide to prevent the growth of microorganisms. The substrate was placed in a 250-ml flask with 10g of immobilized trypsin, immobilized chymotrypsin, or immobilized trypsin-chymotrypsin (1:1 or 1:9, weight ratio). Hydrolysis was carried out under constant agitation (200 rpm) at 55°C for 20 - 140 hr. After hydrolysis, all test samples were dialyzed against distilled water overnight and then freeze-dried.

3.2312   Separation of fractions by salt precipitation

3.23121   7S and 11S protein rich fractions

The fractional extraction of 7S and 11S protein rich fraction (PRF) was performed by the method of Saio et al. (1973) who utilized the phenomenon that the 11S protein was selectively precipitated from the supernatant in the presence of calcium salt. The flowsheet from the soybean isolate is shown in Fig. 1. To 2 L of 10 mM calcium chloride solution, 100g of Promine D were added with stirring. The mixture was mixed for 3 hr and centrifuged at 8,000 x g for 10 min. The extract was adjusted to pH 4.5 and centrifuged to remove whey. The precipitated protein was suspended in water, adjusted to pH 7.0 with 0.1N NaOH or 0.1N HCl and then freeze dried to get the 7S protein rich fraction. The residue was suspended in water at 40°C using 20 times the weight of the residue. The suspension was adjusted to pH

Soybean protein isolate
(Promine D)

Extracted with 10 mM $CaCl_2$

for 3 hr (isolate:solvent = 1:20)

Centrifuged at 8,000 g for 10 min

Residue   Extract

Adjusted pH to 4.5

Suspended in water
(40°C, residue:
water = 1:20)   Whey   Precipitate

Adjusted pH to
8.0 - 8.5

Centrifuged at   Suspended in
8,000 g   water

Adjusted pH to 7
freeze dried

Residue   Extract   7S Protein rich
fraction

Adjusted pH
to 7.0

Freeze dried

11S Protein rich
fraction
(11S PRF)

Fig. 1. Schematic outline for the preparation of 7S and 11S protein
rich fraction from soybean protein isolate.

8.0 to 8.5, stirred for 30 min and centrifuged. The extract was adjusted to pH 7.0 and freeze dried to obtain 11S protein rich fraction.

3.23122   Cold insoluble fraction

The separation of cold insoluble fraction (CIF) was carried out as described by Eldridge and Wolf (1967). The CIF was prepared by extracting the soybean isolate with a mixture of water to isolate of 10:1 at 25°C. After centrifugation at 8,000 x g for 10 min, the clear supernatant was decanted and cooled at 4°C overnight. The precipitate (CIF) was then collected by centrifuging at 4°C and 8,000 x g. The fraction was dissolved in distilled water and dialyzed for one week against distilled water at 4°C. The contents of the dialysis casing were titrated to pH 7.8 with 1.0N NaOH and a small amount of insolubles was removed by centrifugation before solubles were freeze-dried.

3.2313   Controlled hydrolysis of separated fractions

After the partial purification of 7S PRF and 11S PRF, the fractions were further hydrolyzed by the immobilized trypsinchymotrypsin (1:1) at 55°C for 20 hr. Then, the medium was removed from the reactor, followed by dialysis against distilled water overnight and freeze-drying.

3.232   Molecular charge and molecular size modification

A 2.5% (w/v) suspension of soybean proteins was prepared in

deionized water and adjusted to pH 8.0. Succinic anhydride was added to the suspension over a 1 - 2 hr period at levels of 10, 70, and 130% of the weight of protein in the suspension (Beuchat, 1977a). The pH was maintained at 8.0 with 0.1N NaOH and the temperature ranged from 24 to 36°C. The reaction mixtures were dialyzed against deionized water at 4°C for 24 hr and freeze-dried.

Soybean proteins were further modified by succinylation. Weight ratios of 1.3 of succinic anhydride to 1.0 protein were used, followed by hydrolysis with immobilized trypsin/chymotrypsin in a 1.0:1.0 weight ratio for 20 hr. Another modification of soybean proteins was conducted by reverse treatments: hydrolysis and then succinylation.

3.24   Determination of bound protein

Determination of the amount of enzyme protein bound to the supports was performed by a modification of the method of Bliss and Hultin (1977). Known weights of the support-bound enzyme were shaken with 5 ml of 1N NaOH for 4 hr at 50°C, filtered and assayed for protein content. The same procedure was followed with treated supports which had been reacted with glutaraldehyde or other reagents. These served as controls.

The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Sodium citrate was adopted in Reagent B instead of sodium tartrate to give a more stable reagent (Bailey, 1967). Reagents were: A, 2% Na_2CO_3 in 0.1N NaOH; B,

0.5% $CuSO_4 \cdot 5H_2O$ in 1.0% sodium citrate; C, 1.0 ml of Reagent B mixed with 50 ml of Reagent A; D, Folin-Ciocalteu reagent diluted with water to give a solution 1.0N in acid. One ml of the sample containing 100 - 1,000 µg of protein was mixed with 1.0 ml of Reagent C and the solution allowed to stand for 10 min at room temperature. One-tenth ml of mixing and the absorbance at 750 nm read after a time interval of 0.5 - 2.0 hr.

3.25   Evaluation of modification

3.251   Available amino groups

The available amino groups were determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) reaction to measure the number of new amino groups formed (Okuyama and Satake, 1960; Habeeb, 1966; Ozols and Strittmatter, 1966; Lin et al., 1969a; Lin et al., 1969b). To 1 ml of protein filtrate or solution (0.6 - 1.0 mg/ml) were added 1.0 ml of 4% $NaHCO_3$, pH 8.5 and 1.0 ml of 0.1% TNBS. The samples were the incubated in the dark for 2 hr at 40°C. After incubation, 1.0 ml of a 10% sodium dodecyl sulfate solution was added to solubilize the protein and prevent its precipitation on addition of 0.5 ml 1.0N HCl. The absorbance of the solution was read at 340 nm against a blank in which trichloroacetic acid was added prior to the addition of enzyme. The wavelength of 340 nm was chosen since the absorption spectrum of the solution read in the spectrophotometer showed a maximum at 340 nm.

The extinction coefficients of various trinitrophenyl alpha-amino acids had been determined by many workers (Okuyama and Satake, 1960; Ozolos and Strittmatter, 1966; Lin et al., 1969a). This values varied slightly with different alpha-amino acids but have an average value of approximately 1.3×10^4 mole $^{-1}cm^2$. With this value, the results can be expressed in terms of the number of primary amino groups present irrespective of the peptide bond from which they were derived. The molar concentration of cleaved peptide bonds can therefore be calculated from the relationship:

$$NH_2 - terminal, mole/L = A_{340}/1.3 \times 10^4$$

For the degree of proteolysis, the reaction medium was diluted with an equal volume of 10% trichloroacetic acid. After filtration, the filtrate was added to 1:2.5 borate buffer (pH 8.5). An aliquot was then analyzed for the amino groups as previously described.

3.252 Available lysyl groups

The available lysyl groups were estimated by the method of Kakade and Liener (1969) as the degree of succinylation. Briefly, to 1.0 ml of solution containing 1.0 mg native or succinylated protein in 4.0% $NaHCO_3$ at pH 8.5, was added 1.0 ml of a freshly prepared water solution of 0.1% TNBS. The reaction was allowed to proceed at 40°C for 2 hr at which time 3.0 ml of concentrated HCl was added. After the hydrolysate had been allowed to cool to room temperature, 5.0 ml of distilled water was added. Filtration was necessary to remove

insoluble particulate matter. The contents of each tube were transferred to a screw-stopped test tube and extracted twice with 10 ml ethyl ether in order to remove trinitrophenyl-N-terminal amino acids or peptides as well as picric acid, which was also produced during the course of reaction. Residual ether was eliminated from the aqueous phase by placing each tube in hot water for at least 5 min. The aqueous solution was read at 346 nm against a blank carried through the same procedure except that the concentrated HCl was added to the protein solution prior to the addition of the TNBS reagent. The introduction of an ether extraction step in the procedure removed picric acid as well as untreated TNBS. The ϵ-TNP lysine content of the protein was calculated by using a value of 1.46×10^4 $M^{-1}cm^2$ at 346 nm for the molar absorptivity of ϵ-TNP lysine.

3.26   Molecular properties of modified soybean proteins

3.261   Evaluation of molecular sizes

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate and evaluate soybean proteins by the Weber and Osborn method (1969) as well as the Laemmli method (1970), using an anionic detergent, SDS, in sample solutions and gels.

3.2611   Preparation of sample solutions

Proteins were dissolved in a solution (2 mg/ml) consisting of

0.01M sodium phosphate buffer, pH 7.0 with 1.0% SDS and 1.0% 2-mercaptoethanol, and then incubated in a 97°C bath for 2 min (Weber and Osborn, 1969). The samples contained the final concentrations; 0.0625M tris-HCl (pH 6.8), 2.0% SDS, 10% glycerol, 5.0% 2-mercapto-ethanol and 0.001% bromophenol blue as the dye before dissociating completely by immersing the samples for 1.5 min in boiling water (Laemmli, 1970).

3.2612   Preparation of gels

The Weber and Osborn gels (1969) were prepared from 10.10 parts by volume of the acrylamide solution A consisting of 22.2g of acryla-mide, and 0.6g of N,N'-methylenebisacrylamide (BIS) in 100 ml; 3.40 parts by volume of water; 15.00 parts by volume of gel buffer (7.8g of NaH$_2$PO$_4$.H$_2$O, 38.6g of Na$_2$HPO$_4$.7H$_2$O, and 2.0g of SDS in 1,000 ml); 1.50 parts by volume of ammonium persulfate (15 mg/ml); 0.05 parts by volume of N,N,N',N'-tetramethylethylenediamine (TEMED). The Laemmli gels (1970) containing 3.0% acrylamide for the stacking gel, and 7.0% acrylamide for the separating gels were prepared from the stock solu-tion of 30% by weight of acrylamide and 0.8% by weight of BIS. The final concentration in the separating gels were 0.375M tris-HCl (pH 8.8) and 0.1% SDS. The gels were polymerized by 0.25% by volume of TEMED and ammonium persulphate. The stacking gels of 3.0% acrylamide contained 0.125M tris-HCl (pH 6.8) and 0.1% SDS. Eleven cm of gels had 10 cm of the separating gel and 1 cm of the stacking gel in glass

tubes of a total length of 12.5 cm and with an inside diameter of 6 mm.

3.2613   Preparation of samples

For each gel, 5 µl of tracking dye (0.05% bromophenol blue in the stacking gel buffer), 1 drop glycerol, and 5 µl of 2-mercaptoethanol were added to 75 µl of sample or 12 µl of standard protein solutions, and then applied on the gel after mixing (Weber and Osborn, 1969).

3.2614   Electrophoresis

A Biorad Model 155 electrophoretic apparatus was used with a Gelman power supply Model 38520. Electrophoresis was carried out with 8 mA per gel until the bromophenol blue marker reached the bottom of the gel for the Weber and Osborn method (1969). However, for the discontinuous system, electrophoresis was performed at a constant current of 1 mA per stacking gel with the positive electrode in the lower buffer. At the separating gel, the current was increased to 3 mA per gel (360 - 390V). The position of the tracking dye was marked in each gel with a fine wire.

For the staining and destaining gels, the gels were fixed, stained and destained by a series of gradient solutions of the Fair-banks et al. method (1971): (1) 25% isopropyl alcohol, 10% acetic acid, 0.025 - 0.050% coomassie blue for overnight; (2) 10% isopropyl alcohol, 10% acetic acid, 0.0025 - 0.0050% coomassie blue for 6 - 9 hr; (3) 10% acetic acid with 0.0025% coomassie blue for overnight; (4) 10% acetic

acid until the background was clear.

3.2615   Evaluation of gels

The gels stained with coomassie blue were photographed through a skylight filter using Kodak Panatomic-X film. Gel densitometry was done using a Gilford spectrophotometer 250 at 600 nm, and chart speed of 1cm/min.

The relative mobility (R_f) is the ratio between the distance of the band migrated in the separating gel and the distance of the tracking dye migrated. The position of the tracking dye in the stained gel is equal to the position of the dye in the unstained gel multiplied by the ratio of the stained gel length of the unstained gel length (Rodbard and Chrambach, 1971):

$$R_f = \frac{x_2 \, L_1}{L_2 \, x_1}$$

where x_1 = position of dye in unstained gel, x_2 = position of protein in stained gel, L_1 = length of unstained gel, and L_2 = length of stained gel.

3.262   Evaluation of molecular sizes and molecular charges

Polyacrylamide disc-gel electrophoresis (PAGE) was carried out essentially as described by the Davis method (1974) and the modified Davis method (Groves and Kiddy, 1968; Melachouris, 1969). The Davis method (1964) involved an electrophoresis system consisting of various

gel concentrations ranging from 5.25 to 16.00% T in in separating gel (pH 8.9), 2.50% C in stacking gel (pH 6.7), and tris-glycine electrode buffer, pH 8.3, using the terminology introduced by Hjertén (1962). Gel concentration (% T) and crosslinkage (% C) are given as:

$$\% \text{ T} = \left(\frac{\text{g acrylamide + g BIS}}{100 \text{ ml solution}}\right) \times 100$$

$$\% \text{ C} = \left(\frac{\text{g BIS}}{\text{g acrylamide + g BIS}}\right) \times 100$$

3.2621 Preparation of protein solutions

The proteins (1 - 2 mg/ml) were dissolved in 0.062M tris-buffer (pH 6.7) for the Davis method (1964), 0.1% Triton X-100 (Kawasaki and Ashwell, 1976), 1.0% Triton X-100 (Hearing et al., 1976), or 4.0M urea (Hood et al., 1981; Schofield and Baianu, 1982) with or without reducing agents, 1.0% 2-mercaptoethanol or 10 mM dithiothreitol (Draper and Catsimpoolas, 1977) for the Groves and Kiddy method (1968) as well as the Melachouris method (1969). Phenol-acetic acid-water (2:1:1, w/v/v) made in 5.0M urea was used as a solvent system for the Catsimpoolas et al. method (1968). Sample solutions with reducing agents were incubated at 50°C for 30 min just prior to the sample application.

3.2622 Electrophoresis

David gels (1964) were prepared from the following stock

solutions in a tube previously described: solution A (3M tris-HCl, containing 0.23 ml TEMED/100 ml, pH 8.9); solution B (0.49M tris-HCl with 0.46 ml TEMED/100 ml, pH 6.7); solution C (28% acrylamide and 2.5% BIS); solution D (10% acrylamide and 2.5% BIS); solution E (0.004% riboflavin); solution F (40% sucrose); catalyst (0.14 ammonium persulfate/100 ml); reservoir buffer (0.04M tris-glycine buffer, pH 8.3). Separating gels consisted of 1.00 part of solution A, various parts of solution C, 0.16 parts of catalyst, and water to reach 2.94 parts in total, while stacking gels had 1 part of solution B, 2 parts of solution D, 1 part of solution E, and 4 parts of solution F. Gels prepared by the Groves and Kiddy method (1968) as well as the Melachouris method (1969) contained similar components as gels prepared by the Davis method (1964), except that the gel for this research contained 4.0M urea and 7.0M urea, respectively. Furthermore, solution A was replaced by 1.5M tris-HCl buffer, and 4.0M or 7.0M urea was substituted for solution F. The Catsimpoolas et al. method (1968) required the positive electrode in the upper buffer because of the net positive charge of the protein in the latter buffer.

Preparation of samples, electrophoresis, staining, destaining, and evaluation of gels were similar to those of SDS-PAGE.

3.2623   Ferguson plots

When $\log_{10}$ relative mobility for a single protein is plotted versus gel concentration, a linear relationship is found which follows

the equation (Ferguson, 1964):

$$\log_{10} R_f = \log_{10} Y_o + K_R T$$

where R_f is the relative mobility at gel concentration T, Y_o is the extrapolated relative mobility at zero gel concentration, and K_R is defined as the retardation coefficient. The least-square regression line of $\log_{10} R_f$ versus % T was used for the determination of K_R and Y_o (Rodbard and Chrambach, 1971).

3.26231 Average molecular weight

The number average molecular weight ($\overline{M}w$) determined by gel filtration was characterized by its principal statistical moments (Catsimpoolas, 1974):

$$\overline{M} = \sum_{i=1}^{\infty} n_i M_i / \sum_{i=1}^{\infty} n_i$$

where n_i is the concentration number of molecules of the i-th kind per unit volume and M_i is its molecular weight.

Assuming the standard deviation of the concentration distribution of all stained different species to be identical, the concentration of protein or peptide molecules in the i-th interval of a gel is proportional to absorbance (A_i). Therefore, the proportion of the total weight present in the i-th distance (L) is:

$$n_i = \frac{A_i L}{\sum A_i L}$$

Likewise, the proportion of the i-th band is:

$$n_i = \frac{A_{ri}}{\sum A_{ri}}$$

where A_{ri} is area of the i-th band. Therefore, the average molecular weight is:

$$\overline{M}_W = \frac{\sum A_{ri}\, M_i}{\sum A_{ri}}$$

$$\overline{M}_W = \frac{\sum A_i\, M_i}{\sum A_i} \quad \text{(Hsieh et al., 1979)}$$

3.26232   Average molecular charge

In a similar manner as the average molecular weight, the average molecular charge can be derived as:

$$\log \overline{Y}_0 = \frac{\sum A_{ri}\, \log Y_{oi}}{\sum A_{ri}}$$

3.27   Functional properties of modified soybean proteins

3.271   Solubility

To 0.1g of modified soybean proteins, 5.0 ml of 0.07M Sorenson's phosphate buffer (pH 6.5) were added in a weighted centrifuge tube. The tube was agitated on a Vortex mixer (Model K-550-6, Scientific Industries, Inc., Bohemia, N.Y.) set at speed 5 for 2 min after swelling samples for one hour. The sample was centrifuged at 1,300 x g for 10 min. The supernatant was analyzed for amino groups after the dilution of 1:11 ratio with the phosphate buffer.

3.272   Water holding capacity

After decanting the clear supernatant from the solubility determination, the pellet was weighted and the weight of water bound per gram protein was calculated as water holding capacity (Childs and Park, 1976).

3.273   Oil holding capacity

The oil holding capacity was determined in the same manner as the water holding capacity except that 5 ml of 100% corn oil (Best Foods, Englewood Cliffs, N.J.) were substituted for 5 ml of the phosphate buffer (Childs and Park, 1976).

3.274   Relative viscosity

The relative viscosity was determined in an Ostwald viscometer at 27 ± 1°C. Eleven mg of soybean protein was dissolved in 3.3 ml of 0.07M phosphate buffer (pH 6.5) and the relative viscosity of the

product solution was defined as the ratio of the viscosity of the product solution to the viscosity of water.

3.275   Emulsifying ability

The emulsifying ability was estimated by a slightly modified method of Yasumatsu et al. (1972a). Two ml of corn oil was added to 3 ml of 3.3% soybean protein suspension in 0.07M phosphate buffer (pH 6.5). The mixture was emulsified with a vortex mixer for 2 min. The emulsion was centrifuged at 1,300 x g for 10 min. The emulsifying ability was the ratio of the volume of emulsified layer x 100 to the whole volume in the tube.

3.276   Emulsion stability

The emulsion prepared by the procedure for the measurement of emulsifying capacity was heated for 30 min at 80°C, cooled with tap water for 15 min, and then centrifuged at 1,300 x g for 10 min (Yasumatsu et al., 1972a). The emulsion stability was expressed as the volume of the emulsified layer after heating x 100 over the volume of the emulsified layer before heating.

3.277   Foaming ability

A 0.08% of an aqueous suspension of soybean protein was prepared. The suspension was stirred at low speed in a Waring blender (Waring Products Div., New Hartford, Conn.) for 5 min. The content was

immediately transferred to a 100 ml graduated cylinder and the foam volume was measured.

3.278 Foam stability

After standing 30 min, the residual foam volume was estimated (Puski, 1975). The foam stability was the volume of foam after standing x 100 over the volume of foam before standing.

4.0   RESULTS AND DISCUSSION

4.1   Immobilization of proteases

4.11   Binding of enzyme protein and enzyme activity

The amounts of bound enzyme protein, assayed enzyme activities, and the ratio of enzyme activities are summarized in Table 1. According to the results of this research, trypsin immobilized on nylon pellets by 6% glutaraldehyde showed higher enzyme immobilized than trypsin immobilized by the acid azide method. This indicates that glutaraldehyde is the most effective reagent in facilitating intermolecular cross-linking of enzymes (Branner-Jorgensen, 1978). With the isothiocyanate reaction trypsin was unstable. The unstability of immobilized enzyme might be due to the unstable linkages at pH extremes (Weetall, 1975a).

The amount of trypsin immobilized on porous glass was higher in comparison to that of trypsin immobilized on nylon pellets. The dimension of nylon pellets is in the mm range, while the dimension of the porous glass particles is ca. 50 μm. That is reason why, for the same weight, the surface area of the porous glass is much larger than that of nylon pellets. Further, controlled-pore glass particles, by being porous, have an even larger surface area available for immobilizing. Porous glass with 500 Å pore diameter have 27 - 50 m^2 surface area and 0.7 cm^3/g specific volume (Filbert, 1975).

Table 1. Amount of enzyme protein and enzyme activity immobilized on glass or nylon support.

Immobilized enzyme			Protein bound (mg/g support)	Enzyme activity Assayed	Ratio of enzyme activity (%)[a]
Enzyme	Support	Method			
Trypsin	Nylon	Glutaraldehyde	0.146	8.61[b]	84.2
Trypsin	Nylon	Acid azide	0.012	0.11	13.1
Trypsin	Nylon	Isothiocyanate	-	unstable	-
Trypsin	Glass	Glutaraldehyde	9.288	94.27	14.5
Chymotrypsin	Nylon	Glutaraldehyde	0.159	2.43[c]	33.8
Papain	Nylon	Glutaraldehyde	0.022	0.16[d]	10.2

[a] Enzyme activity of immobilized enzyme x 100/enzyme activity of soluble enzyme.

[b] μM BAEE hydrolyzed/min.

[c] μM BTEE hydrolyzed/min.

[d] ml of 0.01 M NaOH required/min.

Therefore, most of the surface available for binding enzymes is owed
to the porous glass internal structure. However, non-porous supports
like nylon have certain advantages such as: no vigorous clotting
reactions occurring as is the case with porous supports; diffusional
effects are minimal; readily reacting with large substrates in
solution (Messing, 1975). Nonetheless, non-porous supports have a
relatively samall surface area for the attachment of enzymes. The
enzyme loading problem may be partially overcome by using fine particles
and fibers. On the other hand, the advantages of porous materials are
very large surface area for immobilization, and protection of the
bound enzyme from removal due to turbulent flow. Major factors that
limit the advantages of porous carriers are that the coupling reagent
must have access to the pores, and that larger enzyme molecule must
also penetrate the internal surface in order to be bonded. A substrate
larger than the enzyme may not penetrate the pores. Consequently, a
large proportion of the enzymes immobilized on porous carriers may
never fully utilized.

With glutaraldehyde as a covalent bonding agent, the immobili-
zation of trypsin was quite similar to that of alpha-chymotrypsin
because purified trypsin and crystallized alpha-chymotrypsin were
used. However, the amount of unpurified papain immobilized was less
than that of trypsin and alpha-chymotrypsin. Indeed, Yokote et al.
(1975) showed that the degree of purification of the enzyme did have
an effect on the level of immobilization attained by glucose

isomerase.

The percent activity of immobilized trypsin to activity of the equivalent amount of soluble trypsin was 84.2% of trypsin immobilized on nylon pellets by glutaraldehyde. Nonetheless, the ratio of immobilized trypsin on nylon pellets by acid azide and on glass support by glutaraldehyde was 13.1% and 14.5%, respectively. This indicated that trypsin with serine-183 residue possibly involved in the active site was bound to the nylon pellets through acid azide, whereas alpha-amino and epsilon-amino groups were utilized to attach trypsin to the pellets through glutaraldehyde (Weetall, 1975a). Furthermore, Keil (1971) suggested that the specific acylation of lysine and tyrosine did not reduce the specific esterolytic activity of trypsin. The low value of the ratio of immobilized trypsin on glass support might be due to the diffusional barriers. For lactose on inorganic support, the coupling efficiencies, which were basically the ratios of enzyme activity of immobilized enzyme to the activity of soluble enzyme, were reduced by loading high concentrations of the initial enzyme (Pitcher et al., 1976).

Marconi et al. (1974) demonstrated that at low concentrations of enzyme immobilized by fibers, the activity of the fibers was approximately 100%. On the other hand, by increasing the amount of immobilized enzyme the activity of the fibers was reduced. This reduction was caused by diffusion barriers which became greater when the amount of immobilized enzyme was increased. It is known that the

external boundary layer resistance to mass transfer can be reduced as the fluid velocity through the immobilized enzyme is increased (Pitcher et al., 1976). The internal diffusion might be significant when the reaction rate varies with an increase in packing density (Vieth and Venkatasubramanian, 1976).

4.12   Effect of glutaraldehyde on immobilization

Glutaraldehyde in 0.5M sodium phosphate buffer was used to examine the effect of glutaraldehyde concentration on the immobilization of trypsin. Fig. 2 shows that the amount of immobilized trypsin is maximum when the concentration of glutaraldehyde is higher than 6%. However, the increment of concentration of glutaraldehyde did not increase immobilization. Smiley et al. (1974) used 6% glutaraldehyde to immobilize alpha-amylase on nylon tube. When the glutaraldehyde concentration was increased by more than 2%, no significant change in the chitin-bound urease was observed (Iyengar and Rao, 1979). When the glutaraldehyde method is used, the specific activity of immobilized enzymes is an indication of the optimum concentration of the reagent, probably due to the changed conformation of enzymes through high concentrations of the bifunctional agents (Weetall, 1975b; Kim et al., 1982). In addition, the formation of intermolecular linkages between enzyme and glutaraldehyde depended on pH (Ueki et al., 1974).

4.13   Treatment of nylon surface: effect on immobilization
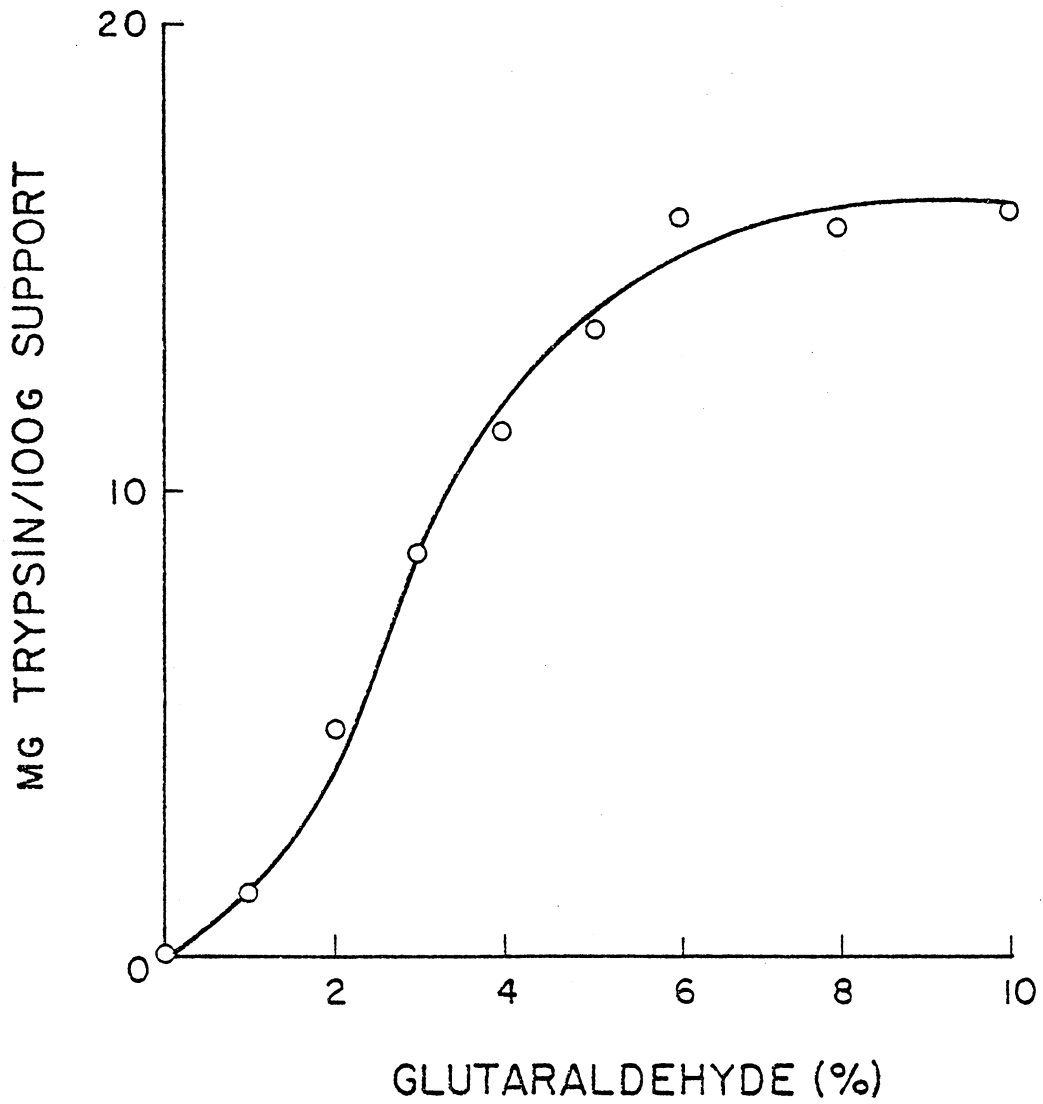
Fig. 2. Effect of glutaraldehyde on the retention of trypsin
on nylon pellets.

The specific activity of immobilized trypsin depends on the type of surface treatment of nylon support, as shown in Table 2. Treatment with a methanolic solution having 18.5g $CaCl_2$, 18.5 ml water and 63.0 ml methanol was essential to maximize the specific activity of trypsin. The bifunctional agent, glutaraldehyde, alone canot immobilize any enzyme. The hydrochloric acid treatment led to immobilizing trypsin having one-half of the activity of the methanolic solution or hydrochloric acid treatment was necessary to immobilize enzymes (Smiley et al., 1974).

Hydrolysis of nylon pellets with hydrochloric acid can be replaced by methanolic solution treatment without affecting immobilization. Thus, the number of free carboxyl and amino groups of nylon pellets before hydrolysis might meet the saturation levels of available amino groups for attaching enzymes. However, Johnson (1979) reported that hydrolysis increased the free carboxyl and amino groups on the surface of nylon.

Nylon treatment with solutions of hydrochloric acid of concentration higher than 3.76N caused nylon pellets to form amorphous structures. The expanded structures possessing large surface area provided larger area for immobilization. The specific activity of the immobilized trypsin was similar to that of enzyme on nylon pellets treated with less than 3.75N hydrochloric acid. This experiment emphasized the importance of immobilization without changing the nylon structure.

Table 2. Effect of nylon treatment on immobilization
of trypsin.

Treatments	Specific Activity[a]
Glutaraldehyde	0
3.65 N Hcl + Glutaraldehyde	31 ± 1.1
Methanolic solution[b] + Glutaraldehyde	57 ± 2.3
Methanolic solution + X N HCl + Glutaraldehyde	
1 N	57 ± 2.4
2 N	58 ± 2.9
3 N	58 ± 2.3
3.65 N	59 ± 2.1
3.75 N	55 ± 2.2

[a] μm BAEE hydrolyzed/(min)(mg enzyme) ± standard deviation.

[b] 18.5 g $CaCl_2$, 18.5 ml water, and 63 ml methanol.

The binding sites of supporting materials for enzyme immobilization is dependent on the kind of buffers. In the presence of a carboxyl, like acetate, coupling of an enzyme would be expected to occur mainly via amino groups, whereas in the presence of an amino group like tris (hydroxymethyl)-aminomethane, the coupling probably occurs through carboxyl groups of the enzyme (Goldstein et al., 1974).

4.14   Effect of available enzyme on immobilization

Stepwise increase in concentrations of soluble enzymes in the coupling medium was used in an attempt to find the optimum limits of the protein-binding capacity on nylon pellets. As the amounts of available soluble trypsin were increased, those of immobilized trypsin were enhanced by up to 0.8g protein/100 ml (Fig. 3). However, the percentages of immobilized trypsin of the total soluble trypsin were maximum when the available concentration of trypsin was 0.2g protein/100 ml. Generally, the immobilization of alpha-chymotrypsin was similar to that of trypsin (Fig. 4). The percentage of immobilized alpha-chymotrypsin was reduced drastically as the amounts of available soluble chymotrypsin were increased. Increase in enzyme concentrations led to having enzyme derivatives with higher amounts of bound protein, showing that the limits of protein binding on nylon pellets had not been reached (Kennedy, 1974). Likewise, Iyengar and Rao (1979) reported that immobilized urease on chitin by glutaraldehyde remained constant beyond the enzyme:carrier ratio of 2:1. In view of
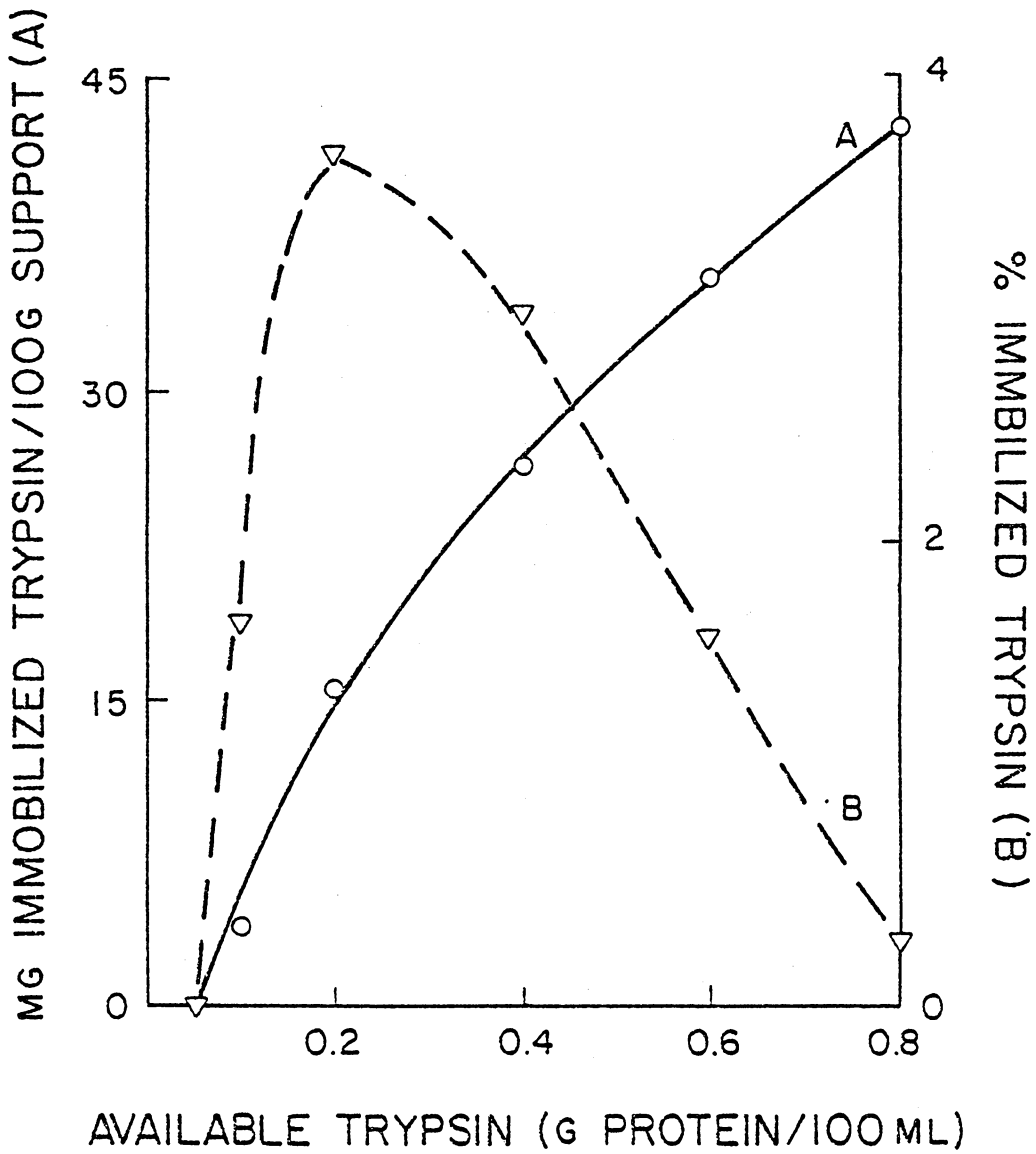
Fig. 3. Amount and yield of bound trypsin as a function of the
concentration of the enzyme.
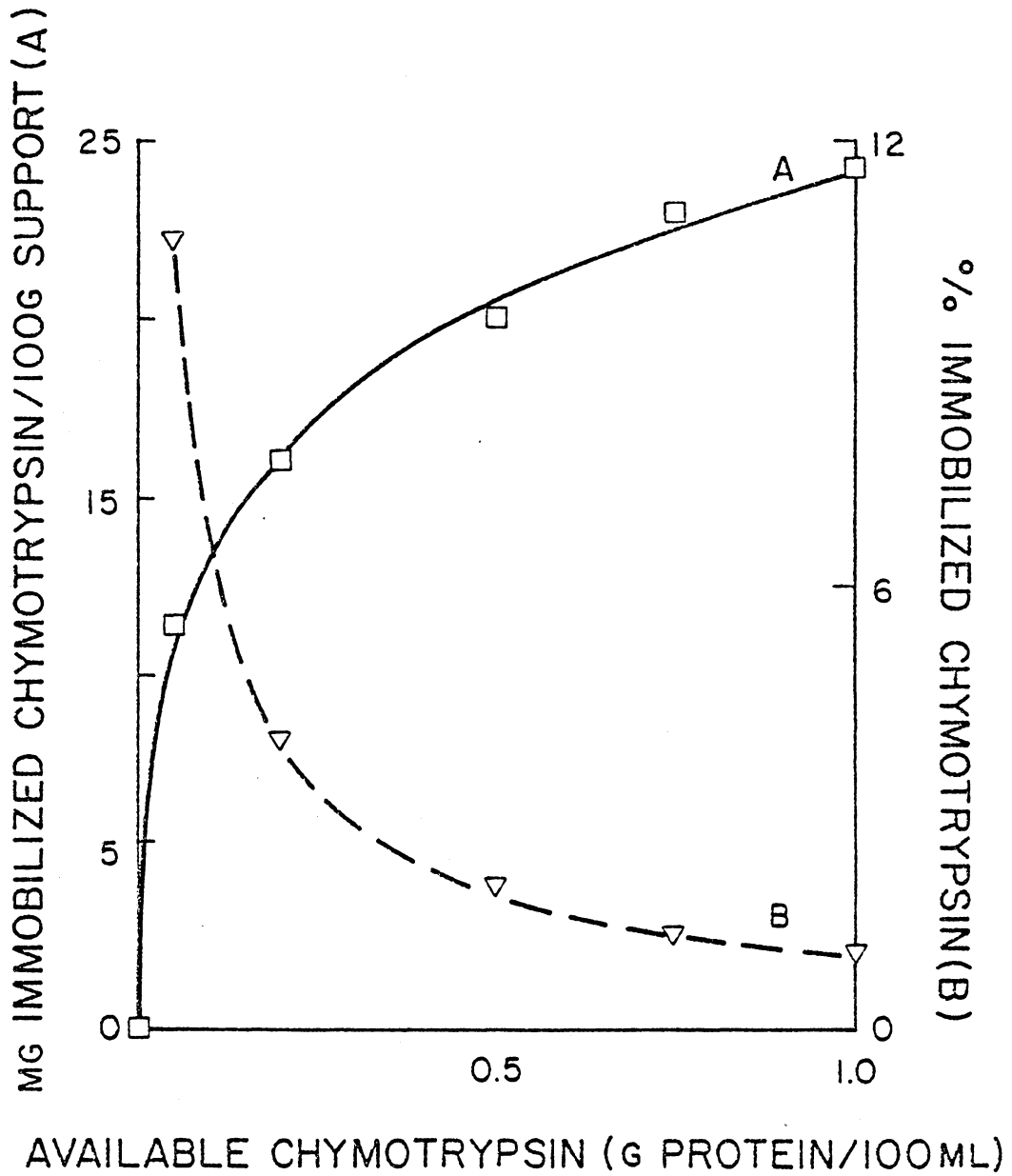
Fig. 4. Amount and yield of bound alpha-chymotrypsin as a
function of the concentration of the enzyme.

the cost of soluble enzymes, the concentration for optimum immobilization has to be determined for each enzyme.

4.15 Michaelis-Menton constants

The determination of the Michaelis constant, Km of a substrate for an immobilized enzyme is one of the most important and useful indicators of the efficiency of enzyme-substrate complex. By definition, Km is the substrate concentration at which the velocity in the Michaelis-Menton reaction is half of the maximum velocity.

The Michaelis constants for immobilized and soluble trypsin are reported in Table 3. With the glutaraldehyde method for immobilization, regardless of types of supporting materials, Km was lower than that of trypsin immobilized by the acid azide method. It seems that the long ligand arms of glutaraldehyde provide flexibility to the immobilized enzyme, resulting in a low Km value. Zemek et al. (1982) reported that the longest mean length of the spacer (about 11 A) of crosslinked polyethylenimine expressed the best binding properties towards various size molecules. It was also known that reducing the physical size of a particle containing a covalently bounded enzyme decreased the Km value by reducing the diffusional limitation (Chibata, 1978). The high Km value of the substrate for the immobilized enzyme showed that the saturation of an immobilized enzyme occurred at a higher substrate concentration than that normally required for saturation of the freely soluble

Table 3. Michaelis-Menton constant (Km) of soluble and
          immobilized trypsin using different methods
          and support materials.

Support	Method	K_m (M)
Nylon	Glutaraldehyde	6.25×10^{-5}
Nylon	Acid azide	1.54×10^{-4}
Glass	Glutaraldehyde	6.62×10^{-5}
Soluble trypsin		2.88×10^{-5}

enzyme (Zaborsky, 1974a).

4.16   pKm

The logarithm of Km values, pKm of immobilized trypsin and
alpha-chymotrypsin show no change as a function of pH (Fig. 5).
Therefore, it might be assumed that the uniform pKm values mean that
there are no ionizing groups in the immobilized enzyme controlling
the binding of the substrate. The facts that velocity varies with pH
and that there is little variation in Km of immobilized trypsin and
alpha-chymotrypsin, are indications that there are ionizing groups in
both the enzyme and enzyme-substrate complex where pKa values are
relatively unaffected by binding the substrate (Roberts, 1977). The
pK value is defined as a logarithmic transformation of a dissociation
constant.

In soluble chymotrypsin, the carboxyl group of Asp-194 and the
N-terminal amino group of Ile-16 form an ionic bond to maintain the
stable conformation of the active site (Whitaker, 1972). Although
neither group is directly involved in the binding of the substrate
or in the catalytic mechanism, any change in the structure of the
active site is reflected in changes in the kinetic parameters such as
Km and reaction rate. Trypsin has three important carboxyl groups,
an imidazole group, a seryl hydroxy group and an alpha-amino group,
all of which have been shown to be important in the catalytic
mechanism. The kinetics of hydrolysis of a number of substrates
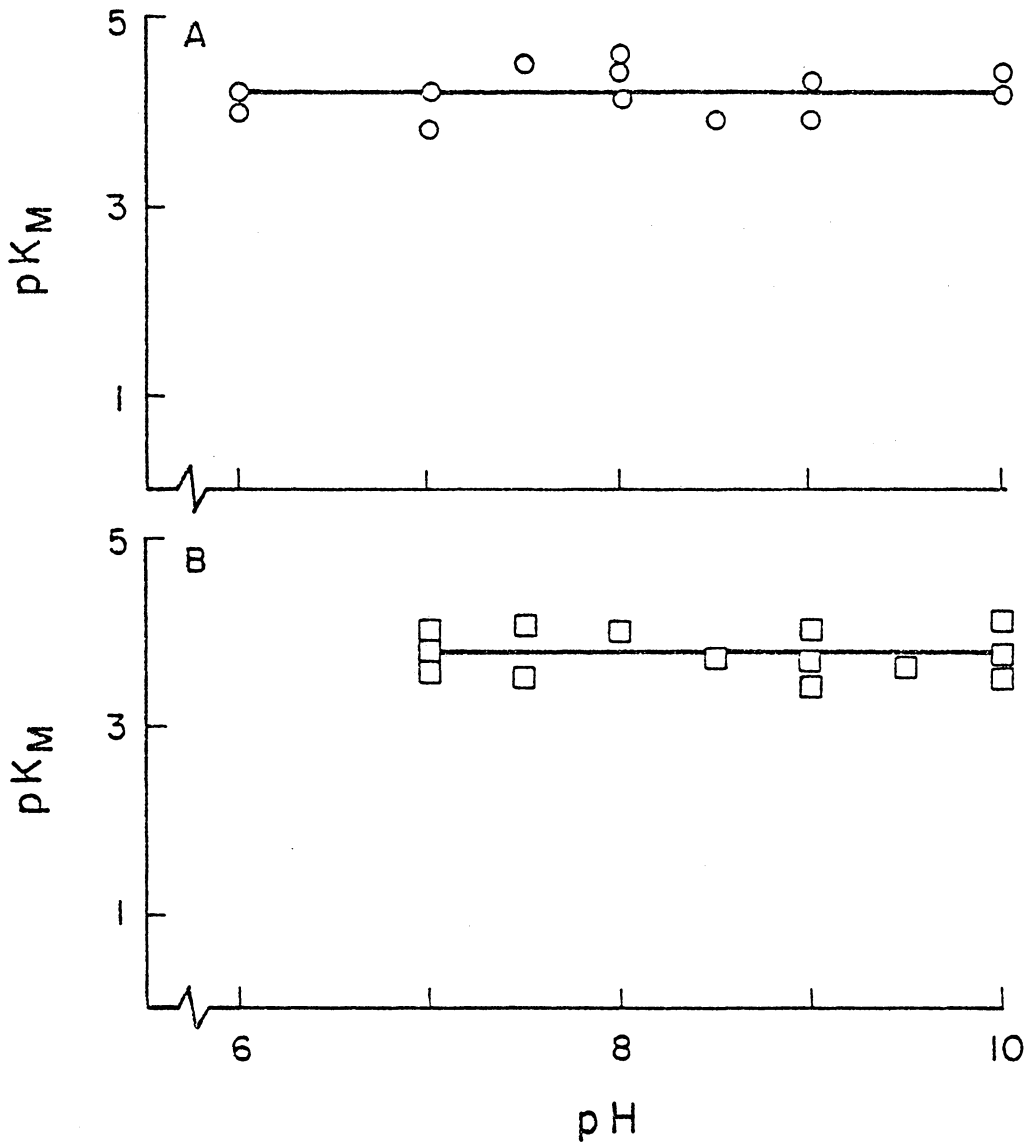
Fig. 5. pKm versus pH profiles for BAEE and BTEE shown by immo-
bilized trypsin (A) and chymotrypsin (B), respectively.

depends upon only two groups with pKa values of approximately 6 - 7
and 9.5 - 10.5 (Keil, 1971).

The pKa values vary with the substrate since the nature of the
substrate alters the polarity of the active site and consequently
alters the observed pKa value for the ionizing group. Indeed, soluble
alpha-chymotrypsin indicates that the Km values are fairly constant
for pH values ranging from 4.5 to 9.0 (Whitaker, 1972). Above pH 9.0,
Km of soluble chymotrypsin increases one order of magnitude. High
pH values lead to forming unprotonated amino groups and to changes in
the conformation of the enzyme.

4.17   Activation energy

The variation of reaction rate with temperature is shown in Fig.
6. The activation energy of trypsin immobilized on nylon pellets by
glutaraldehyde was calculated to be 12.5 kcal/g-mole. This activation
energy is higher than that of soluble trypsin in the presence of
calcium according to Sipos and Merkel (1970) who reported that the
activation energy of soluble trypsin was 8.2 kcal/g-mole at temperatures
below 40°C. The low activation energy value of trypsin immobilized on
glass supporting materials seemed to be controlled by the diffusional
resistance. The apparent activation energy of immobilized lactase was
equal to half of the intrinsic activation energy (Ford and Pitcher,
1975). Trypsin immobilized on nylon pellets through acid azide
exhibited 0.7 kcal/g-mole of activation energy.
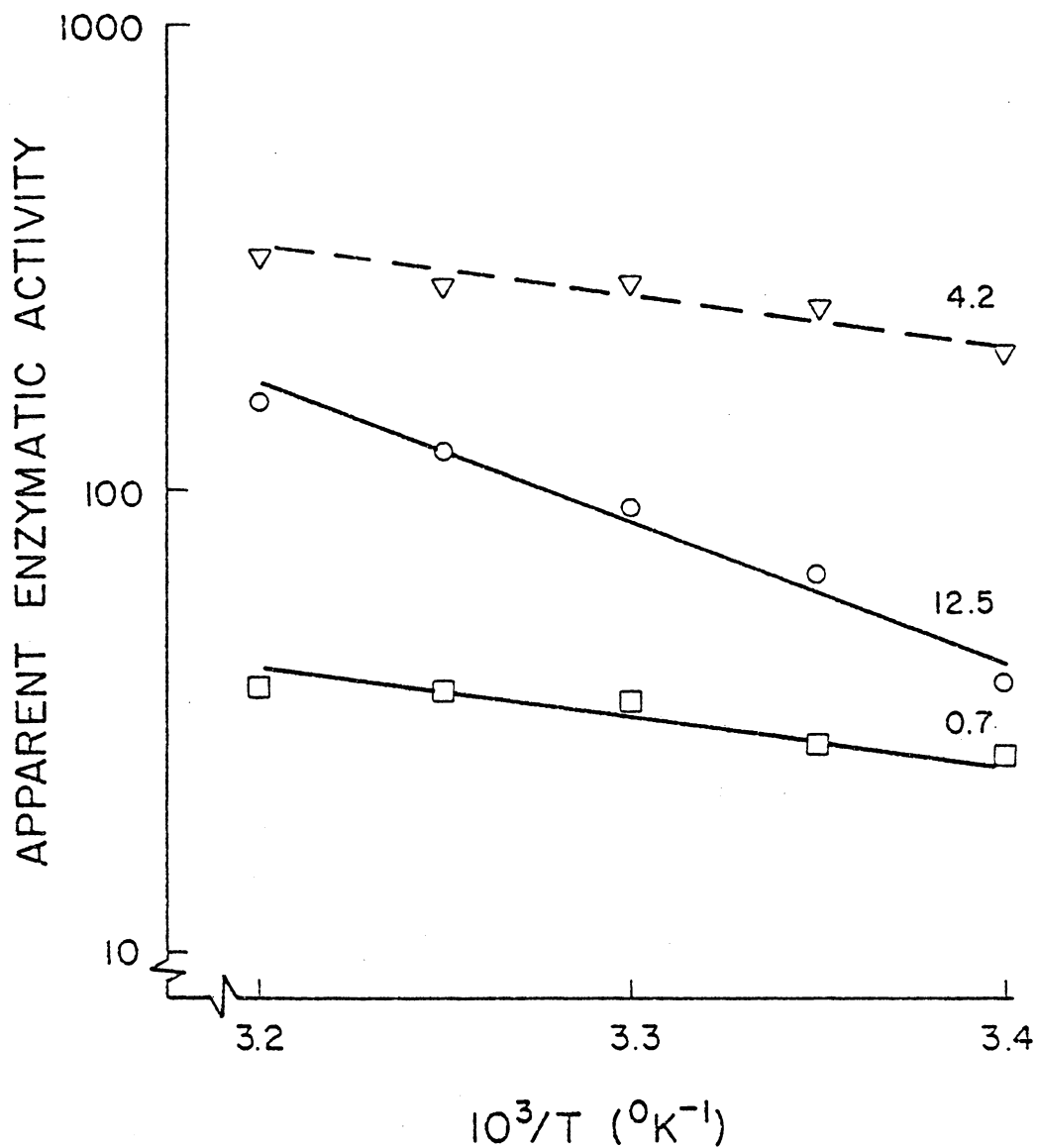
Fig. 6. Effect of temperature on the hydrolysis of BAEE by
immobilized trypsin with nylon-acid azide (□———□),
nylon-glutaraldehyde (O———O), and glass-glutar-
aldehyde (▽— —▽). Numbers refer to activation
energies (kcal/mole).

A low activation energy means that the activity of immobilized
enzyme is less sensitive to temperature changes (Marconi et al.,
1974). This makes more favorable the use of immobilized enzymes in
processes operating at lower temperatures. By increasing temperatures,
high activity of immobilized enzymes with a high activation energy
can be achieved. Obviously, immobilized enzymes are useful in
operation at high temperatures. Therefore, with proper selection of
methods of immobilization and support, the activation energy of
immobilized enzymes can be tailored to meet specific needs.

4.18   Optimum pH

Optimum pH of immobilized trypsin was expressed in percentage of
maximum activity under standard assay conditions except for the use
of buffers of different pH values. As shown in Fig. 7, the optimum
pH values of soluble and immobilized trypsin were 8.5, except for
trypsin immobilized on nylon pellets through acid azide. The similar
pH-activity profile of the immobilized enzymes was due to the net
neutral character of nylon pellets after hydrolysis. Urease immobilized
on nylon tube by glutaraldehyde had a similar pH-activity curve to
that of soluble urease (Sundaram and Hornby, 1970). Trypsin
immobilized on nylon pellets by acid azide shifted the optimum pH of
immobilized trypsin to the alkaline side by 0.5 units because during
immobilization by acid azide, the amino groups newly liberated from
the nylon surface were destroyed by treatment with nitrous acid
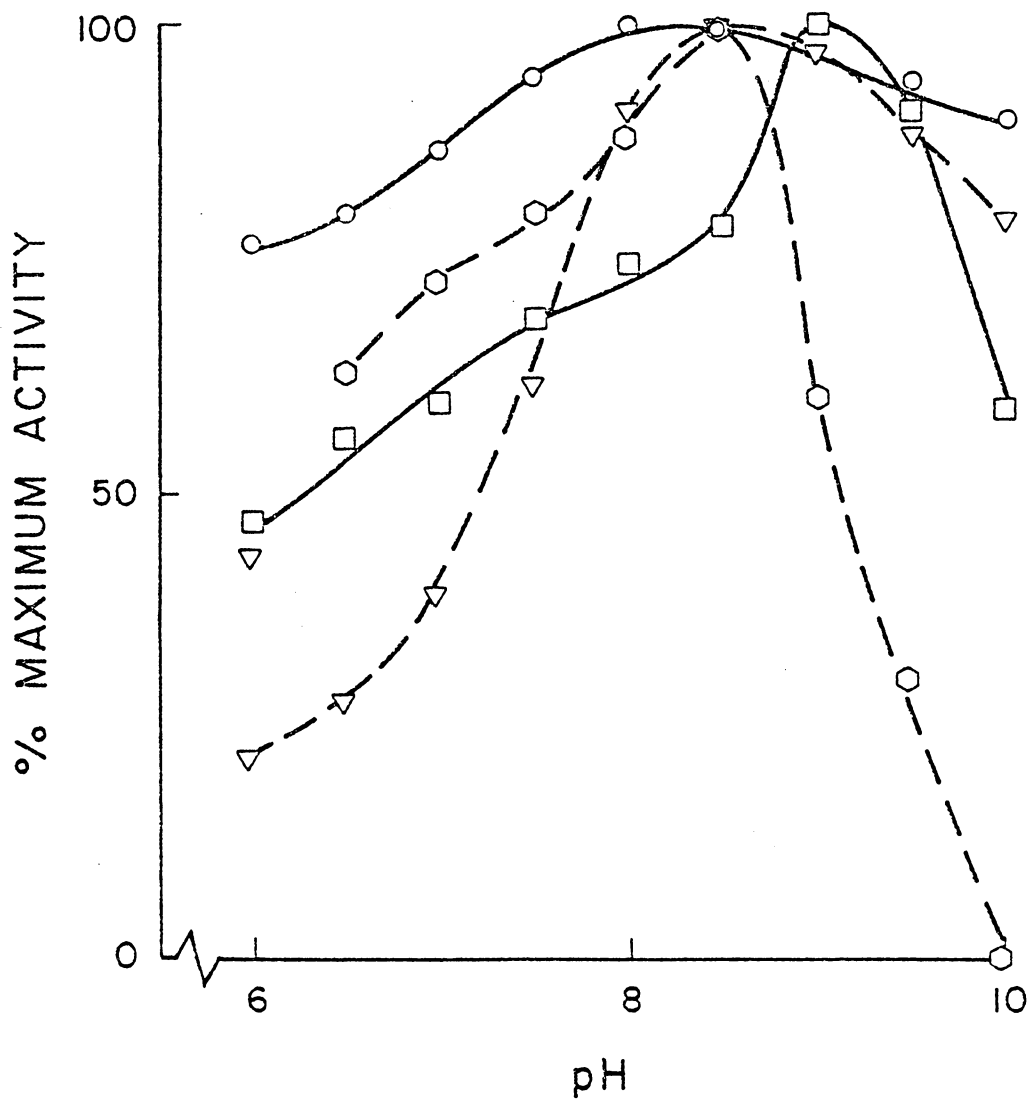
Fig. 7. Effect of pH on the hydrolysis of BAEE by soluble
(▽— —▽) and immobilized trypsin by nylon-acid azide
(□———□), nylon-glutaraldehyde (○———○), and glass-
glutaraldehyde (⬡— —⬡).

(Chibata, 1978). The remaining carboxyl groups on the nylon surface were thus utilized to immobilize enzymes. The pH shift to the alkaline side may have been caused by an increase in negative charges due to carboxyl groups on the support. The microenvironment of immobilized enzymes becomes more acidic than that of the external solution. Furthermore, the pH shifts of the immobilized enzymes might be caused by the chemical modification of the enzyme (Puvana-krishnan and Bose, 1980).

Fig. 8 shows that the pH activity profile of immobilized chymotrypsin is broader than that of soluble trypsin. The flatten-ing of the pH activity curve is the result of the predominant diffusional barriers of the substrate and the differences in activity due to pH variations. The deviation of the pH activity curve of immobilized enzyme from that of soluble trypsin would be due to changes at the surface of the carrier (Line et al., 1971). Components such as other proteins, peptides, and low molecular weight materials were coupled to the supporting materials. The bound materials may place the attached enzyme in a microenvironment, very different from that of the soluble enzyme. It has also been shown that urease had different pH optima depending on the type of buffer used (Iyengar and Rao, 1979).

4.19 Stability
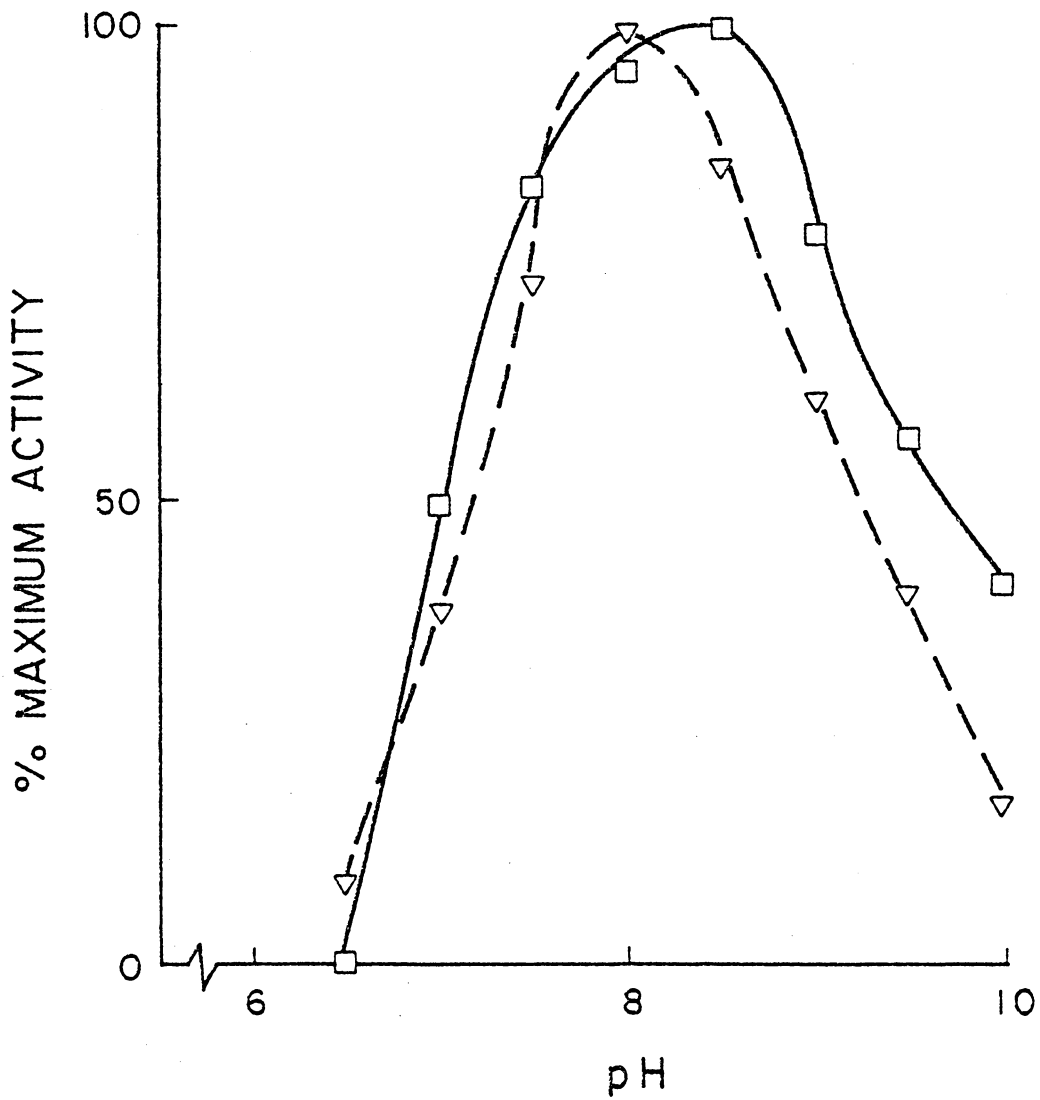
Fig. 8. Effect of pH on the hydrolysis of BTEE by soluble
(▽———▽) and immobilized alpha-chymotrypsin
(□———□).

4.191   Temperature stability

The heat stability of immobilized trypsin on nylon pellets by
glutaraldehyde is superior to that of the native enzyme as shown in
Fig. 9. Above 80°C, immobilized trypsin and soluble trypsin suffered
drastic reduction in their enzymatic activities. As illustrated in
Fig. 10, immobilized alpha-chymotrypsin has slightly higher heat
stability than the soluble enzyme. Above 80°C, both immobilized and
soluble chymotrypsin did not show any enzymatic activity. Therefore,
bifunctional reagents like glutaraldehyde enhanced the thermal
stability of the enzyme. The enhanced thermal stability of the enzyme
crosslinked by glutaraldehyde was presumably attributed to appropriately
positioned intermolecular cross-links (Zaborsky, 1974b; Zaborsky, 1974c).
However, the resulting enzyme conformation must retain sufficient
flexibility to allow for movement of the polypeptide chains necessary
for catalytic activity. Also, intra-molecular crosslinks could
contribute to the enhanced stability of those conjugates by inducing
favorable protein-protein interactions between adjacent sites of two
or more bonded enzyme molecules. On the other hand, Puvanakrishnan
and Bose (1980) reported that the enhanced thermal stability of the
immobilized trypsin might reflect the prevention of autodigestion.
Overall, both soluble and immobilized trypsin showed higher thermal
stability than immobilized chymotrypsin did.

The thermal stability of an enzyme is decided by the primary
structure of a protein, and by the specific secondary and tertiary
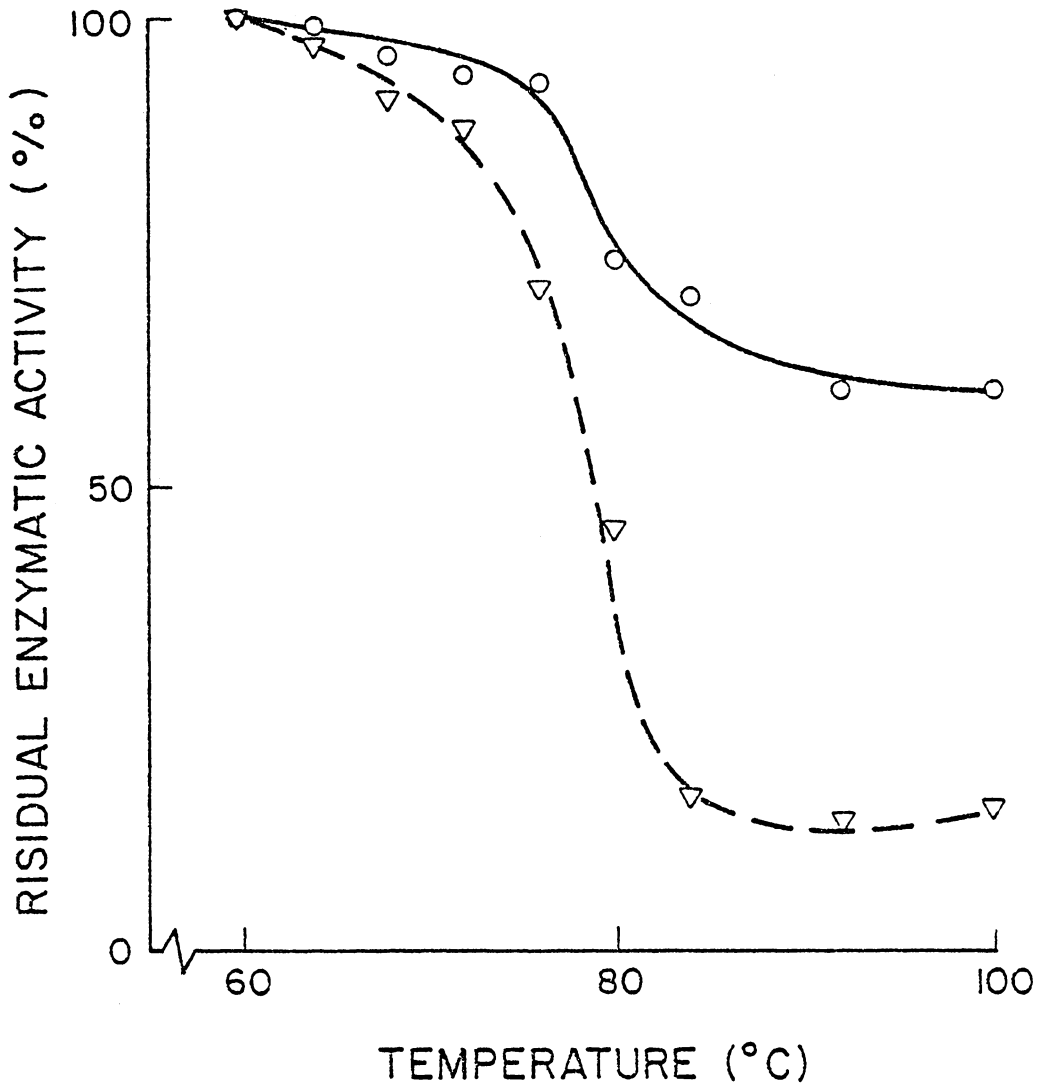
Fig. 9. Heat stability of soluble (▽— —▽) and immobilized
(O———O) trypsin, heated at different temperatures
for 30 min.
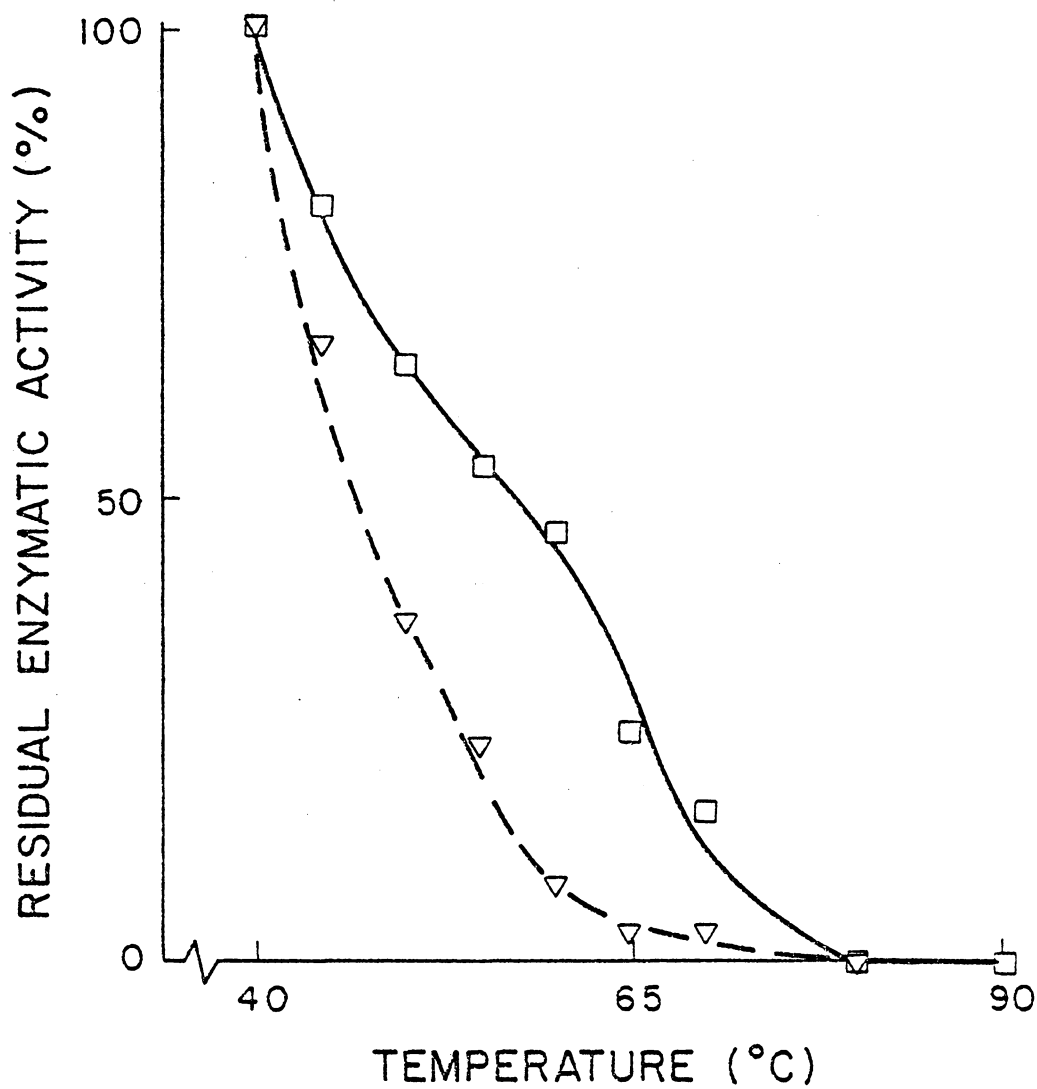
Fig. 10. Heat stability of soluble (▽— —▽) and immobilized
(□————□) alpha-chymotrypsin, heated at different
temperatures for 30 min.

conformation derived from its amino acid sequence (Okada and Urabe, 1975). The number of disulfide bonds contributing to crosslinking within protein molecules and the length of polypeptides may be considered important factors in heat stability. Trypsin contains 229 amino acid residues with six disulfide linkages (Keil, 1971), while alpha-chymotrypsin has 245 amino acid residues with four disulfide bonds (Wilcox, 1970). The larger an enzyme and the more complex its structure are, the more susceptible it is to high temperatures. Integrity can be provided by the disulfide bonds. For example, many of the extracellular low molecular weight proteins contain disulfide bonds and normally these proteins are resistant to denaturation (Whitaker, 1972). Generally, an enzyme is protected by the presence of colloidal materials.

4.192   Storage stability

The stability of immobilized trypsin at 4°C given in Fig. 11. The stability of immobilized trypsin on nylon pellets, regardless of the methods of immobilization, was higher than that of immobilized trypsin on the glass supporting material. A wet cake of immobilized trypsin on nylon pellets can be stored at 4°C for two months without significant loss of activity. This observation is in agreement with the findings of Goldstein et al. (1974) who reported the results about an aqueous suspension of succinyl trypsin on nylon tube. Some nylons, such as Nylon 6 with its hydrophilic character, can support
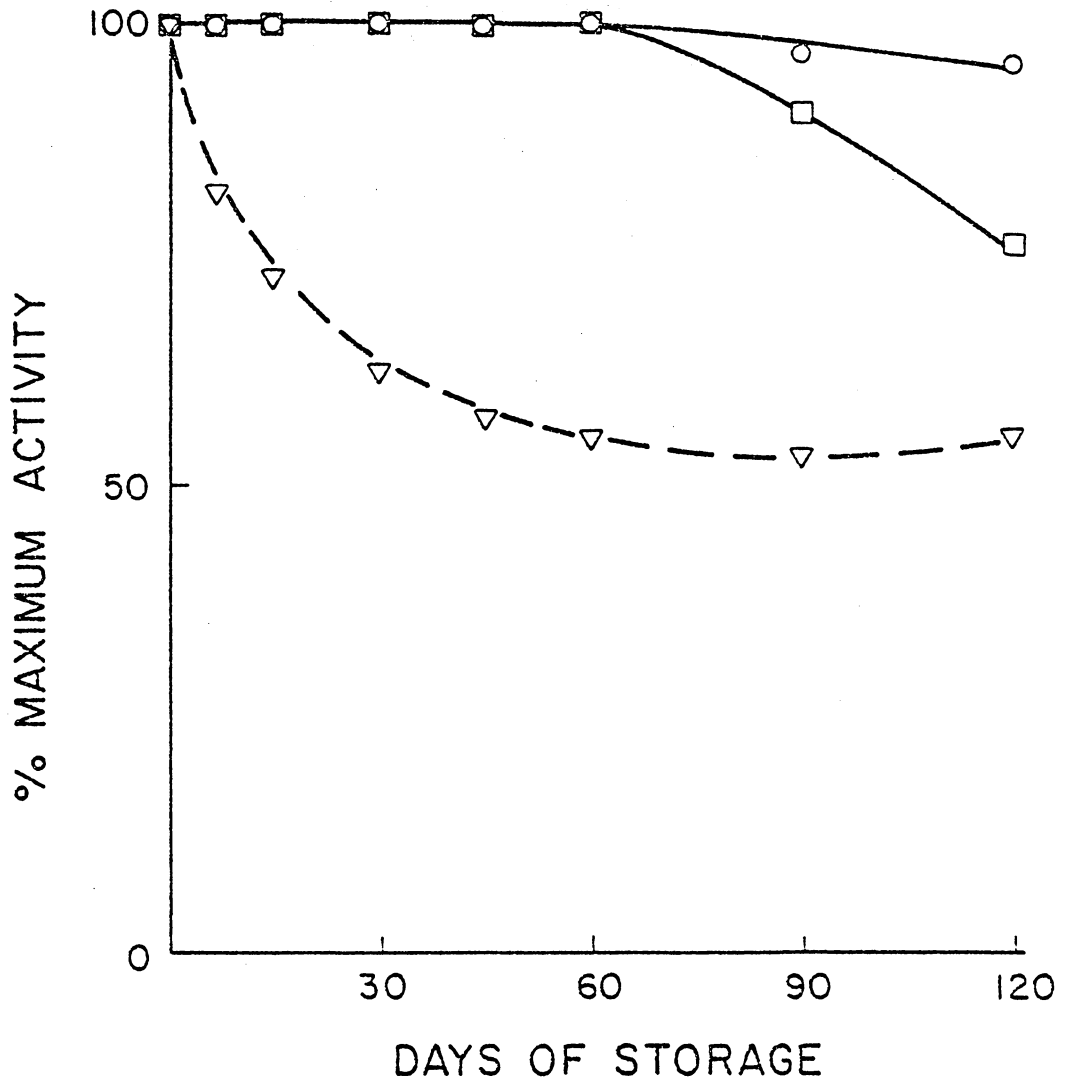
Fig. 11. Storage stability of immobilized trypsin: nylon-acid azide (□———□); nylon-glutaraldehyde (○———○); glass-glutaraldehyde (▽——▽).

an environment conducive to the stability of the protein of immobilized enzymes (Hornby and Goldstein, 1976). Trypsin immobilized by glutaraldehyde on the glass support showed a reduction in its enzymatic activity as a function of storage time. This may be explained by either conformational changes in the enzyme during the storage period, incomplete immobilization of the enzyme, immobilization of different conformation of enzyme protein, or leakage of enzyme from the immobilized preparation (Chibata, 1978).

Decreases in storage stability upon immobilization were reported by Goldstein et al. (1974). They found that naringinase on isobutyl-vinyl ether-maleic anhydride and styrene-maleic anhydride copolymers showed 50% of the original activity after storage at 4°C for 7 days. Regarding storage stability, inorganic materials such as porous glass are superior to organic materials such as cellulose as carriers for immobilization of enzymes. Of the binding methods to the organic carrier, the glutaraldehyde method was superior to the acid azide method. On inorganic carriers diazo binding was superior to any other binding methods insofar as enzyme activity stability after storage (Chibata, 1978).

If immobilized enzymes are intended for reuse, half-lives are very useful parameters. To calculate this, a semilogarithmic plot of activity as a function of time can be obtained. Assuming that the reaction is of the first order, then the slope of this line will be $-k/2.303$; k is the rate constant (Kilara and Shahani, 1979). The

half-life of an immobilized enzyme is a function of temperature.

4.2   Hydrolysis of soybean proteins

Fig. 12 shows the rate of hydrolysis of soybean protein by immobilized proteases. The immobilization method was the glutaraldehyde linkage using nylon pellets as supporting materials. Within the same amount of enzyme-nylon complexes, the immobilized trypsin hydrolyzed a large amount of 1.25% soybean proteins at pH 7.5, followed by immobilized alpha-chymotrypsin. Several factors contributed to high hydrolytic efficiency by immobilized trypsin. The thermal stability of immobilized trypsin is higher than that of immobilized alpha-chymotrypsin (Figs. 9 and 10). This process was conducted at 55°C to reduce possible microbial contamination. Thus, the thermally stable immobilized form was preferred for use during extended periods of operation at high temperature.

As a substrate, soybean protein contains 2 - 6 times lysine and arginine than aromatic amino acids in the first 20 - 30 N-termianl residues of the four acidic subunits (Iyengar and Revestein, 1981). Moreira et al. (1979) also found that basic subunits have two residues of arginine and lysine with no aromatic acids in the 17 residues of the N-terminal sides. This suggests that peptide bonds from the carbonyl groups contributed by lysyl or arginyl residues are more available to trypsin than those bonds from the carbonyl groups furnished by tyrosyl, phenylalanyl, and tryptophanyl residues
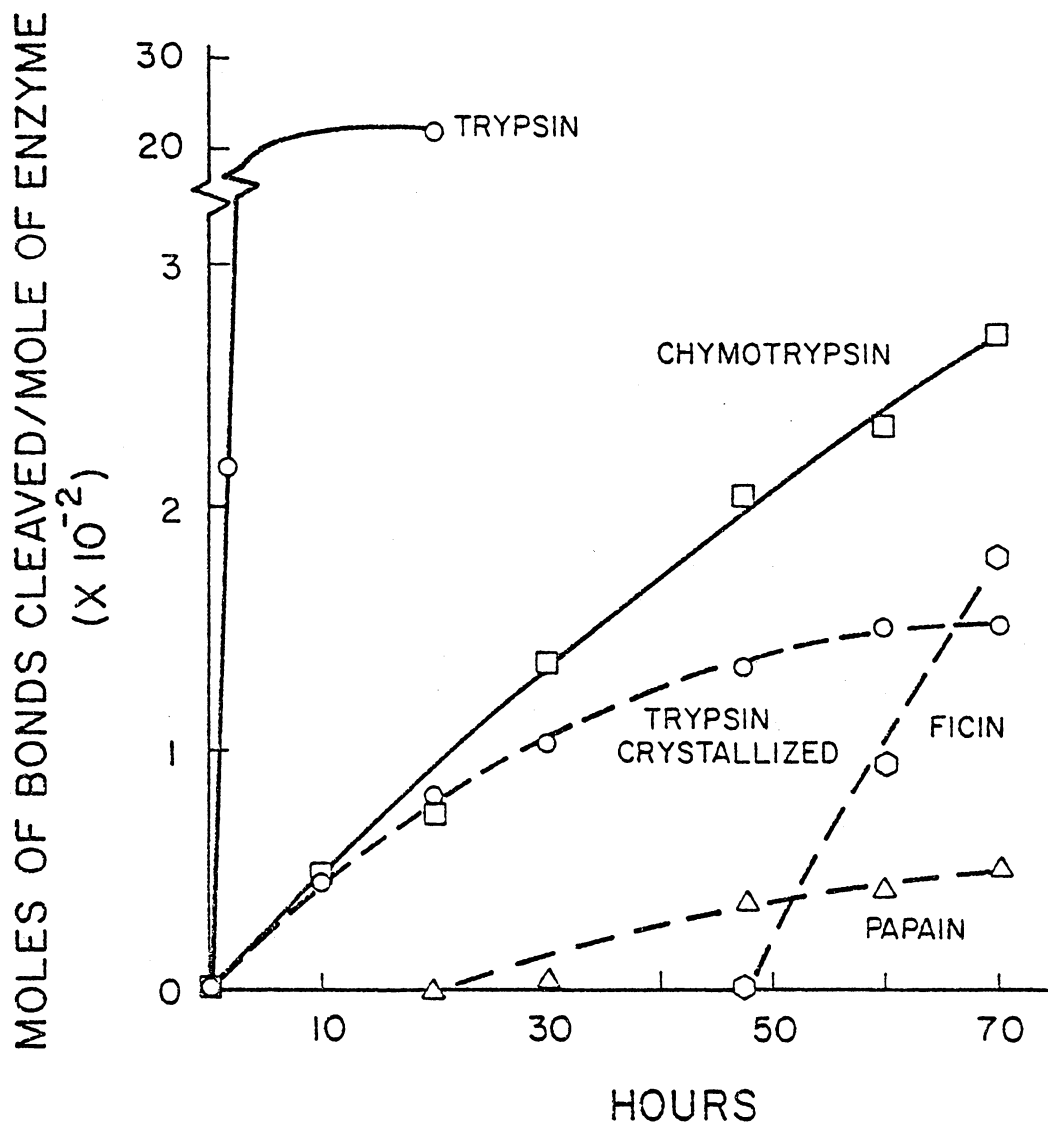
Fig. 12. Hydrolysis of 1.25% soybean protein isolate with immo-
bilized proteases.

available to alpha-chymotrypsin.

Crystallized trypsin immobilized on nylon pellets through glutaraldehyde hydrolyzed less soybean protein than immobilized crude trypsin. This is related to the heat stability of the crude form of the enzyme immobilized on the supporting material. Usually, most of the increased stability of less purified preparations over purified preparations is attributed to protection of enzymes by the presence of some colloid materials such as proteins, carbohydrates, pectins, etc. (Whitaker, 1972).

The low activity of immobilized papain might be caused by either of two factors (Arnon, 1970). Papain requires a free sulfhydryl group for this catalytic activity produced by treatment with mild reducing agents like cysteine, sulfide and cyanide. Heavy metal ions, Cd^{++}, Zn^{++}, Fe^{++}, Cu^{++}, and Pb^{++} are inhibitory. By protein analysis, papain showed 14.4% of protein in this soluble form.

Ficin exhibits enhanced activity in the presence of metal chelating agents such as EDTA, particularly when it is employed in conjunction with one of a variety of reducing agents such as cyanide, cysteine, 1,2-dimercaptoethanol, or 2-mercaptoethanol, having activity mechanisms similar to papain (Liener and Friedenson, 1970). The ficin preparation used contained 28.9% protein which indicated presence of impurities in the enzyme.

4.21 <u>Hydrolysis of soybean proteins as a function of pH</u>

Hydrolysis of 2.5% soybean protein by immobilized trypsin and alpha-chymotrypsin as a function of pH is shown in Fig. 13. Compared with synthetic substrates, immobilized trypsin with soybean protein showed a narrow pH-activity curve, whereas immobilized chymotrypsin with soybean protein had a broad pH activity curve. Both immobilized trypsin and chymotrypsin have optimum pH at 7.5. However, the extent of hydrolysis is very different. Immobilized trypsin released alpha-amino groups of soybean protein larger by one order of magnitude than immobilized chymotrypsin. The preferential hydrolysis of soybean protein by immobilized trypsin was discussed in previous section 4.2. The apparent pK's of both immobilized trypsin and chymotrypsin have the same ranges, 6 - 7 and 8 - 9. This indicated that there was no major change in ionization after immobilization. However, pK_2 of immobilized trypsin moved to the acidic side by 1.0 unit. The group of alpha-chymotrypsin with a pK of 9 was assumed to be involved with Km (whitaker, 1972). It is known that the enzyme undergoes a conformational change in the alkaline region and that the pK of the group involved in this conformational change is 8.5 - 9.0. This group is the alpha-amino group of the N-terminal side which interacts electrostatically with the carboxyl group of aspartic acid to maintain the conformation of the active site. At high pH, the conformational form can no longer bind the substrate. The prototrophic group of soluble chymotrypsin with pK of 6.7 is involved in reaction rate (Whitaker, 1972). Therefore, the groups of immobilized trypsin and alpha-
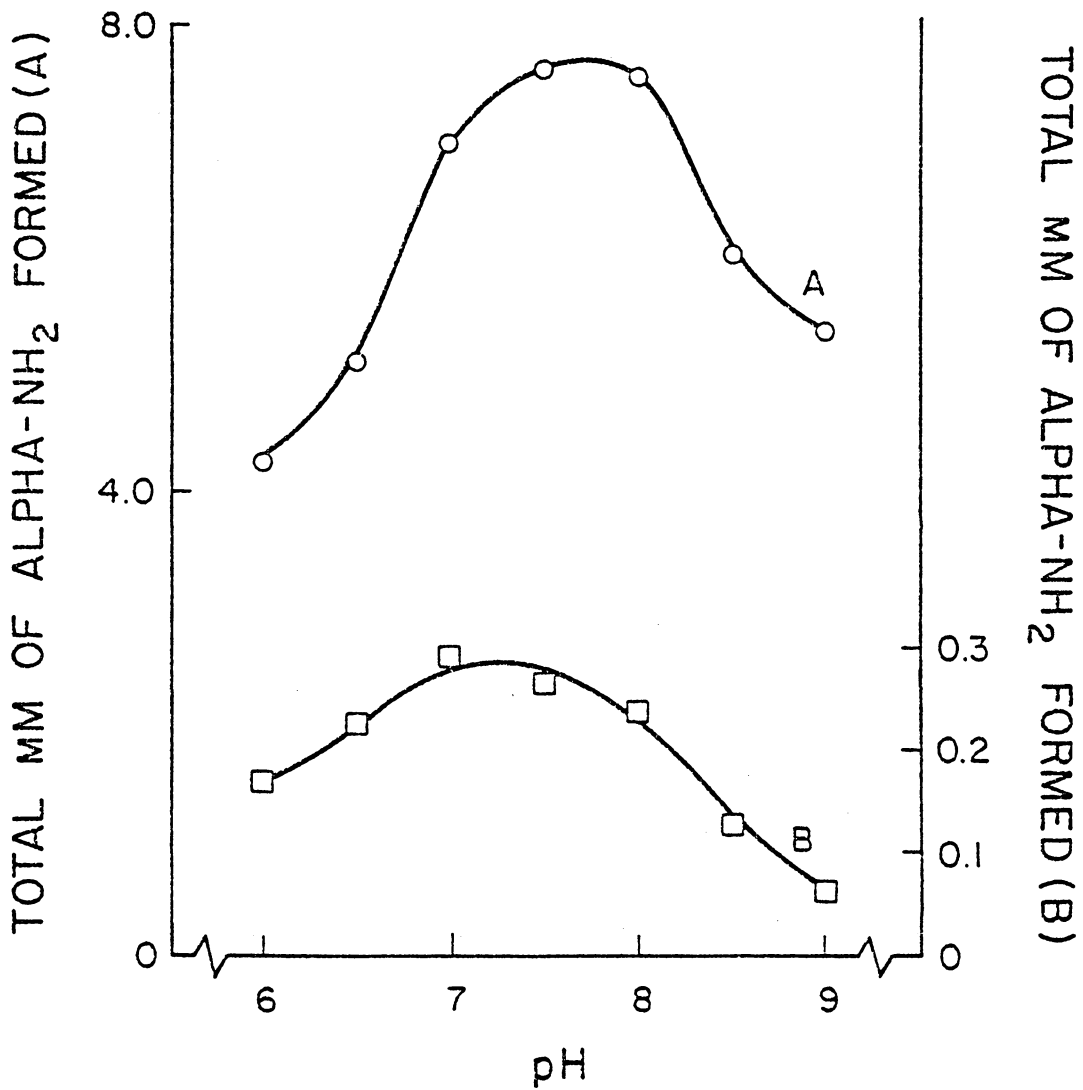
Fig. 13. Hydrolysis of 2.5% soybean protein by immobilized trypsin (O———O) and alpha-chymotrypsin (□———□) as a function of pH.

chymotrypsin seem to be the imidazole group of the histidine residue. Both trypsin and chymotrypsin have histidine residues at the 46 and 57 positions, respectively (Keil, 1971). The imidazole group of histidyl-57 of chymotrypsin acts as a general base to remove the proton from the hydroxyl group of the seryl-195 residue, thus facilitating the nucleophilic attack of the oxygen of the seryl group at the carbonyl of the substrate (Whitaker, 1972). In the deacylation steps, the imidazole group serves as a general base to extract a proton from water to facilitate the attack of the hydroxide ion at the carbonyl group of the acyl-enzyme.

4.22 Immobilized multi-enzymes

Table 4 shows the extent of hydrolysis of 2.5% soybean protein expressed as actual activity in percentage over added theoretical activity of each proportion of enzymes. Two enzyme systems enhanced the hydrolysis of soybean protein more than the expected values calculated from each amount of enzyme. Particularly, when high amounts of immobilized chymotrypsin were included in the system, high values of hydrolysis were observed. This indicates that higher activity of chymotrypsin than that of trypsin is essential for the high degree of hydrolysis because the high proportion of immobilized chymotrypsin may stimulate the availability of labile bonds of substrate for each enzyme. The enhanced hydrolysis of soybean protein was observed after 20 - 140 hr of operational time. Mosbach et al. (1974)

Table 4. Enhanced hydrolysis expressed in percentage of 2.5% soybean protein hydrolyzed by immobilized trypsin/ chymotrypsin systems.

Ratio of enzyme		Operation time (Hours)			
Trypsin	Chymotrypsin	20	80	120	140
		(%)	(%)	(%)	(%)
0.5	9.5	163[a]	162	163	247
1.0	9.0	135	156	122	170
5.0	5.0	126	149	121	132

[a] Expressed as actual activity x 100/added expected theoretical activity of each proportion of enzymes.

reported that the behavior of multistep-enzyme systems was greatly

influenced by the ratio of the activities of the participating enzymes.

In the immobilized multi-enzyme system, the product from the first

enzyme-catalyzed reactions is available in a high concentration around

the second enzyme. Additional advantages of multi-enzyme systems are

the causing or enhancement of reactions by aggregation, the stabiliza-

tion of cofactors, the establishment of a hydrogen ion gradient or a

redox potential, and the provision of hydrophobic areas of reactions

(Hultin, 1974). Several soluble enzymes behind a semi-permeable

membrane have similar advantages. However, enzymes on different

supports would be less efficient than soluble enzymes because of the

fact that when the enzymes are on different particles, the product of

the first reaction will have to diffuse from one particle to another.

Nevertheless, soluble enzymes are generally less stable at high

temperatures than immobilized enzymes. Therefore, binding of enzymes

to separate particles provides better control of the binding as well as

the reaction by adjusting the amounts of enzymes, and the ratios of

one immobilized enzyme to another.

4.3   Functional properties of soybean proteins modified by

      hydrolysis with immobilized protease(s)

4.31   Solubility

      Solubility profiles expressed as alpha-amino groups of soybean

proteins modified by immobilized trypsin and/or alpha-chymotrypsin systems are shown in Fig. 14. The alpha-amino groups of soybean proteins in the supernatant of phosphate buffer (pH 6.5) increased as a function of the degree of hydrolysis expressed as moles of bonds cleaved/mole of enzyme. The higher proportion of trypsin in the multi-enzyme systems increased the degree of hydrolysis and of total alpha-amino group more than a high proportion of chymotrypsin. As pointed out by Moreira et al. (1979), this may be due to the high thermal stability of immobilized trypsin as well as to the high proportions of available lysyl and arginyl groups in the acidic and basic subunits of the major soybean proteins. Within the same degree of hydrolysis of soybean proteins, immobilized multi-enzymes increased more the total alpha-amino groups than immobilized trypsin or alpha-chymotrypsin did. This suggests that the hydrolyzed components of soybean proteins are heterogeneous in terms of molecular size and molecular charge because of different ratios of specificities of enzymes.

Phillips and Beuchat (1981) discussed that hydrolysis increased the number of polar groups such as amino and carboxyl, increased the hydrophilicity of the product and decreased molecular weight of the polypeptides. Obviously, it is not surprising that the high degree of hydrolysis is provided the high available alpha-amino groups in the supernatant.

The pH 6.5 of phosphate buffer was selected because pH values of most animal proteins are close to that pH (Jay, 1978). The pH and
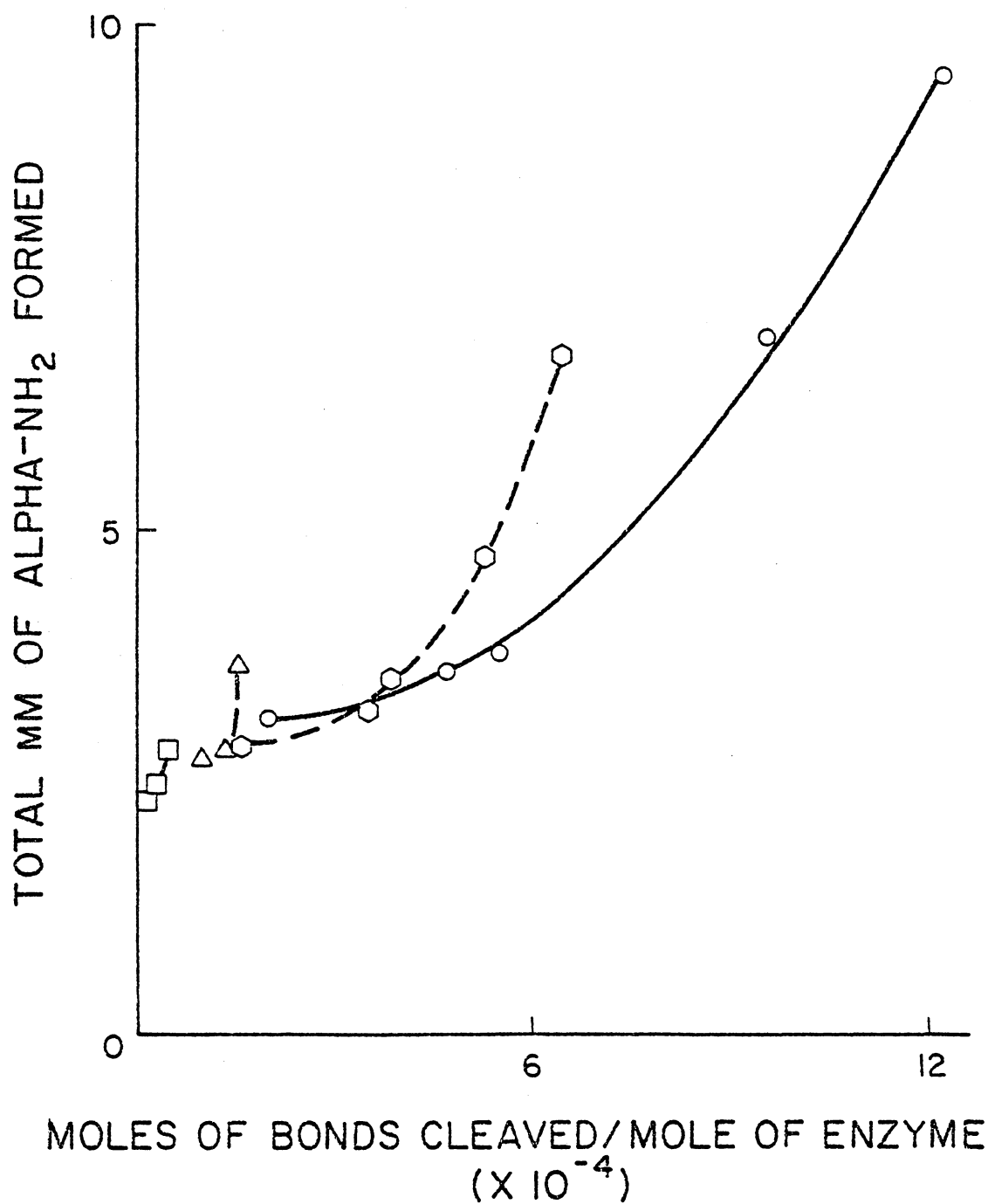
Fig. 14. Increase in solubility of modified soybean protein
produced by immobilized enzyme(s): trypsin (O——O);
chymotrypsin (□——□); trypsin/chymotrypsin (1:1)
(O— —O); trypsin/chymotrypsin (1:9) (△— —△).
Each data point is the average of four determinations.

ionic strength of the aqueous solvent have the most significant effect on the solubility of soybean proteins. The ionization of proteins by added ions according to the Hoffmeister series plays a major role in solubility at a particular pH (Shen, 1981).

Solubility of proteins was increased significantly by enzymatic hydrolysis. The major effects of hydrolysis would be reduced size and changed ionization of protein molecules (Phillips and Beuchat, 1981). The formation of cationic amino groups and anionic carboxyl groups did not cause the net charge of proteins to change substantially after hydrolysis. Puski (1975) increased solubility of soybean protein isolate at pH 4.5 by treatment with a neutral protease from Aspergillus oryzae. This indicates that the size of the molecules plays an important role in solubility at pI. The pH values below are above the isoelectric points of the proteins lead to little difference in solubility between hydrolyzed and controlled peanut proteins. Hermansson et al. (1974) suggests that solubility depends on the molecular size and molecular charge of the protein under the particular ionizing conditions, like pH.

In the presence of 0.03M Ca^{++}, the size of peanut protein molecules is important at pH values above pI, while the charge of the molecules is not important at pH values below pI (Beuchat et al., 1975). Therefore, when the net charge of a protein at a particular pH is minimized either by approaching the pI or by adding limited amounts of ions of opposite charge, the size of the molecules is a critical

factor affecting solubility. Conversely, when the net charge of a

protein is significant either by being quite shifted from pI or by

adding similar ions, the charge of the protein molecules is the major

factor controlling solubility. Under given conditions, thus, the

solubility depends on the sizes and charges of protein molecules. As

a practical application, for the supplement of proteins with ions or

charged molecules, the size of the protein molecules is an alternative

parameter to get desirable solubilities. Solubility of modified soy-

bean protein relates directly to many important functional properties

such as solubility in acid foods, emulsification, foaming capacity, and

gelation (Kinsella, 1976). In fact, the nitrogen solubility levels

were frequently used as indicators of protein functional properties

and potential end use (McWatters and Holmes, 1979a).

4.32   Water holding capacity

The water holding capacities of soybean proteins modified by

immobilized trypsin and/or chymotrypsin are shown in Fig. 15. All

immobilized protease(s) caused the soybean protein to absorb more

water than the non-treated control up to a specific degree of

hydrolysis. The increased water absorbing capacities of immobilized

enzyme(s)-treated soybean proteins were probably related to increased

numbers of polar hydrophilic sites, such as carboxyl and amino groups,

which increased as a result of hydrolysis. Water binding of soybean

protein isolate increased proportionally to the extent of enzyme treat-
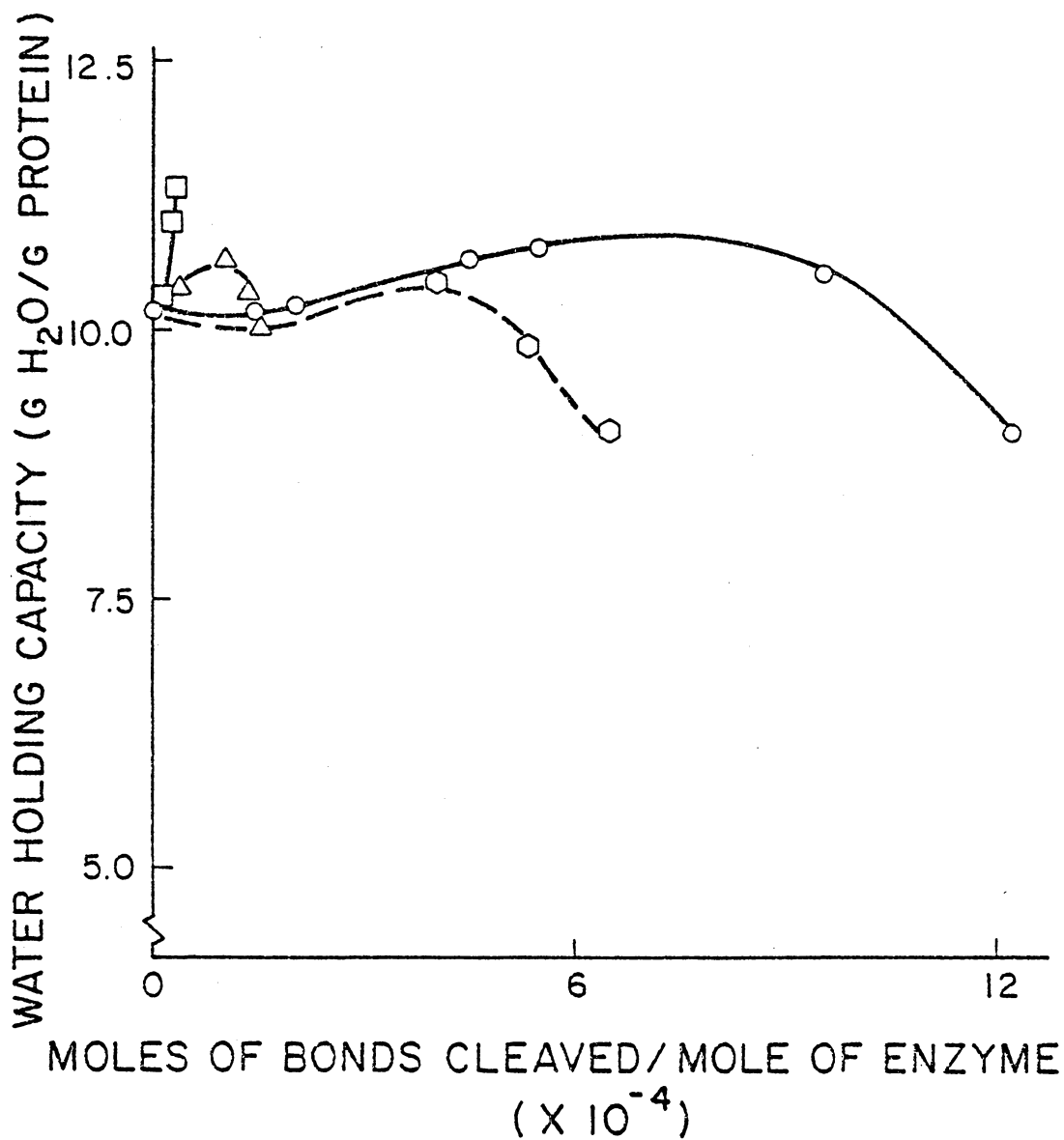
Fig. 15. Effect of immobilized enzyme(s) on water holding capacity of modified soybean proteins: trypsin (O———O); chymotrypsin (□———□); trypsin/chymotrypsin (1:1) (O— —O); trypsin/chymotrypsin (1:9) (△— —△).

ment. On the other hand, the reduction of available polar sites by
succinylated and oxidized globulin led to decreasing water absorption
due to the degree of polymerization and the associated structural
changes in proteins (Sathe and Salunkhe, 1981).

The maximum values for water holding capacity of modified soybean
protein were affected by the relative ratio of immobilized chymotrypsin
to immobilized trypsin in the systems. Soybean protein modified by
immobilized trypsin had the maximum water holding capacity at 7.0×10^4
moles of bonds cleaved/mole of enzyme as a degree of hydrolysis.
However, excessive hydrolysis reduced each water holding capacity.
The high proportion of immobilized alpha-chymotrypsin to immobilized
trypsin enhanced water holding capacity at low degrees of hydrolysis.

Excessive hydrolysis removes the spatial arrangement existing
among protein molecules which is of primary importance to the water
holding capacity of muscle protein (Fennema, 1977). Since water
holding capacity includes entrapped water, extensive hydrolysis can
disrupt the spatial arrangement of proteins resulting in reduced
entrapment of water. The excessive water method for determining water
holding capacity used in the research reported here, involves exposure
of the sample to excess water and application of mild centrifugal force
to separate retained water from the free water. This method does not
account for the protein solubilized by the procedure or for the low-
density components that float on the supernatant surface (Hutton and
Campbell, 1981). For this reason, it is suggested that the excess

water method is suitable only for use on proteins that are mostly
insoluble. Water binding occurs at polar side chanins under low water
activity conditions in the order of amino, carboxyl, and hydroxyl
groups, followed sequentially by peptide linkages at intermediate water
activity and finally by multilayers at higher water activity.

By enzymatic hydrolysis, the transition of the protein molecule
from compact, globular conformation to a random coil conformation
results in the exposure of previously buried peptide bonds and amino
acid side chains so that they may now contact with the aqueous environ-
ment (Kinsells, 1979). Therefore, only controlled hydrolysis of
protein increases water holding capacity. Variation of pH affects the
ionization of amino acid groups. At the isoelectric point where the
net charge is zero, the molecules normally exhibit minimal hydration
and swelling; protein matrices shrink and water holding capacity is at
a minimum (Fennema, 1977). The molecular size and the molecular charge
of a particular protein are important to control water holding capacity
under certain conditions. Water holding capacities relate to other
functional properties such as color, flavor, gelation and mouthfeel.

4.33 Oil holding capacity

The oil holding ability of protein is measured by the processes
of adding excess liquid oil to a protein powder, mixing, centrifuging
the mixture and then determining the amount of oil adsorbed. Fig. 16
shows oil holding ability of modified soybean proteins, hydrolyzed by

Fig. 16. Effect of immobilized enzyme(s) on oil holding capacity
of modified soybean proteins: trypsin (O———O); chymo-
trypsin (□———□); trypsin/chymotrypsin (1:1) (⬡— —⬡);
trypsin/chymotrypsin (1:9) (△— —△).

immobilized trypsin and/or immobilized chymotrypsin. The oil holding
ability of soybean proteins could be maximized by controlling the
hydrolysis of immobilized proteases(s). At 2.0 x 10^4 moles of bonds/
mole of enzyme, the soybean proteins modified by immobilized trypsin
had the maximum oil holding ability. The proteins modified by a high
proportion of immobilized alpha-chymotrypsin in relation to immobilized
trypsin had the maximum oil holding capacity at a lower degree of
hydrolysis than the high proportion of immobilized trypsin or immobil-
ized trypsin only. At the same time, the maximum values of oil holding
capacity were reduced somewhat.

Extensive hydrolysis by immobilized protease(s) reduced the oil
holding capacity, as it did the water holding capacity. The excessive
hydrolysis and the resultant reduction in oil holding capacity depended
on the composition of immobilized proteases. A high proportion of
immobilized chymotrypsin enhanced hydrolysis far more than a low ratio
of immobilized chymotrypsin to immobilized trypsin, or than immobilized
trypsin alone. The controlled hydrolysis of protein probably transforms
the compact globular conformation of native proteins to a random coil
conformation, and exposes buried hydrophobic amino acid side chains of
the proteins (Kinsella, 1979). This would be conducive to absorbing
more oil than in non-hyrolyzed samples. However, extensive hydrolysis
excessively increased the polar hydrophilic groups such as carboxyl
and amino groups and the number of small sizes of molecules which
negatively affected the physical entrapment of oil. Similar results

were obtained by Beuchat et al. (1975) who showed that peanut flour

treated with bromelain for 10 min at 50°C had maximum oil holding

capacity; hydrolysis for more than 50 min at 50°C reduced oil holding

capacity of the peanut flour. The ability of protein to bind fat is

very important for such applications as meat replacers and extenders

because it enhances flavor retention and improves mouth-feel.

4.34   Relative viscosity

The relative viscosities of the soybean proteins modified by

immobilized chymotrypsin and/or immobilized trypsin are given in Fig.

17. Diluted protein solutions (0.33%) below 6.0 x 10^4 moles of bonds

cleaved/mole of enzyme had high relative viscosity as compared to that

of water. The overall trends of relative viscosity were quite similar

to each other regardless of types of immobilized protease(s). The

initial hydrolysis might be expected to change many factors such as

conformation, hydration, exposure of hydrophobic groups and charge

distribution (Shen, 1981), which would contribute to the intermolecular

interactions, resulting in increased viscosity. The soluble protein

molecules, having exposed hydrophobic surface areas, would have greater

protein-protein interactions. This was demonstrated for heat denatured

proteins (Catsimpoolas and Meyer, 1970; Wu and Inglett, 1974; Mori et

al., 1981a; German et al., 1982; Peng et al., 1982).

A more extensive hydrolysis of soybean proteins by immobilized

trypsin showed that the viscosity was inversely correlated with

Fig. 17. Effect of immobilized enzyme(s) on relative viscosity
of modified soybean proteins: trypsin (○———○);
chymotrypsin (□———□); trypsin/chymotrypsin (1:1)
(⬡— —⬡); trypsin/chymotrypsin (1:9) (△— —△).

solubility. Protein molecules of small size cause reduced viscosity
because soybean proteins have less tendency to act as if they had high
specific volume. Viscosity is an indication of the changes that take
place in protein systems during processing, such as in pumping, spray
drying, and heat exchanging (Kinsella, 1976).

4.35 Emulsifying ability

Emulsifying ability of soybean proteins was evaluated by following
the method explained in the experimental section 3.275. Results are
presented in Fig. 18. With immobilized trypsin, either as a single
enzyme or in a multienzyme system, the emulsifying ability (volume
of the emulsion layer expressed as a percentage of the total volume
of the centrifugate) increased when hydrolysis was limited to about
4.0×10^4 moles of bonds cleaved/mole of enzyme. However, soybean
proteins modified by immobilized chymotrypsin always had reduced
emulsification ability. Similarly, hydrolysis beyond the limited
specified above caused by immobilized trypsin/chymotrypsin, or by
immobilized trypsin singly resulted in emulsifying capacity below
that of soybean protein unhydrolyzed. Puski (1975) theorized that
enzymatic digestion of proteins increased the number of polypeptide
molecules, resulting in the emulsification of more oil because a
larger amount of the polypeptide emulsifying agent was available at
the oil/water interface. Enzyme treatment may expose buried hydrophobic
groups which improve the hydrophilic-lipophilic balance (HLB) causing
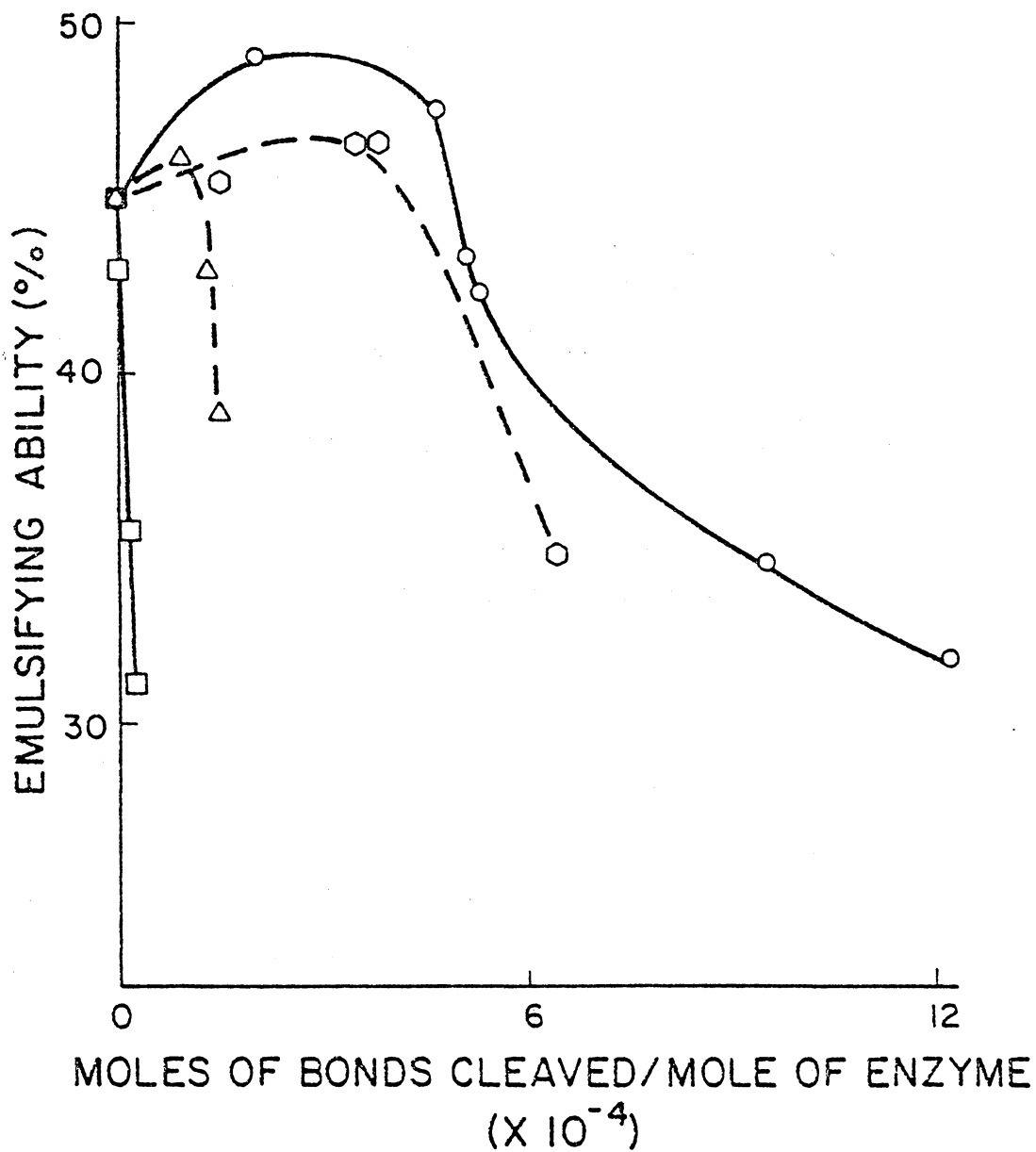
Fig. 18. Effect of immobilized enzyme(s) on emulsifying ability of modified soybean proteins: trypsin (O———O); chymotrypsin (□———□); trypsin/chymotrypsin (1:1) (⬡— —⬡); trypsin/chymotrypsin (1:9) (△— —△).

better emulsification. Phillilps and Beuchat (1981) found that the emulsifying ability of whey proteins decreased as proteolysis continued, suggesting that there was an optimum mean molecular size of the whey proteins contributing to emulsification. Enzymatic hydrolysis of proteins destroyed the emulsifying capacity of the peanut protein (Beauchat et al., 1975).

Apparently, digestion substantially affected the surface activity of proteins and their ability to stabilize emulsions. Moreover, the excessive formation of carboxyl and amino groups after hydrolysis and breakage of peptide bonds might change the hydrophilic-hydrophobic balance, thus affecting emulsifying ability. Therefore, the extent to which emulsion ability of soybean proteins is affected depends on the proportion between enzyme and protein, system conditions, methods used to form the emulsion, and the origin of the protein.

4.36   Emulsion stability

Protein stabilized emulsions are often exposed to heat during food manufacturing and this determination simulates this exposure by measuring emulsion stability for 30 min at 80°C. The emulsion stability of soybean proteins modified by immobilized chymotrypsin and/ or immobilized trypsin are shown in Fig. 19. The high proportion of immobilized trypsin in the multi-enzyme system, or immobilized trypsin singly had no effect on emulsion stability of modified soybean proteins under limited hydrolysis, but caused sharp decreases in stability
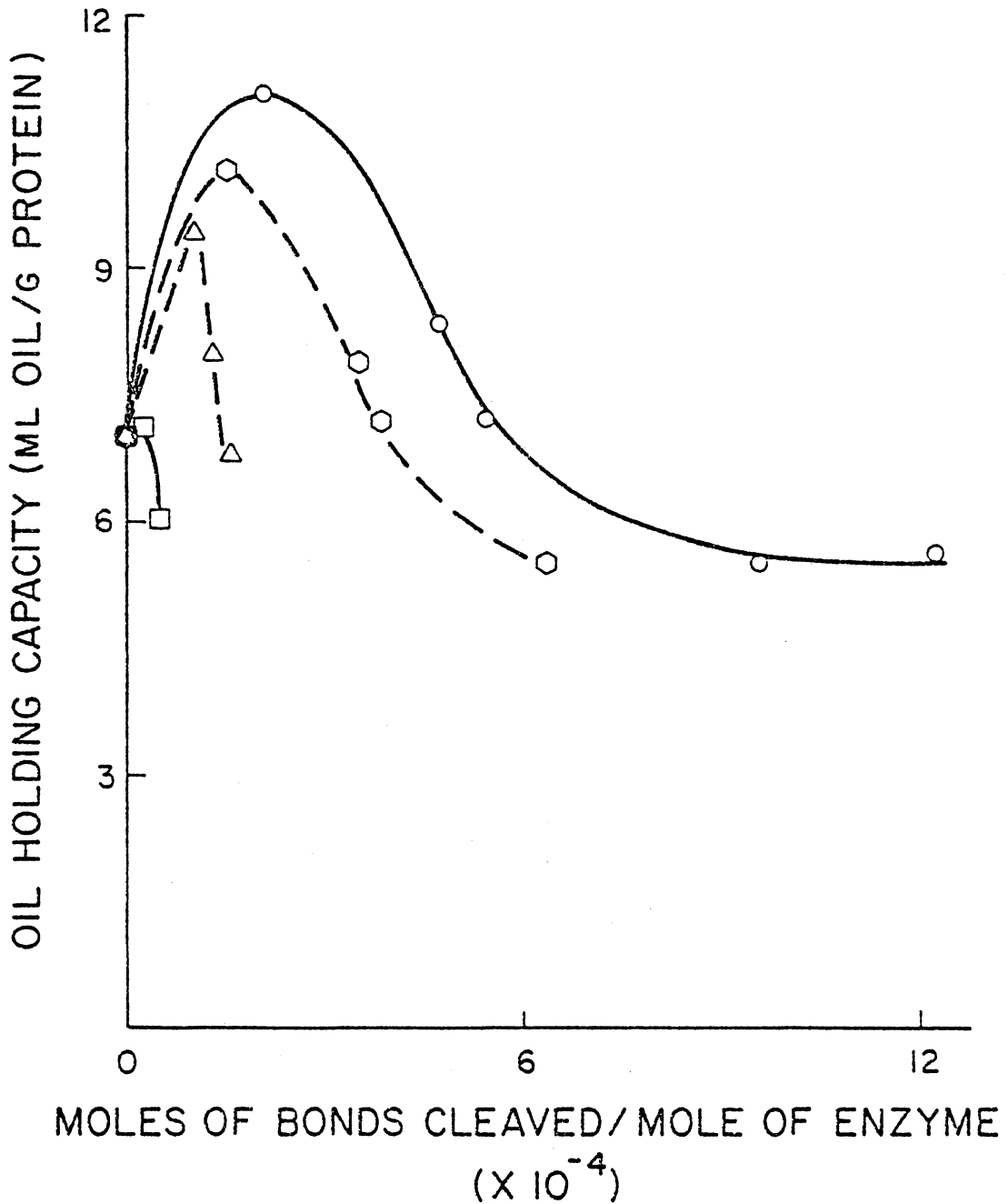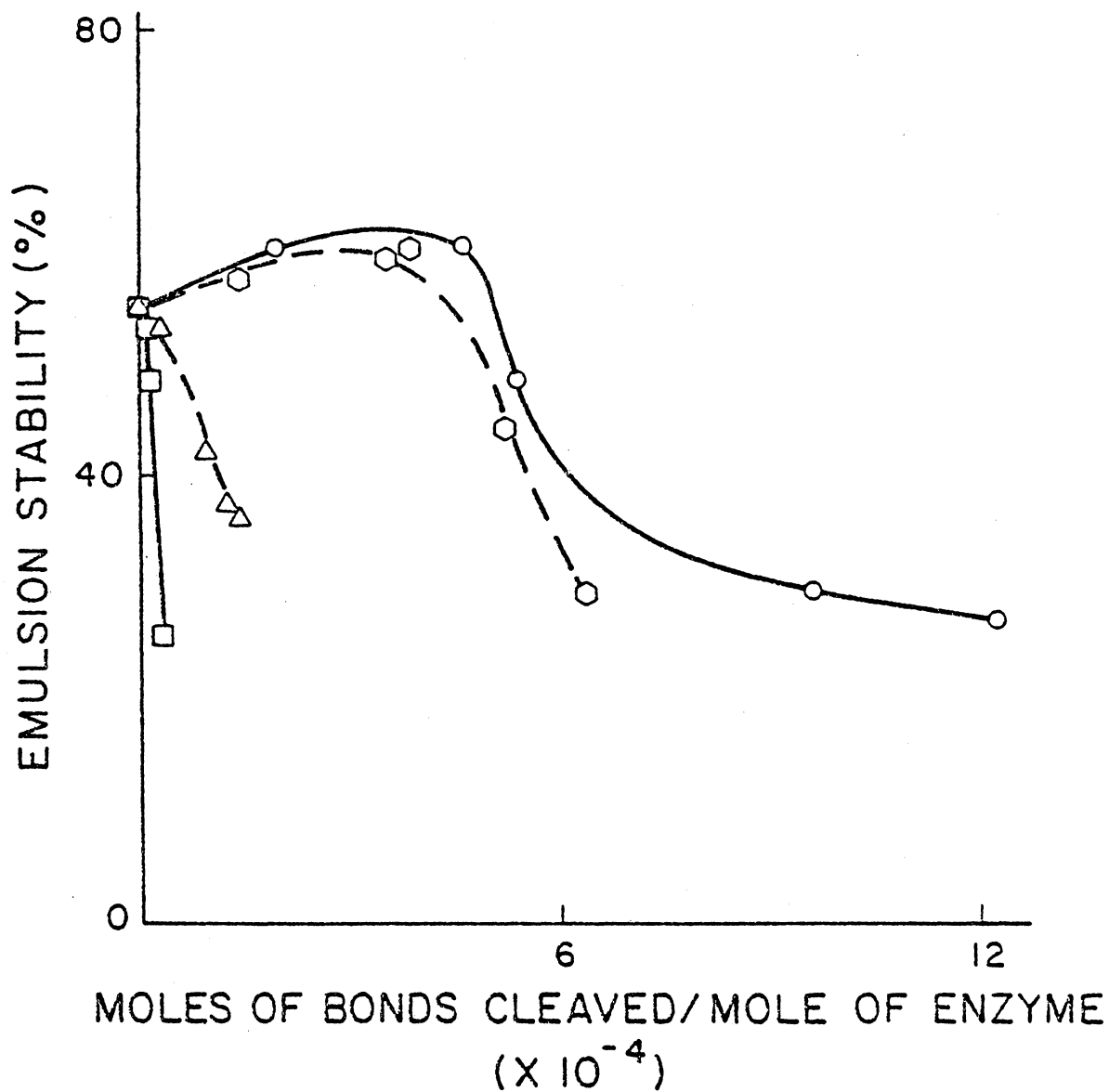
Fig. 19. Effect of immobilized enzyme(s) on emulsion stability of
modified soybean proteins: trypsin (O——O); chymo-
trypsin (□——□); trypsin/chymotrypsin (1:1) (⬡— —⬡);
trypsin/chymotrypsin (1:9) (△— —△).

beyond about 5.0×10^4 moles of bonds cleaved/mole of enzyme.
Chymotrypsin or trypsin/chymotrypsin (1:9) caused a sharp drop in
emulsion stability with only slight hydrolysis.

Puski (1975) demonstrated that the emulsion stability of soybean
protein isolate progressively hydrolyzed by soluble protease was lower
than those of less-hydrolyzed or non-hydrolyzed samples. This indicated
that since the peptides were smaller and less globular after hydrolysis,
they formed a thin protein layer around the oil droplets, reflecting an
emulsion with less stability. Therefore, the more globular compact
structures of proteins that were available at the oil/water interface,
the higher the emulsion stability of proteins that could be achieved.

4.37   Foaming ability

The data in Fig. 20 show that the presence of immobilized trypsin
in the multienzyme system, or of immobilized trypsin singly increases
foaming abilities. However, soybean proteins modified by immobilized
chymotrypsin had a decreased foaming capacity. The increased or
decreased foaming ability probably results from changes in molecule
to molecule interactions as hydrolysis proceeds. These changes could
involve exposure of hydrophobic groups, reduction in molecular size or
molecular charge, aggregation effects, etc. The degree of exposure of
hydrophobic groups together with reduction of molecular size and
molecular charge are no doubt important to achieving optimum foaming
capacity.
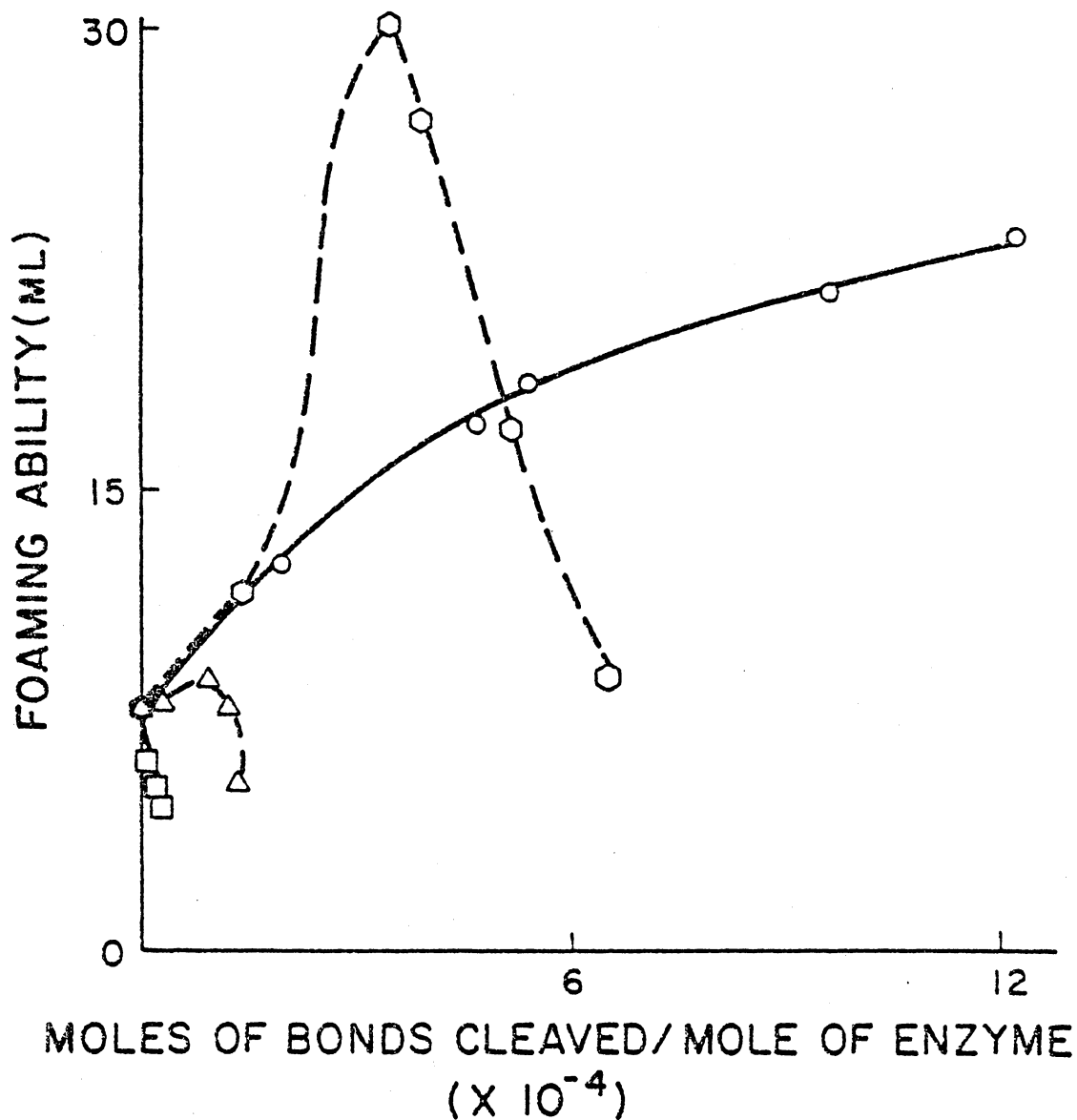
Fig. 20. Effect of immobilized enzyme(s) on foaming ability of
modified soybean proteins: trypsin (O——O); chymo-
trypsin (□———□); trypsin/chymotrypsin (1:1) (O— —O);
trypsin/chymotrypsin (1:9) (△— —△).

4.38   Foam stability

Foam stability of soybean proteins modified by immobilized chymotrypsin/trypsin and/or immobilized trypsin is shown in Fig. 21. Generally, soybean proteins hydrolyzed by immobilized protease(s) had lower foam stability than that of the control. The decrease in foam stability caused by hydrolysis might be due to the change of molecular structure. Evidently, unhydrolyzed soybean proteins have compact, globular structures which are conducive to form thick interfaces.

4.4   Available amino and lysyl groups of succinylated soybean proteins

As a charge modification, succinic anhydride was utilized to convert cationic amino groups of soybean proteins to anionic charged residues. Amino groups soluble in 0.07M phosphate buffer are reduced by treating them with increasing amounts of succinic anhydride, as in the case of both soybean protein isolate and 11S protein rich fraction (Table 5). Amino groups of 11S PRF were more reactive with succinic anhydride than was soybean protein isolate during a 2 hr experiment. Available lysyl groups of soybean protein isolate and 11S PRF fraction were lost equally. Therefore, succinylation removed alpha-amino and epsilon-amino groups.

In this experiment, the soybean protein isolate was hydrolyzed by immobilized trypsin/chymotrypsin (1:1) and succinylated; the ratios of succinic anhydride to protein were 0.1, 0.7, and 1.3, respectively.
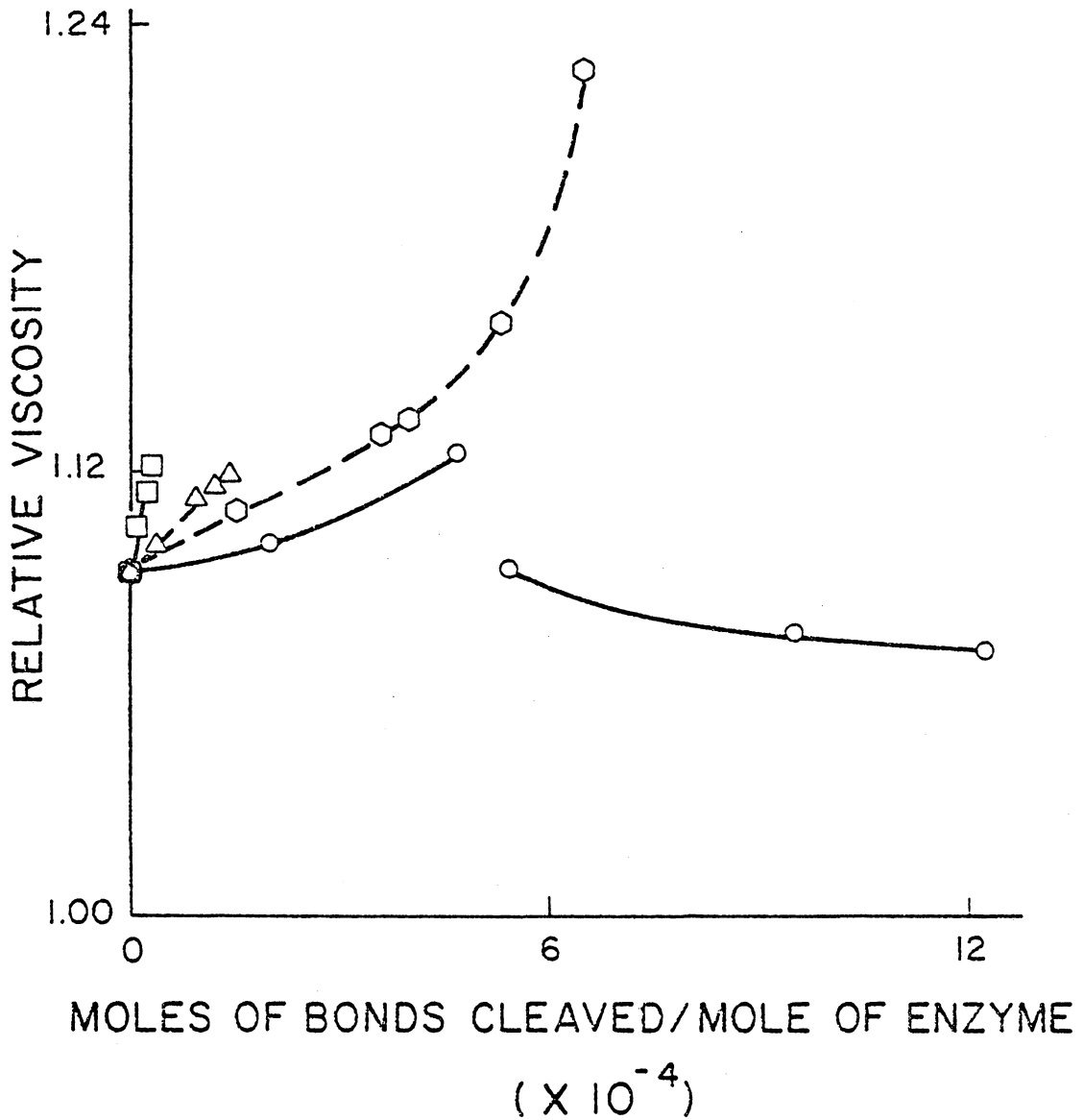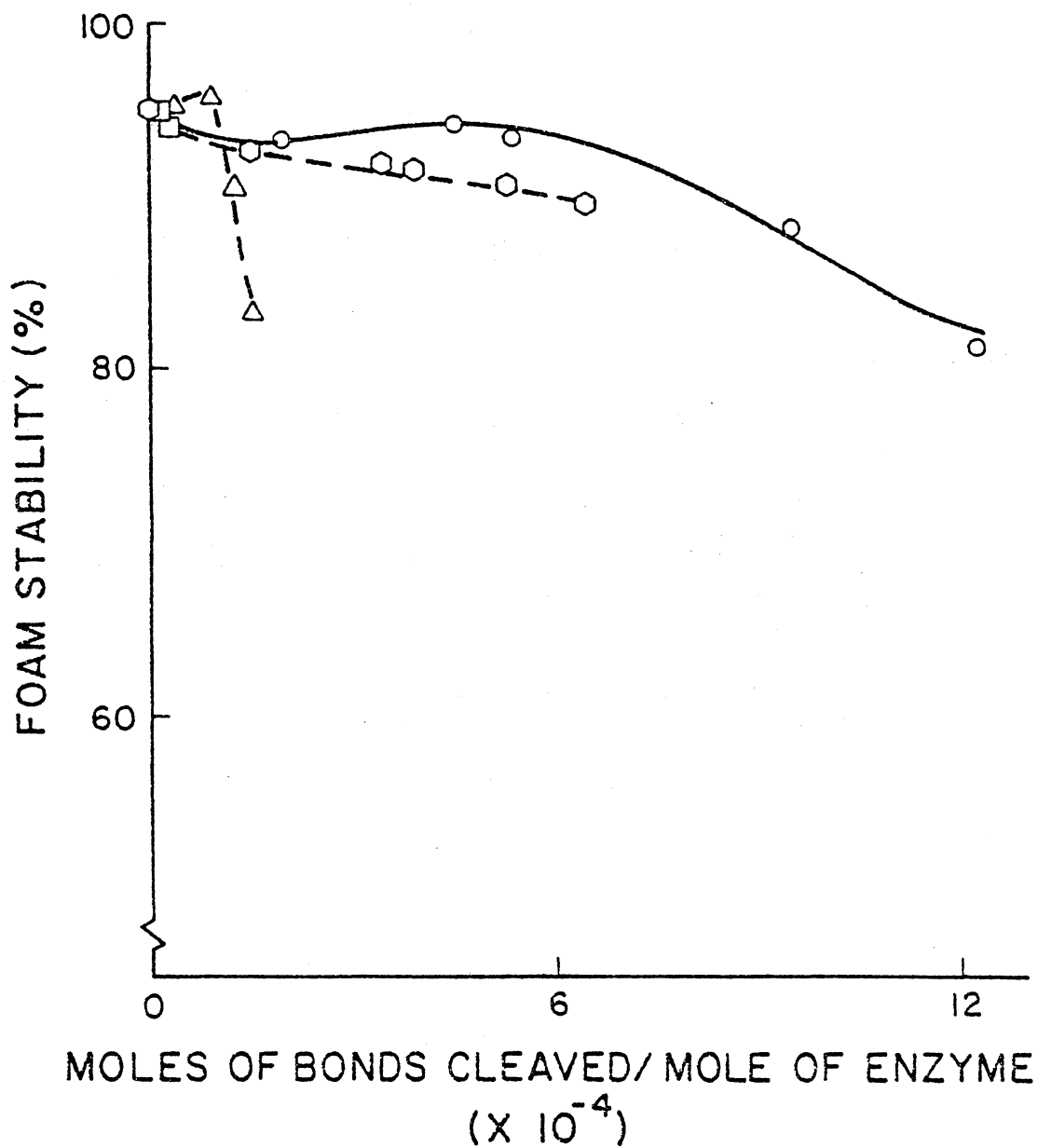
Fig. 21. Effect of immobilized enzyme(s) on foam stability of modified soybean proteins: trypsin (O———O); chymotrypsin (□———□); trypsin/chymotrypsin (1:1) (⬡——⬡); trypsin/chymotrypsin (1:9) (△— —△).

Table 5. Effect of succinylation on available amino and lysyl
groups of soybean proteins.

Succinylation (succinic anhydride:protein)	Soybean protein isolate		11S protein rich fraction	
	Amino group	Lysyl group (g/100g protein)	Amino group (mM)	Lysyl group (g/100 g protein)
0:00	2.81	4.60	3.06	4.30
1:10	1.81	4.31	0.96	4.02
4:10	1.05	4.09	0.93	4.00
7:10	0.98	4.07	0.90	3.64
10:10	0.95	3.27	0.83	3.47
13:10	0.93	1.85	0.80	2.90

Then, the available amino and lysyl groups of the modified proteins were measured (Table 6). Available amino groups of soybean protein isolate were high when they were treated with succinic anhydride and then with immobilized trypsin/chymotrypsin. This was due to the formation of alpha-amino groups after hydrolysis at the second stage. Modified soybean proteins having a low amount of succinic anhydride maintained a high number of available amino groups regardless of the order in which hydrolysis and succinylation were done. However, the reduction of available lysyl groups of soybean proteins treated by hydrolysis and then by succinylation was similar to that obtained by succinylation and then hydrolysis. This result showed that the order in which hydrolysis and succinylation were done led to forming different available amino groups of soybean proteins. As soybean protein isolate contains nucleic acid, trinitrobenzene sulfonic acid can react with guanidine and ribose moieties of nucleic acid; therefore, the absolute numbers of available amino and lysyl groups of protein must be critically evaluated (Puski, 1975).

4.5 Effect of fractionation and hydrolysis on the functional properties of modified soybean proteins

The average molecular size of soybean proteins can be altered by partial fractionation and hydrolysis with immobilized trypsin and/or immobilized chymotrypsin. The solubility, water holding capacity, relative viscosity, emulsifying ability, emulsion stability, foaming

Table 6. Effect of hydrolysis and succinylation on available amino and lysyl groups of soybean protein isolate.

Treatment		Amino group (mM)	Lysyl group (g/100 g protein)
First	Second		
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (1:10)	0.68	3.62
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (7:10)	0.50	2.45
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (13:10)	0.49	2.25
Succinic anhydride /protein (1:10)	Trypsin/chymo-trypsin (1:1)	1.36	3.30
Succinic anhydride /protein (7:10)	Trypsin/chymo-trypsin (1:1)	1.08	2.77
Succinic anhydride /protein (13:10)	Trypsin/chymo-trypsin (1:1)	1.05	2.63

ability and foam stability of modified soybean proteins are shown in
Tables 7 and 8. Basically there was no difference in solubility of
proteins (amino groups) in 0.07M phosphate buffer (pH 6.5) between
fractions. However, an increase in the amino groups of soybean proteins
hydrolyzed by the immobilized trypsin and/or immobilized chymotrypsin
for 20 hr was observed. During the hydrolysis on soybean protein
isolate, 6.3 x 10^4 moles of alpha-amino groups per mole of enzyme were
formed. The high proportion of immobilized trypsin in the multi-enzyme
system or in the immobilized trypsin singly remarkably enhanced the
solubility of amino groups. Therefore, it is desirable to utilize
immobilized trypsin singly or a high proportion of immobilized trypsin
to immobilized chymotrypsin on nylon pellets to obtain a high solubility
of proteins in acid foods. Data showing the high solubility of 7S PRF
in comparison with that of 11S PRF is supported by Aoki et al. (1980).

The 11S PRF had a high water holding capacity, while 7S PRF
absorbed lower amounts of water. Hydrolysis of 7S and 11S PRF by
immobilized enzyme(s) reduced water holding capacity. This might be
due to the excess formation of hydrophilic groups and to the weakened
physical entrapment property of the modified proteins (Fennema, 1977;
Hansen, 1978a; Chou and Morr, 1979).

The 7S PRF and cold insoluble fractions of soybean proteins
showed high oil holding capacity. Nevertheless, hydrolysis of soybean
proteins by immobilized enzyme(s) reduced their oil holding capacity
because of the reduced entrapment of oil in the protein matrix.

Table 7. Effect of fractionation and hydrolysis on the functional properties of soybean proteins[a].

Treatment		Solubility (mM of NH_2)	Water holding capacity (ml H_2O/g protein)	Oil holding capacity (ml oil/g protein)	Relative viscosity[b]
Fraction	Hydrolysis				
7S PRF[c]		3.40 ± 0.68	9.01 ± 0.86	9.8 ± 0.3	1.05 ± 0.02
7S PRF	Trypsin/chymo-trypsin (1:1)	10.48 ± 0.51	8.64 ± 0.47	6.5 ± 0.4	1.05 ± 0.02
CIF[d]		3.55 ± 0.67	9.81 ± 0.85	9.7 ± 0.3	1.09 ± 0.01
11S PRF		3.06 ± 0.45	11.04 ± 0.45	8.9 ± 0.3	1.09 ± 0.01
11S PRF	Chymotrypsin	4.09 ± 0.51	10.08 ± 0.76	8.4 ± 0.4	1.14 ± 0.01
11S PRF	Trypsin/chymo-trypsin (1:9)	5.09 ± 0.46	11.02 ± 0.43	8.6 ± 0.5	1.08 ± 0.01
11S PRF	Trypsin/chymo-trypsin (1:1)	11.55 ± 0.35	10.20 ± 0.38	5.5 ± 0.4	1.09 ± 0.01
11S PRF	Trypsin	16.43 ± 0.43	9.27 ± 0.42	5.3 ± 0.4	1.10 ± 0.01

[a] Average of 4 determinations ± standard deviation.

[b] Relative to water.

[c] PRF = Protein rich fraction.

[d] CIF = cold insoluble fraction.

Table 8. Effect of fractionation and hydrolysis on the functional properties of soybean proteins[a].

Treatment		Emulsion		Foam	
Fraction	Hydrolysis	Ability (%)	Stability (%)	Ability (ml)	Stability (%)
7S PRF[b]		45.2 ± 4.3	86.7 ± 3.6	4.6 ± 1.9	91.7 ± 4.1
7S PRF[b]	Trypsin/chymo-trypsin (1:1)	18.9 ± 2.5	66.6 ± 6.0	8.7 ± 1.3	74.4 ± 3.0
CIF[c]		51.2 ± 2.5	94.3 ± 4.1	9.5 ± 2.5	91.6 ± 3.0
11S PRF		37.5 ± 3.5	86.7 ± 4.7	5.0 ± 1.6	86.4 ± 4.2
11S PRF	Chymotrypsin	30.0 ± 4.1	82.3 ± 4.2	7.1 ± 1.7	88.6 ± 3.3
11S PRF	Trypsin/chymo-trypsin (1:9)	14.4 ± 2.3	77.5 ± 3.7	4.0 ± 1.5	92.5 ± 3.7
11S PFR	Trypsin/chymo-trypsin (1:1)	11.2 ± 2.1	31.2 ± 5.1	4.5 ± 1.6	70.8 ± 4.3
11S PRF	Trypsin	8.7 ± 1.8	33.3 ± 3.1	11.0 ± 2.1	87.6 ± 4.8

[a] Average of 4 determinations ± standard deviation.

[b] PRF = protein rich fraction.

[c] CIF = cold insoluble fraction.

Among the partially purified fractions, the cold insoluble and 11S
PRF fractions had higher values of relative viscosity as compared to
water. Immobilized chymotrypsin caused increased relative viscosity of
the 11S PRF fraction.

The highest emulsifying ability values among the partially
purified fractions were obtained by the cold insoluble fraction, as
shown in Table 8. A comparison of Table 7 with Table 8 calls attention
to the fact that the emulsifying ability of partially purified fractions
correlates with their solubility. It was suggested that alfalfa proteins
soluble in pH 5 buffer had a higher emulsifying capacity than those
solubilized above or below this pH value (Wang and Kinsella, 1976).
McWatters and Holmes (1979a) indicated that the high levels of nitrogen
solubility were not necessarily associated with the maximum emulsifying
capacity. After hydrolysis of soybean proteins by immobilized trypsin
and/or immobilized chymotrypsin for 20 hrs, the modified soybean proteins
had a low emulsifying capacity. This suggested that excessive hydrolysis
formed small protein molecules with the unbalanced hydrophilic-lipophilic
groups. Further molecular size and charge studies of proteins are
needed to explain this apparent discrepancy.

The cold insoluble fraction had high emulsion stability. After
being hydrolyzed by the immobilized enzyme(s), the 7S PRF and 11S PRF
fractions had low emulsion stability. Particularly, the immobilized
trypsin/chymotrypsin lowered the emulsion stability of the 11S PRF
fraction more than did the immobilized trypsin. This might be attri-

buted to the smaller and less globular structure of proteins which
formed a thinner interface between oil and water.

The foaming ability of the modified soybean proteins depends upon
the kind of treatments with immobilized enzyme(s) as indicated in Table
8. The cold insoluble fraction had high foaming ability. Fractions such
as 7S PRF and 11S PRF, after treatment with immobilized trypsin/
chymotrypsin (1:1) or immobilized trypsin singly had higher foaming
ability than the non-hydrolyzed fractions. Treatment of the 11S PRF
with immobilized chymotrypsin increased foaming ability. However, the
multi-enzyme systems with the immobilized trypsin/chymotrypsin decreased
the foaming ability.

The cold insoluble fraction had a high foam stability. After
hydrolysis of soybean proteins by immobilized protease(s), the 7S PRF
and 11S PRF showed low foam stability. Nevertheless, the immobilized
trypsin/chymotrypsin (1:9) enhanced the foam stability of the 11S PRF.
Hence, heterogeneous components of modified soybean proteins showed
different surface property values. This indicates the need for evaluat-
ing the molecular sizes and charges of soybean proteins.

4.6   Effect of fractionation and succinylation on the functional
      properties of modified soybean proteins

Soybean proteins were partially purified and succinylated for the
purpose of further changing molecular sizes and molecular charges.
Tables 9 and 10 show the functional properties of modified soybean

Table 9. Effect of fractionation and succinylation on the functional properties of modified soybean proteins[a].

Treatment		Solubility (mM of NH_2)	Water holding capacity	Oil holding capacity	Relative viscosity[b]
Fraction	Succinic anhydride:protein		(ml H_2O/g protein)	(mg oil/g protein)	
11S PRF[c]	1:10	0.93 ± 0.12	9.01 ± 0.23	9.0 ± 0.3	1.14 ± 0.01
11S PRF	7:10	0.61 ± 0.09	1.27 ± 0.18	9.2 ± 0.4	1.16 ± 0.01
11S PRF	13:10	0.54 ± 0.10	1.33 ± 0.25	10.6 ± 0.4	1.19 ± 0.02
S.P.I.[d]	1:10	2.25 ± 0.38	4.39 ± 0.43	9.0 ± 0.3	1.09 ± 0.01
S.P.I.	7:10	0.59 ± 0.08	3.22 ± 0.32	10.4 ± 0.5	1.30 ± 0.02
S.P.I.	13:10	0.46 ±·0.08	3.17 ± 0.55	9.6 ± 0.2	1.31 ± 0.01

[a] Average of 4 determinations ± standard deviation.

[b] Relative to water.

[c] PRF = protein rich fraction.

[d] soybean protein isolate.

Table 10. Effects of fractionation and succinylation on the functional properties of modified soybean proteins[a].

Treatment		Emulsion		Foam	
Fraction	Succinic anhy-dride:protein	Ability (%)	Stability (%)	Ability (%)	Stability (%)
11S PRF[b]	1:10	8.7 ± 1.3	78.4 ± 4.3	25.6 ± 2.2	91.4 ± 3.5
11S PRF	7:10	14.4 ± 1.4	75.6 ± 4.7	14.2 ± 1.5	91.9 ± 2.8
11S PRF	13:10	21.2 ± 1.8	63.6 ± 4.1	10.1 ± 1.8	97.0 ± 2.3
S.P.I.[c]	1:10	43:3 ± 4.1	38.2 ± 3.2	8.3 ± 1.2	90.3 ± 2.1
S.P.I.	7:10	42.1 ± 4.6	41.9 ± 3.7	16.4 ± 2.2	94.6 ± 2.6
S.P.I.	13:10	27.9 ± 3.9	53.2 ± 4.3	11.0 ± 1.6	90.9 ± 2.0

[a] Average of 4 determinations ± standard deviation.

[b] PRF = protein rich fraction.   [c] S.P.I. = soybean protein isolate.

proteins by fractionation and succinylation. The solubliity of soy-
bean proteins expressed as available amino groups was reduced linearly,
as the high amount of succinic anhydride was included in the reaction
medium. Obviously, the low value of available amino groups was due to
the binding of succinic anhydride. A decrease in water binding capacity
of 11S PRF and soybean protein isolate was observed upon succinylation.
However, the oil holding capacity of succinylated 11S PRF and soybean
protein isolate was high. This suggested that the mechanisms involved
in water holding capacity and oil holding capacity of soybean proteins
were not the same. The unfolded protein molecules and the exposed
hydrophobic residues would presumably enhance entrapment of oil. None-
theless, Canella et al. (1979) illustrated that the decreased water
absorption capacity of succinylated sunflower protein was ascribed to
a certain degree of polymerization in the modified sunflower proteins.

Relative viscosities of the succinylated soybean proteins were
increased significantly. Succinylation may cause unfolding and
exapnsion of the globular structures of protein molecules, resulting in
higher intrinsic viscosity because of the expanded molecular specific
volume (Konings et al., 1969).

The emulsifying ability and emulsion stability of the succinylated
11S PRF and soybean protein isolate are given in Table 10. The
emulsifying capacity of the succinylated 11S PRF increased with increas-
ing amount of succinic anhydride used, whereas the emulsifying ability
of the succinylated soybean protein isolate decreased. The emulsion

stability of succinylated 11S PRF decreased as 11S PRF was treated with high amounts of succinic anhydride. However, the emulsion stability of the succinylated soybean protein isolate increased. With increasing amounts of succinic anhydride, the foaming ability of the succinylated 11S PRF was reduced, while the foam stability of the succinylated 11S PRF was increased. The foaming ability and stability of the succinylated soybean proteins were maximum when the soybean proteins were treated with the ratio, 0.7 of succinic anhydride to protein. Franzen and Kinsella (1967a) reported the improved foaming capacity and foam stability of the succinylated soybean protein isolate. Thus, various functional properties of succinylated soybean proteins depend on which of the fraction is succinylated, as well as the ratio of succinic anhydride to protein, indicating the importance of molecular sizes and molecular charges for the expression of functional properties.

4.7 Effect of hydrolysis and succinylation on the functional
     properties of modified soybean proteins

Succinylation and hydrolysis with immobilized trypsin/chymotrypsin were carried out to modify the molecular size and molecular charge of soybean proteins. The effects of the hydrolysis and the succinylation of the soybean proteins on functional properties are shown in Tables 11 and 12. Available amino groups of soybean protein succinylated and then hydrolyzed by immobilized trypsin/chymotrypsin were higher than those hydrolyzed by immobilized enzyme(s) and then succinylated. This

Table 11. Effect of hydrolysis and succinylation on the functional properties
of modified soybean proteins from soybean protein isolate[a].

Treatment		Solubility (mM of NH_2)	Water holding capacity (ml H_2O/g protein)	Oil holding capacity (ml oil/g protein)	Relative Viscosity[b]
First	Second				
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (1:10)	1.54 ± 0.25	1.87 ± 0.41	7.6 ± 0.2	1.12 ± 0.01
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (7:10)	0.74 ± 0.14	1.81 ± 0.37	8.5 ± 0.2	1.15 ± 0.02
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (13:10)	0.73 ± 0.17	1.75 ± 0.48	10.9 ± 0.3	1.20 ± 0.02
Succinic anhydride /protein (1:10)	Trypsin/chymo-trypsin (1:1)	4.14 ± 0.45	0.90 ± 0.25	8.8 ± 0.2	1.06 ± 0.01
Succinic anhydride /protein (7:10)	Trypsin/chymo-trypsin (1:1)	3.06 ± 0.43	0.75 ± 0.30	8.4 ± 0.2	1.05 ± 0.01
Succinic anhydride /protein (13:10)	Trypsin/chymo-trypsin (1:1)	3.04 ± 0.48	0.73 ± 0.35	8.3 ± 0.3	1.05 ± 0.02

[a] Average of 4 determinations ± standard deviation.   [b] Relative to water.

Table 12. Effects of hydrolysis and succinylation on the functional proper-
ties of modified soybean proteins from soybean protein isolate[a].

| Treatment | | Emulsion | | Foam | |
First	Second	Ability (%)	Stability (%)	Ability (%)	Stability (%)
Trypsin/chymo-trypsin (1:1)	Succinic anhydride /protein (1:10)	11.0 ± 1.7	81.8 ± 5.6	9.5 ± 2.7	90.7 ± 3.7
Trypsin/chymo-trypsin (1:1)	Succinic anydride	34.3 ± 2.3	32.6 ± 4.3	66.0 ± 2.8	92.4 ± 4.1
Trypsin/chymo-trypsin (1:1)	Succinic anhydride	38.5 ± 2.5	30.8 ± 3.2	53.5 ± 1.7	95.6 ± 4.3
Succinic anhydride /protein (1:10)	Trypsin/chymo-trypsin (1:1)	4.9 ± 0.8	99.2 ± 6.4	106.7 ± 5.1	47.1 ± 1.7
Succinic anhydride /protein (7:10)	Trypsin/chymo-trypsin (1:1)	9.6 ± 1.6	70.6 ± 5.0	103.4 ± 4.3	42.2 ± 1.5
Succinic anhydride	Trypsin/chymo-	11.0 ± 1.8	26.1 ± 4.6	45.0 ± 1.5	40.8 ± 2.0

[a] Average of 4 determinations ± standard deviation.

suggested that hydrolysis released the available amino groups of the soybean proteins, especially during the second stage hydrolysis. Some of the available amino groups of the soybean protein still remained even after the second succinylation treatment.

Soybean proteins modified by immobilized proteases and succinylated had higher water holding capacity and relative viscosity than those that were succinylated and then hydrolyzed by immobilized proteases. There was little difference in oil holding capacity between the two treatments. Therefore, the molecular expansion of the hydrolyzed soybean protein isolate enhanced water holding capacity and relative viscosity.

Emulsifying ability and foam stability of the modified soybean protein isolate were high when treated by immobilized enzymes and then succinylation. However, maximum emulsion stability and foaming ability of the modified soybean proteins were observed when the soybean proteins were treated with a ratio of 0.1 succinic anhydride to protein and then hydrolysis by immobilized trypsin/chymotrypsin. Nonetheless, Groninger and Miller (1975) reported that a product prepared by the succinylation (66% level) of fish protein mildly hydrolyzed by soluble bromelain (enzyme:protein, 1:165) had essentially similar aeration properties as a product prepared by the mild hydrolytic treatment of the succinylated protein. Furthermore, Miller and Groninger (1976) modified fish proteins by various degrees of succinylation and then by two degrees of hydrolysis with soluble bromelain. They found that the fish protein modified by the moderate succinylation and then by hydrolysis showed high

emulsifying capacity, water and fat absorption, as well as gelatin, while the proteins modified by high succinylation and then hydrolysis indicated high aeration (foaming) capacity. Thus, the order of the treatments consisting of immobilized trypsin/chymotrypsin and then succinylation differently affected the molecular size and molecular charge as well as the various functional properties of the soybean proteins.

4.8   Evaluation of molecular sizes and molecular charges

4.81   Evaluation of molecular sizes by SDS-PAGE

4.811   Partially purified fractions

Figs. 23 and 24 show sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) patterns of partially purified fractions. The major protein bands of soybean proteins were made up of three components with molecular weights of 87,000, 28,000, and 13,000 as shown by gel A peaks in Fig. 29. The fraction, 7S PRF shown in Fig. 24, developed bands indicating a molecular weight of 87,000. Together, the cold insoluble fraction and the 11S PRF distinctly showed high peaks with molecular weights of 28,000 and 13,000. This is due to the cold insoluble fraction (Eldridge and Wolf, 1967) and the 11S PRF (Saio et al., 1973) having 65.0% and 61.8% of 11S fraction components, respectively. Consequently, the two bands with molecular weights of 28,000
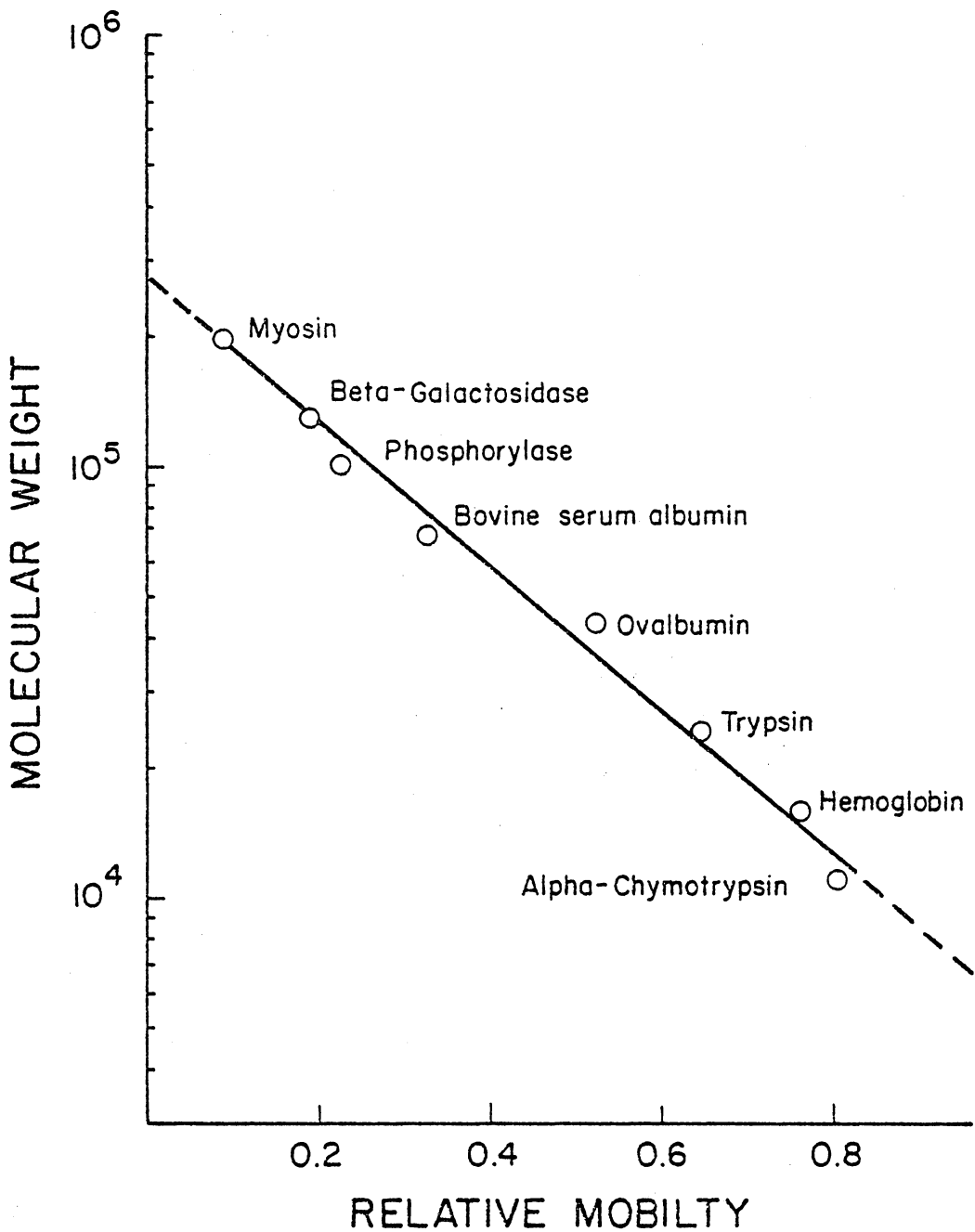
Fig. 22. Comparison of the molecular weights of the standard
marker proteins in 7.5% gels: myosin (200,000); beta-
galactosidase (130,000); phosphorylase (100,000);
bovine serum albumin (68,000); ovalbumin (43,000); tryp-
sin (23,000); hemoglobin (15,000); chymotrypsin (11,000).
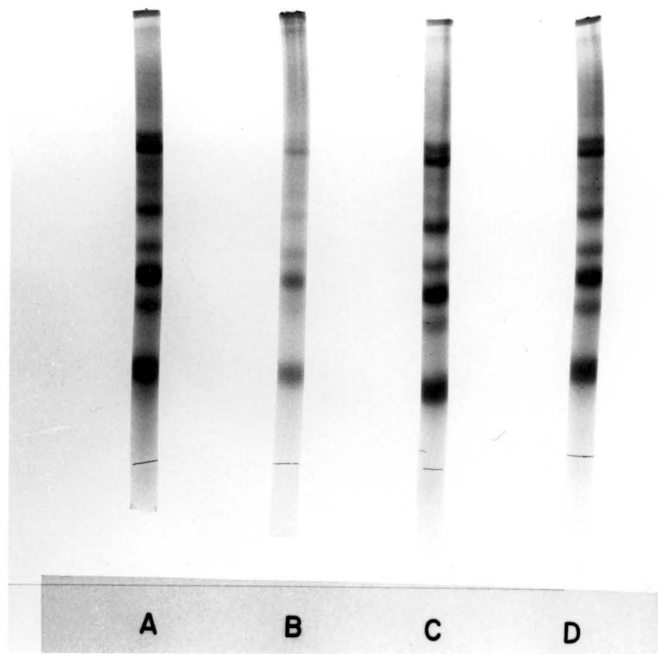
Fig. 23. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis disc patterns of partially purified fractions: (A) 7S protein rich fraction; (B) 11S protein rich fraction; (C) cold insoluble fraction; (D) soybean protein isolate (control).

Fig. 24. Densitometer tracings of sodium dodecyl sulfate (SDS) polyacrylamide gel electro-phoretic patterns of soybean proteins soluble in SDS and 2-mercaptoethanol: (A) 7S protein rich fraction; (B) cold insoluble fraction; (C) soybean protein isolate; (D) 11S protein rich fraction.

and 13,000 were parts of 11S components which were presumed to be
acidic and basic subunits of the 11S fraction of soybean proteins.
Iyengar and Ravestein (1981) concluded that the molecular weights of
acidic and basic subunits of the 11S soybean protein fraction had molec-
ular weights ranging from 28,000 to 42,000 and from 17,000 to 20,000,
respectively. The mobility of the commercially available soybean
proteins showed that the molecular weights of 11S components depended
on the source of isolates, indicating the effects of different process-
ing conditions, such as heat, on the mobility (Peng et al., 1982).

4.812   Succinylated soybean proteins

The SDS electrophoretic patterns of succinylated 11S PRF and
soybean protein isolate, as well as the combined treatments of succinyl-
ation and hydrolysis by the immobilized trypsin/chymotrypsin, are
shown in Figs. 25 and 26. Succinylation converts amino groups of
proteins to anionic groups. Moreover, sodium dodecyl sulfate confers
anionic charges on the proteins. Therefore, succinylation and SDS
completely remove charge differences of proteins, resulting in complete
separation of 11S components into subunits and possibly also molecular
expansion (increase in specific molecular volume via unfolding) by
disrupting electrostatic interactions between the charged residues of
the proteins. For example, by comparing the electrograms in gel B,
Fig. 26 and gel A, Fig 29, it can be suggested that the bands of com-
ponents with molecular weights of 22,000 and 28,000 were converted via
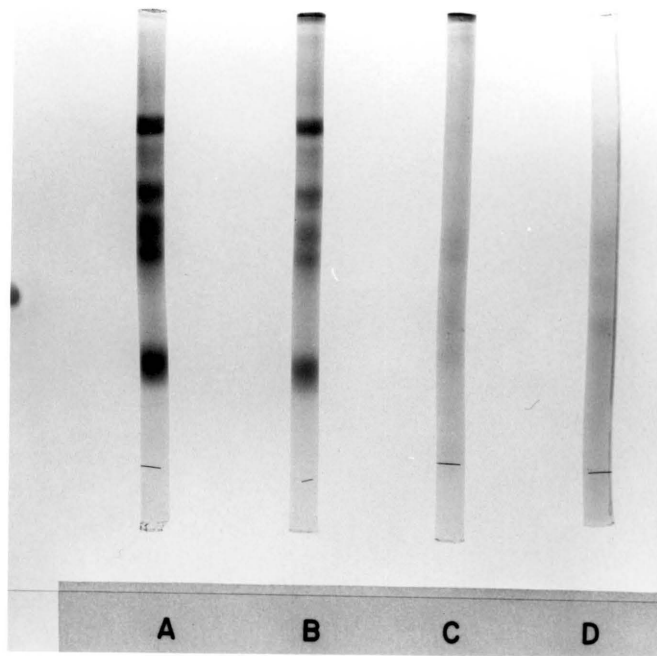
Fig. 25. Sodium dodecyl sulfate-polyacrylamde gel electrophoresis
disc patterns of succinylated soybean proteins: (A)
succinylated 11S protein rich fraction; (B) succinylated
soybean protein isolate; (C) hydrolyzed and succinylated
soybean protein isolate; (D) succinylated and hydrolyzed
soybean protein isolate.

Fig. 26. Densitometer tracings of sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of succinylated soybean proteins: (A) 11S protein rich fraction; (B) soybean protein isolate; (C) hydrolyzed and succinylated isolate; (D) succinylated and hydrolyzed isolate. Numbers refer to molecular weights (x 1/1,000).

succinylation into components with mobilities corresponding to molecular

weights of 35,000 and 43,000, respectively. Fig. 27 shows that

succinylation results in high molecular expansion (increases in

apparent molecular weight) of all the major soybean proteins. Overall,

this indicates that succinylation does cause increases in specific

molecular volume, as shown by the reduced mobility and high apparent

molecular weight.

Hydrolysis by immobilized trypsin/chymotrypsin followed by

succinylation reduced peak heights of all the components of soybean

proteins with the remaining proteins having an average molecular weight

of 75,000. Succinylation followed by hydrolysis with the immobilized

proteases also removed most of the major and minor components of the

soybean protein isolate, but the average molecular weight was reduced

to 37,000 in this case. This lower average molecular weight in the

second case could be explained if succinylation results in molecular

expansion via unfolding and enhances the splitting of peptide bonds

by hydrolysis.

4.813   Soybean protein isolate modified by hydrolysis with immo-

bilized protease(s)

The electrophoretic patterns of soybean proteins hydrolyzed by

immobilized protease(s) are shown in Fig. 28. The densitometric

tracings of the soybean proteins hydrolyzed by the immobilized chymo-

trypsin showed a low degree of hydrolysis, but reduction of each peak
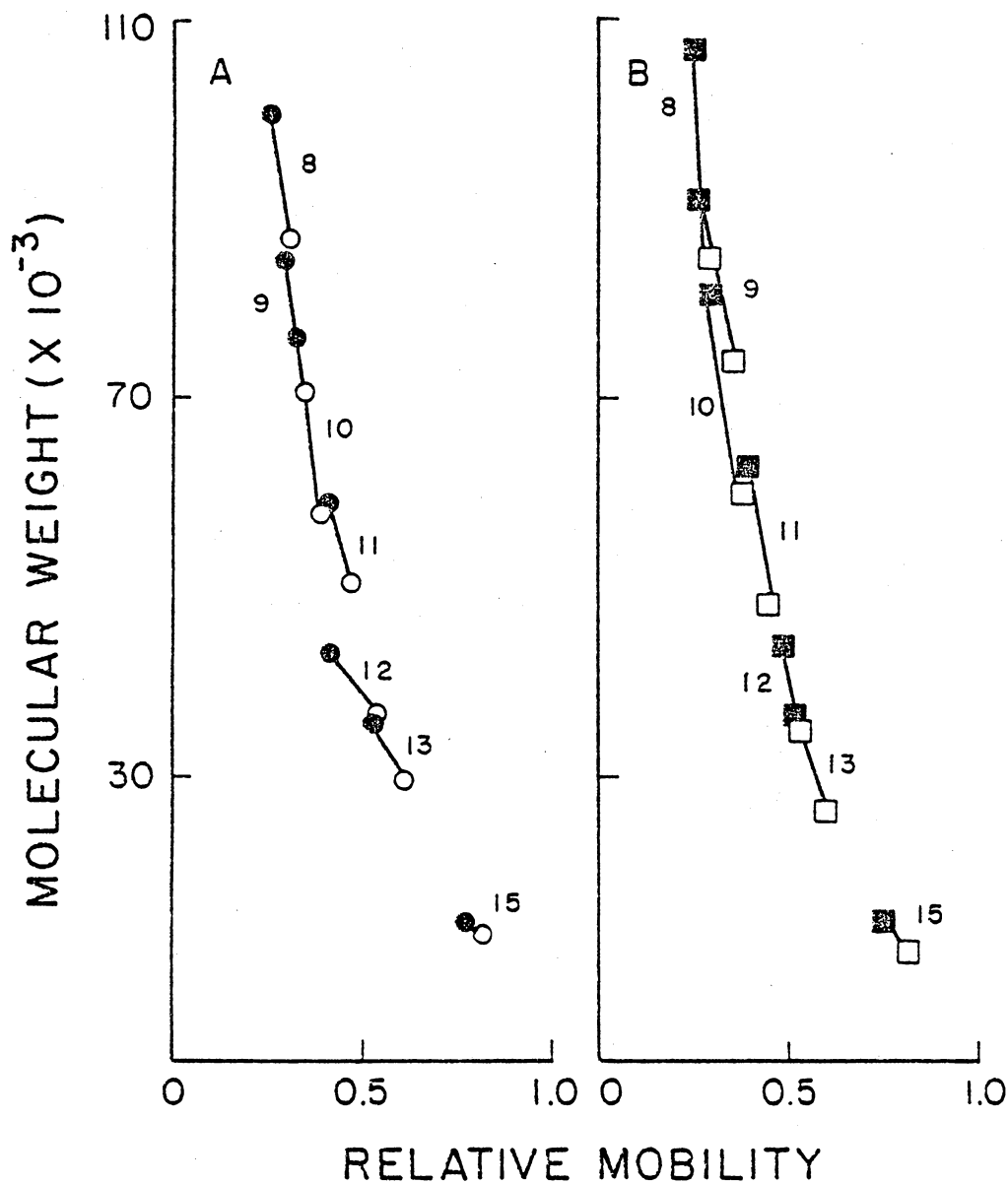
Fig. 27. Molecular expansion of each band of 11S protein rich
fraction of soybean proteins (A) and soybean protein
isolate (B) after succinylation: untreated (○,□);
treated (●,▨). Numbers refer to bands from the
cathode.
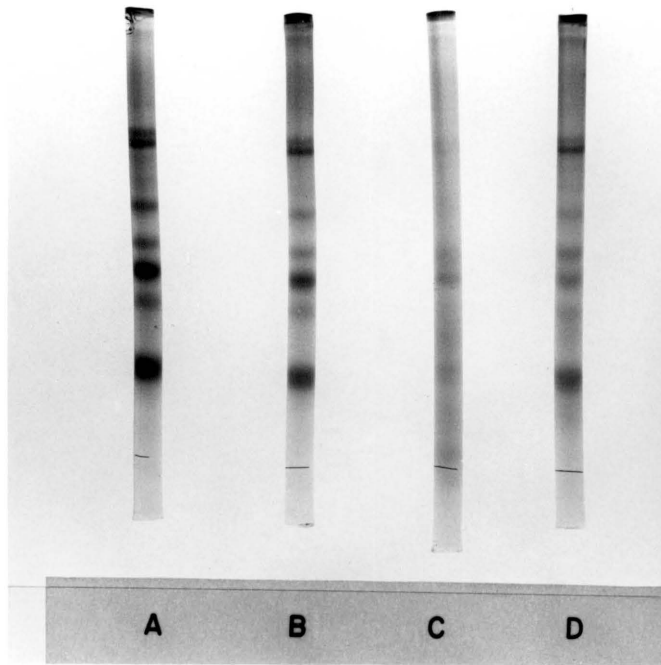
Fig. 28. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
disc patterns of soybean protein isolate hydrolyzed by
immobilized protease(s): (A) control; (B) chymotrypsin;
(C) trypsin; (D) trypsin/chymotrypsin (1:9).

height and width after hydrolysis (Fig. 29, gel B). This indicates

that the immobilzed chymotrypsin hydrolyzed all bands at the same rate.

The low degree of hydrolysis of soybean protein isolate by immobilized

chymotrypsin was attributed to the preferred cleavage sites buried in

the hydrophobic interior of the protien molecules, making them less

accessible to the enzyme (Kimball et al., 1981). However, immobilized

trypsin-chymotrypsin mixtures selectively reduced the peak of the

acidic subunit (Fig. 29, gel C).

Hydrolysis with immobilized trypsin for 20 hr removed all

components of soybean protein isolate except for the residues of the

acidic subunit. Romero and Ryan (1978) found a number of relatively

large trypsin-resistant peptides in the G_1 fraction from the bean,

Phaseolus vulgaris. They showed that the conformation of the polypep-

tides might be a factor affecting the rate of hydrolysis after heat

treatment.

4.814   Soybean protein fractions modified by hydrolysis with

        immobilized trypsin/chymotrypsin

The SDS-electrophoresis patterns shown in Figs. 30 and 31

demonstrate the different molecular weight distribution of the soybean

proteins modified by the immobilized trypsin/chymotrypsin (1:1).

When partially hydrolyzing with immobilized trypsin/chymotrypsin, the

acidic subunits with molecular weight of 28,000 were hydrolyzed to a
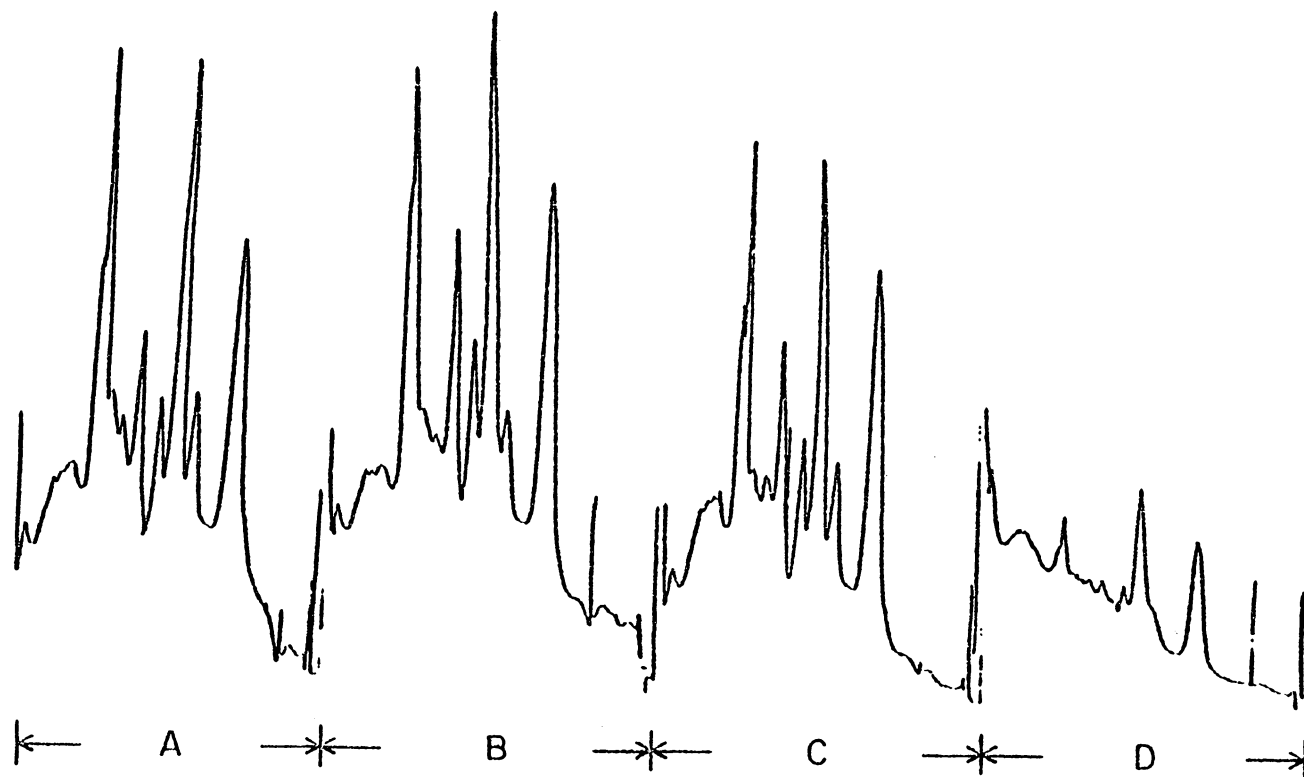
larger extent than the basic subunits, as shown in gel A, Fig. 31.
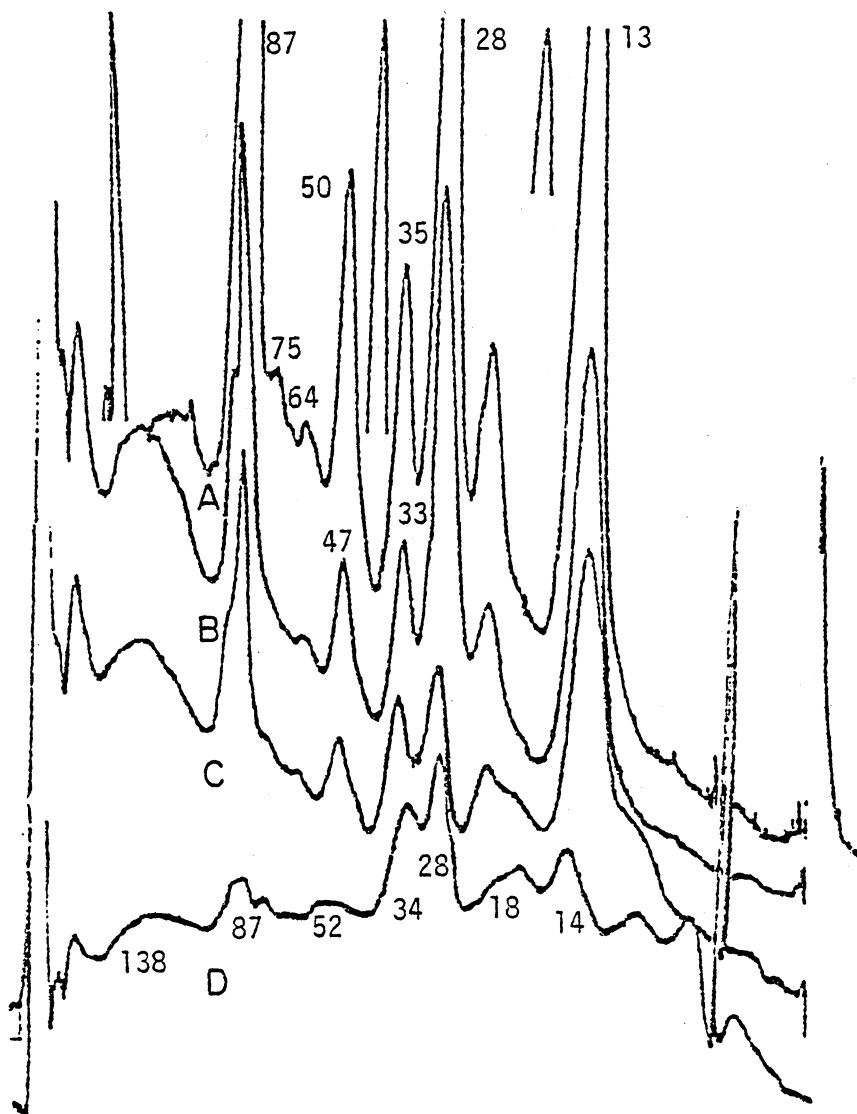
Fig. 29. Densitometer tracings of sodium dodecyl sulfate-polyacrylamide gel electrophoretic patterns of soybean protein isolate modified by immobilized protease(s): (A) control; (B) chymotrypsin; (C) trypsin/chymotrypsin (1:9); (D) trypsin. Numbers refer to molecular weights (x 1/1,000).
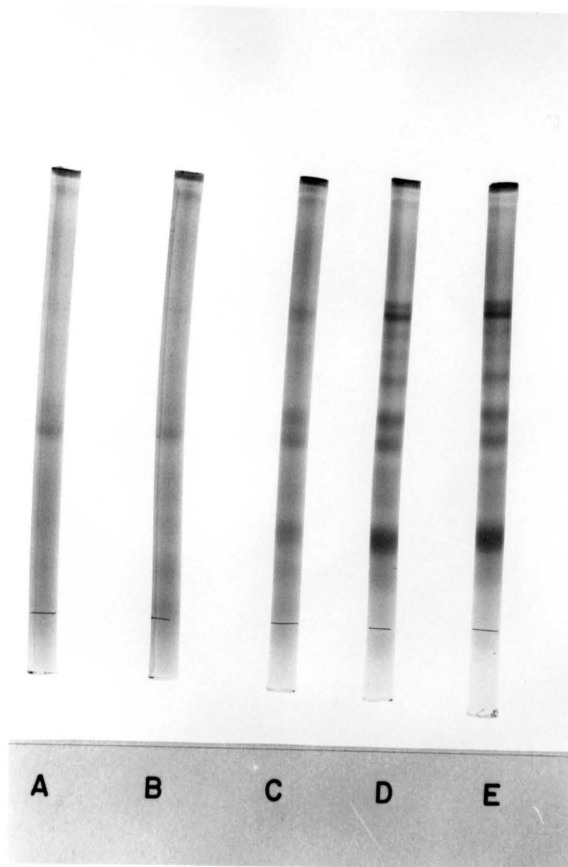
Fig. 30. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis disc patterns of soybean proteins hydrolyzed by immobilized trypsin/chymotrypsin (1:1): (A) 7S protein rich fraction; (B) 11S protein rich fraction; (C) soybean protein isolate; (D) and (E) partially hydrolyzed soybean protein isolate.
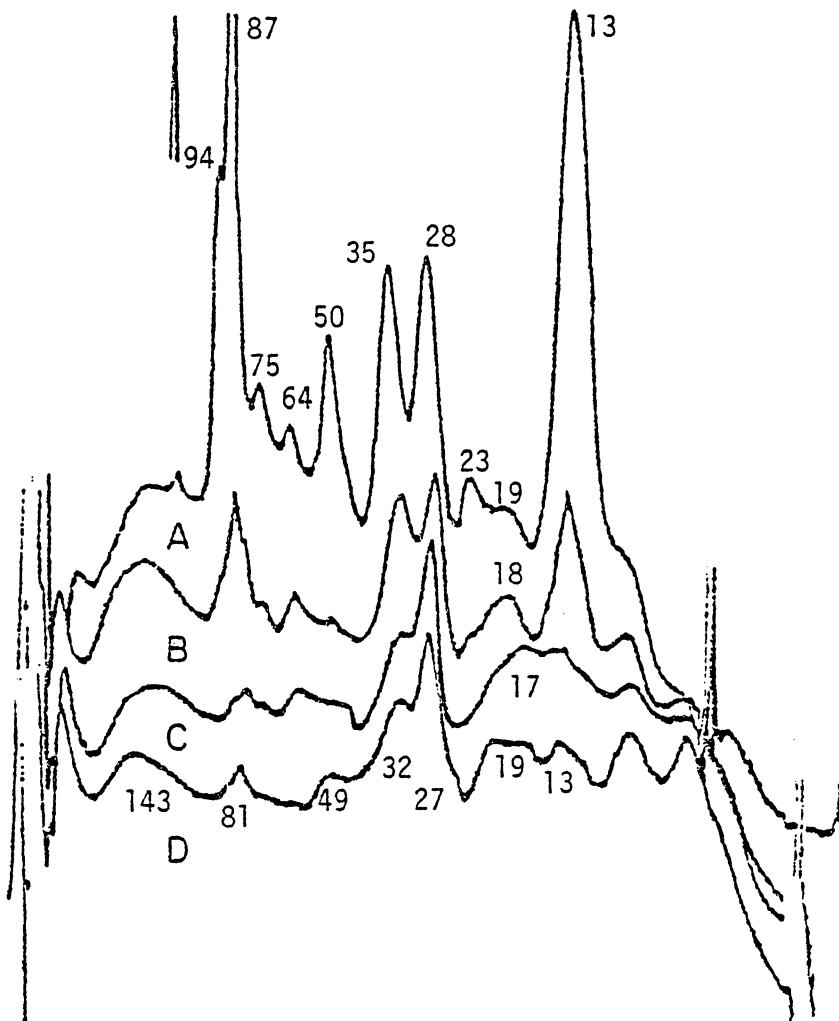
Fig. 31. Densitometer tracings of sodium dodecyl sulfate-
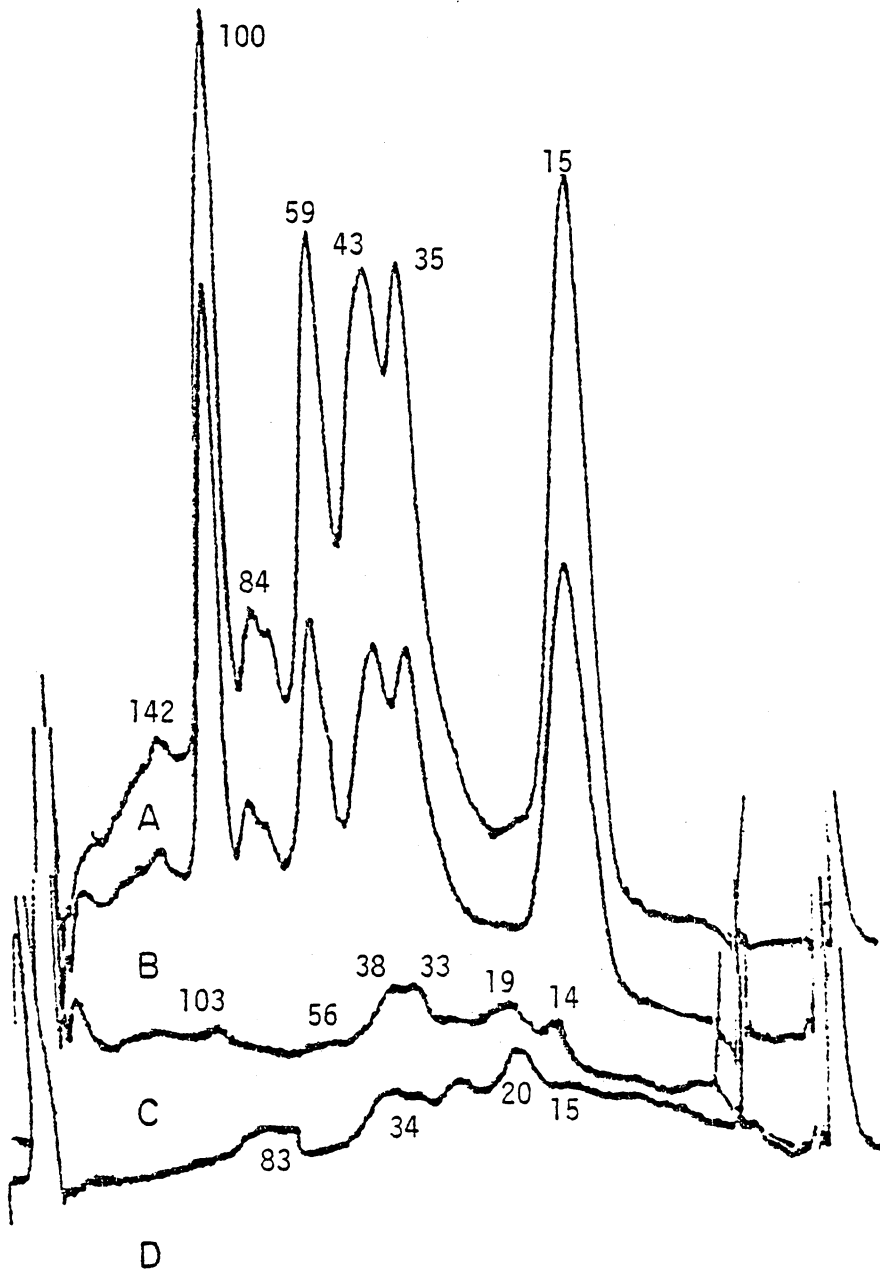polyacrylamide gel electrophoretic patterns of
soybean proteins hydrolyzed by immobilized trypsin/
chymotrypsin (1:1): (A) partially hydrolyzed soybean
protein isolate; (B) soybean protein isolate: (C)
7S protein rich fraction; (D) 11S protein rich
fraction. Numbers refer to molecular weights
(x 1/1,000).

The soybean protein isolate hydrolyzed by the immobilized trypsin/chymotrypsin showed some electrophoretic bands indicative of some residues of 7S as well as of acidic and basic subunits of 11S proteins leftover after enzymatic hydrolysis (Fig. 31, gel B). This might be due to the residual activities of protease inhibitors in the soybean protein isolate, active even after heat treatment (Baintner, 1981; Koshiyama et al., 1981b). The immobilized trypsin/chymotrypsin removed 7S and basic components of 11S PRF. When partially purified isolate fractions were hydrolyzed by immobilized trypsin/chymotrypsin, there was evidence that during the purification procedure there was selective removal of protease inhibitors, evidenced by the greater hydrolysis of the purified fractions.

4.82   Evaluation of molecular sizes and molecular charges by PAGE

4.821   Sample and gel preparation for gel electrophoresis

For the purpose of evaluating the molecular charges of soybean proteins, several methods of polyacrylamide gel electrophoresis (PAGE) were followed. The electrophoretic patterns of the soybean protein isolate are shown in Fig. 32. Using Davis procedure (1964), soybean proteins were separated into 2 - 3 bands. As a comparison, the Laemmli method (1970) utilizing 0.1% and 2.0% of SDS in 7.0% gels and samples, respectively, provided 4 - 5 bands of soybean proteins with a maximum component near the tracking dye, bromophenol blue. Following
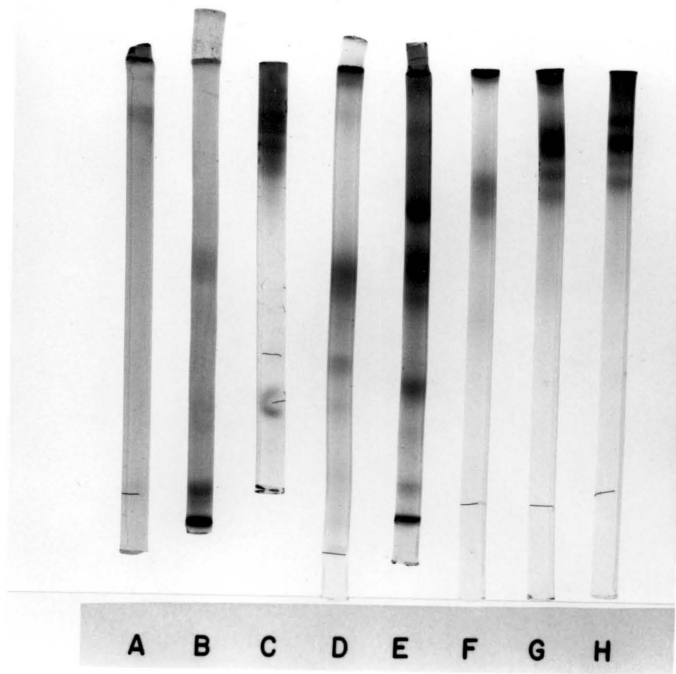
Fig. 32. Polyacrylamide gel electrophoresis disc patterns of soybean protein isolate by various methods: (A) Davis (1964) .procedure, 14.0% acrylamide; (B) Laemmli (1970) procedure, 7.0% acrylamide; (C) Catsimpoolas et al. (1968) procedure, 7.5% acrylamide; (D) Groves and Kiddy (1968) procedure, 7.0% acrylamide gel containing 4.0M urea; (E) Melachouris (1969) procedure, 7.0% acrylamide gel containing 7.0M urea; (F) Weber and Osborn (1969) procedure, 7.5% gel without sodium dodecyl sulfate; (G) same as in F, 7.5% gel containing 4.0M urea; (H) same as in F, 7.5% acrylamide gel containing 7.0M urea.

the Catsimpoolas et al. procedure (1968), it was possible to clearly
distinguish 6 - 7 bands of soybean protein isolate, but their mobili-
ties were quite low during the 3 hr of electrophoresis that the proce-
dure recommended. When protein samples were dissolved in 1.0% Triton
X-100 and 1.0% 2-mercaptoethanol, the use of the Groves and Kiddy
procedure (1968), which made use of the Davis method (1964) with 4M
urea in gels, resulted in one major and four to five minor bands. When
following the Melachouris procedure (1969) with a high concentration of
urea (7M) in the gels, nine to ten bands of soybean proteins were
separated. When the concentration reached more than 7M urea, however,
the gel buffer began to crystallize. In the absence of SDS, by apply-
ing the Weber and Osborn procedure (1969) it was possible to achieve a
better degree of resolution of the proteins with increasing amounts of
urea in the gel (Fig. 32, gels, F, G, and H). In contrast, the
mobilities of the protein bands were lower than those obtained by the
Melachouris procedure. A good resolution of soybean protein was found
in the starch gel electrophoresis when urea was incorporated into gel
and buffer (Puski and Melnychyn, 1968). It is concluded that the
choice of gel preparation for the evaluation of molecular charge and
molecular size of soybean proteins is gels prepared according to the
Davis procedure, but as modified by Melachouris to include 7M urea in
the gels.

4.822   Modified sample preparation for gel electrophoresis

Several neutral solubilizing agents were utilized in an attempt
to evaluate the molecular charge of modified soybean proteins in
absence of SDS as illustrated in Fig. 33. Gels A and B represent soy-
bean proteins undissociated into their subunitts. The nonionic deter-
gent, Triton X-100 is recommended because of excellent protein solu-
bilizing properties, the preservation of protein configuration, and its
non-ionic nature, which allows molecular weight and charge estimation
(Hearing et al., 1976). In the presence of 2-mercaptoethanol, several
major components were separated as shown in gels C and D, Fig. 33.
The concentration of solubilizing agents did not affect the separation
of soybean proteins. With 10 mM dithiothreitol, which was commonly
used in the method of Draper and Catsimpoolas (1977), the electro-
phoretic patterns of soybean proteins were similar to those with 2-
mercaptoethanol (gel E). Therefore, the use of dithiothreitol instead
of 2-mercaptoethanol was not advantageous. The solubilizing agents
composed of 1.0% Triton X-100 and 4.0M urea with or without reducing
agents showed several major components and no slow moving components.
This suggested that disulfide bonds were not important to maintain
quaternary structures. Urea at concentration ranging from 3 to 12M
has been used to render soluble certain classes of proteins by dis-
rupting hydrogen and possibly hydrophobic bonds of proteins (Gordon,
1970; Anglemier and Montgomery, 1976; Clatterbuck et al., 1980).
However, urea is not as good a protein solvent as Triton X-100 and
usually results in the loss of protein conformation and function
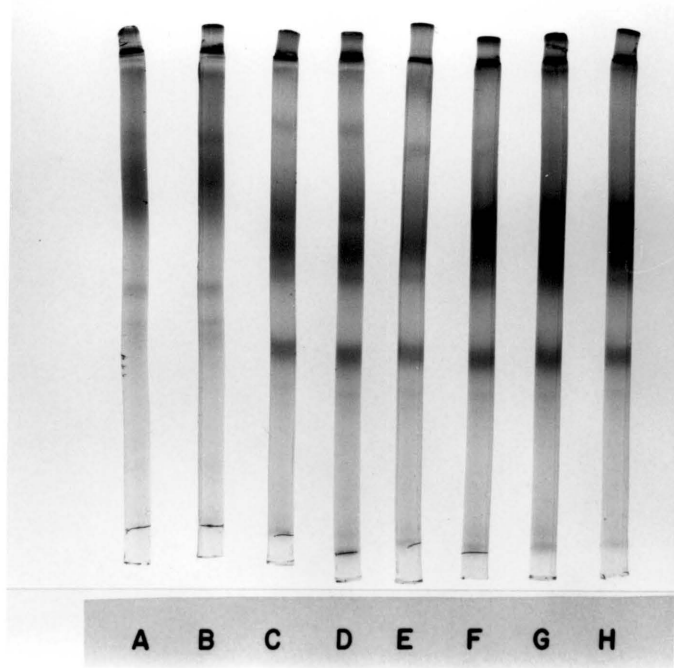
Fig. 33. Polyacrylamide gel electrophoresis disc patterns of
soybean protein isolate in the presence of various
dissociating agents in 7.0% gels with 4.0M urea: (A) 0.1%
Triton X-100; (B) 1.0% Triton X-100; (C) 1.0% Triton
X-100 and 1.0% 2-mercaptoethanol; (D) 0.1% Triton
X-100 and 0.1% 2-mercaptoethanol; (E) 1.0% Triton
X-100 and 10.0 mM dithiothreitol; (F) 1.0% Triton
X-100 and 4.0M urea; (G) 1.0% Triton X-100, 1.0% 2-
mercaptoethanol and 4.0M urea; (H) 1.0% Triton X-100,
10.0 mM dithiothreitol and 4.0M urea.

(Hearing et al., 1976). Based on the results shown in Fig. 33, soybean proteins were dissolved in 1.0% Triton X-100 and 2-mercaptoethanol to measure their molecular sizes and molecular charges.

4.823 Retardation coefficient and relative free mobility

In Fig. 34, retardation coefficients (K_R) for marker proteins are plotted against their molecular weights. The retardation coefficients of proteins were obtained from the slopes of the Ferguson plot (1964) which is the logarithm of the relative mobility of marker proteins on the ordinate and the percent acrylamide in gels on the abscissa. The standard proteins soluble in 1.0% Triton X-100 and 1.0% 2-mercapto-ethanol showed relationshops between molecular weights and retardation coefficients. In addition, a limited linear regression if (retardation coefficient)$^{1/2}$ against (molecular weight)$^{1/3}$ was found by Kawasaki and Ashwell (1976). According to Frank and Rodbard (1975), the molecular weight obtained for rhodopsin in gels at a single % T was invalid. They noted that the most appropriate measurement of molecular weight using SDS-PAGE was obtained from a plot showing retardation coefficient as a function of molecular weights.

Since the retardation coefficient (K_R) and relative free mobility (Y_0) are independent variables (Rodbard and Chrambach, 1971), it is possible to have anomalous behavior of a protein in PAGE so that K_R has an appropriate relationship to molecular weight, but Y_0 differs significantly from the standards. In the equation, $\log_{10} R_f = \log_{10}$
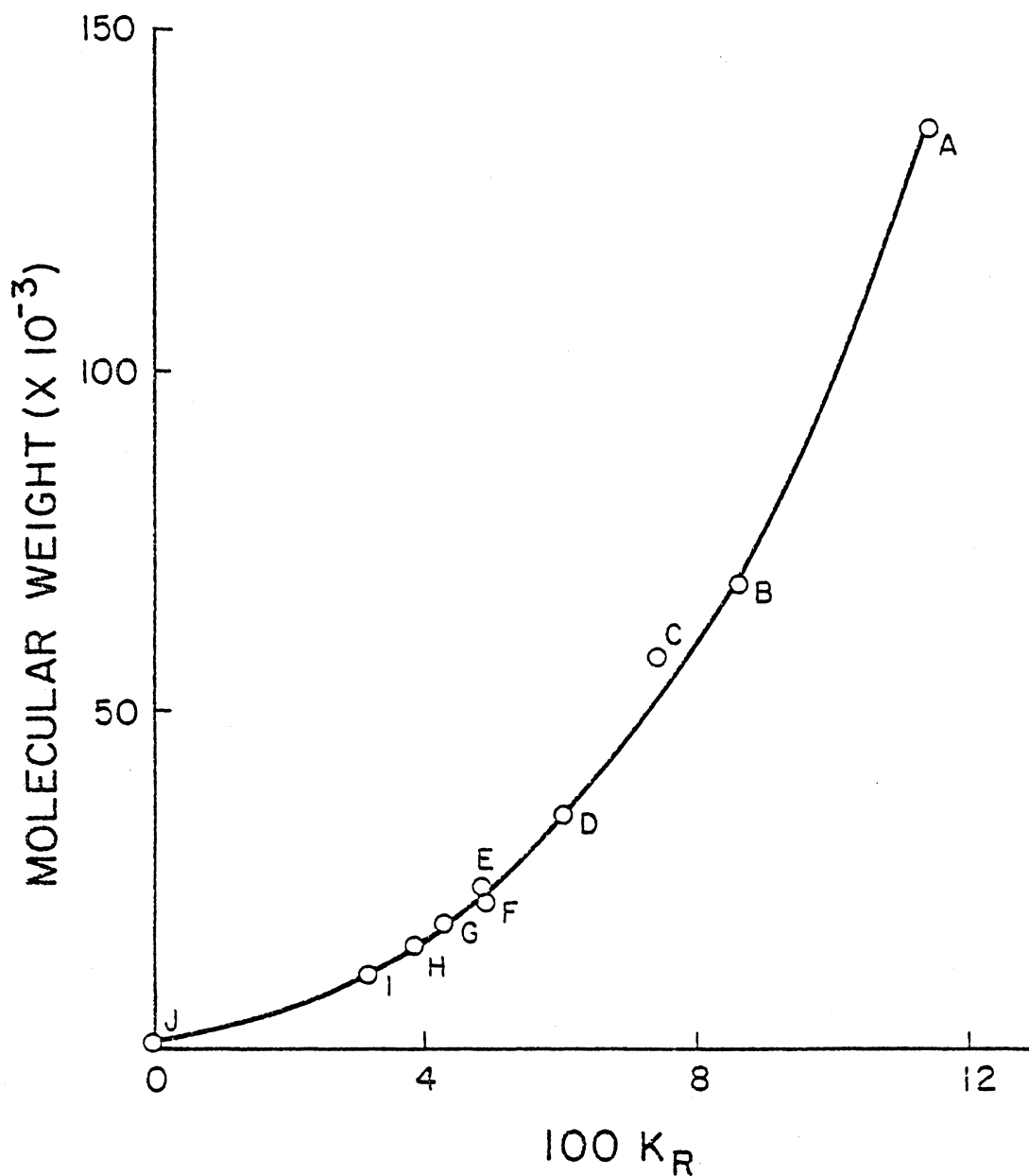
Fig. 34. Relationship between K_R and molecular weight for
standard proteins: data of proteins in 1.0% Triton
X-100 and 1.0% 2-mercaptoethanol. A = bovine serum
albumin (dimer); B = bovine serum albumin (monomer);
C = catalase; D = pepsin; E = trypsin; F = trypsin
inhibitor; G = beta-lactoglobulin; H = hemoglobin;
I = chymotrypsin; J = bromophenol blue.

$Y_0 - K_R T$, K_R is the slope of $\log_{10} R_{f1} - \log_{10} R_{f2}/(T_1 - T_2)$, where Y_0 is the relative mobility (R_f) of the components at 0% T. Therefore, this discussion is based on the molecular weights obtained from the K_R value, and $\log_{10} Y_0$ which is the logarithm of the relative mobility at 0% T.

The slow moving components of soybean proteins are separated efficiently at low gel concentration, whereas fast moving components are easily distinguished from other components at high gel concentrations as shown in Fig. 35.

4.824   Partially purified fractions

Figs. 36 and 37 show polyacrylamide gel electrophoretic patterns of partially purified fractions of soybean proteins in 6.10% and 12.00% gels. Each soybean protein has its distinctive peak heights and areas as seen in the series of analysis of densitometer tracings (Fig. 38), Ferguson plot (Fig. 39) and physical constants of the soybean proteins (Appendices I, II, III and IV). The components with molecular weights of 63,500 and 3,000 were high in 7S PRF. The cold insoluble fraction and 11S PRF had high peaks of bands with molecular weights ranging from 44,000 to 56,000. Apparently, the components with molecular weights of 44,000 to 56,000 mentioned in Appendices I, II, III and IV are intermediate subunits. According to Iyengar and Rvestein (1981), the disulfide bonds of the glycinin monomer are stable, but the disulfide bridges responsible for polymer formation were reduced at
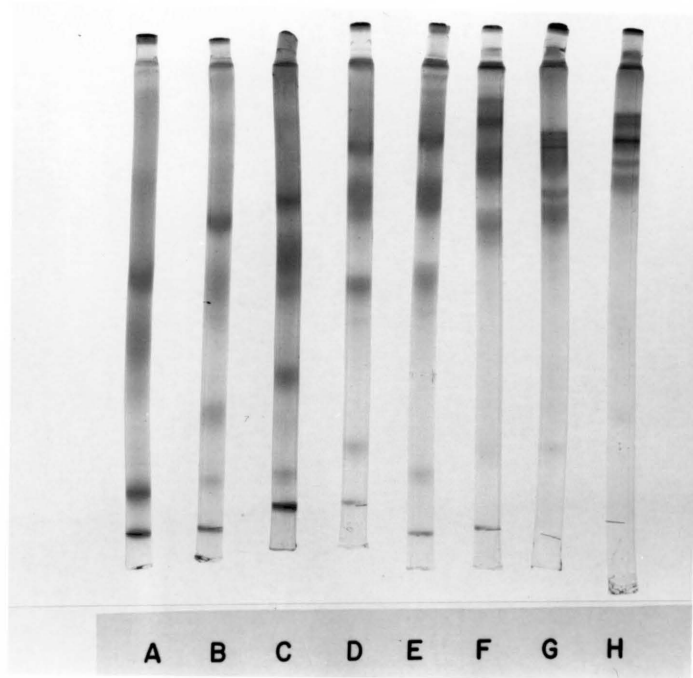
Fig. 35. Polyacrylamide gel electrophoresis disc patterns of
soybean protein isolate solubilized in 1.0% Triton X-100
and 1.0% 2-mercaptoethanol with various gel concentrations:
(A) 5.25%; (B) 6.10%; (C) 7.00%; (D) 8.70%; (E) 10.50%;
(F) 12.00%; (G) 14.00%; (H) 16.00%.
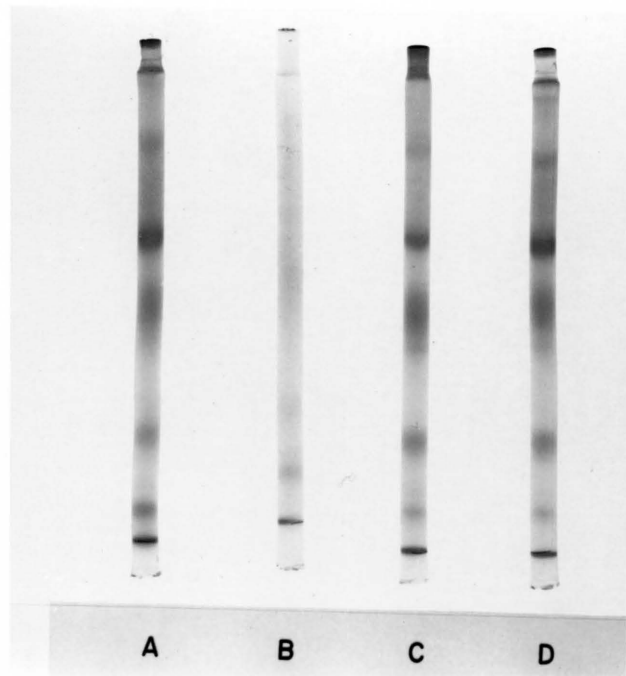
Fig. 36.   Polyacrylamide gel electrophoresis disc patterns of
partially purified soybean proteins in 6.1% gels: (A)
7S protein rich fraction; (B) 11S protein rich fraction;
(C) cold insoluble fraction; (D) soybean protein isolate.
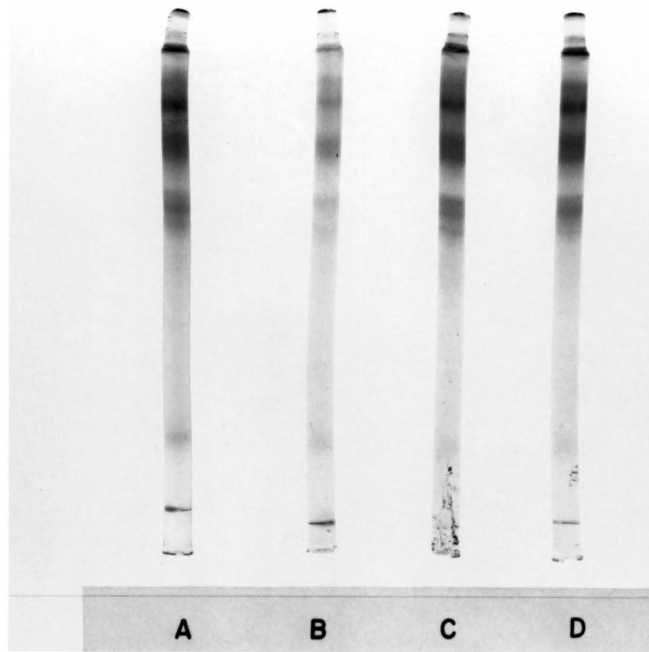
Fig. 37. Polyacrylamide gel electrophoresis disc patterns of
partially purified soybean proteins in 12.0% gels: (A)
7S protein rich fraction; (B) 11S protein rich fraction;
(C) cold insoluble fraction; (D) soybean protein isolate.
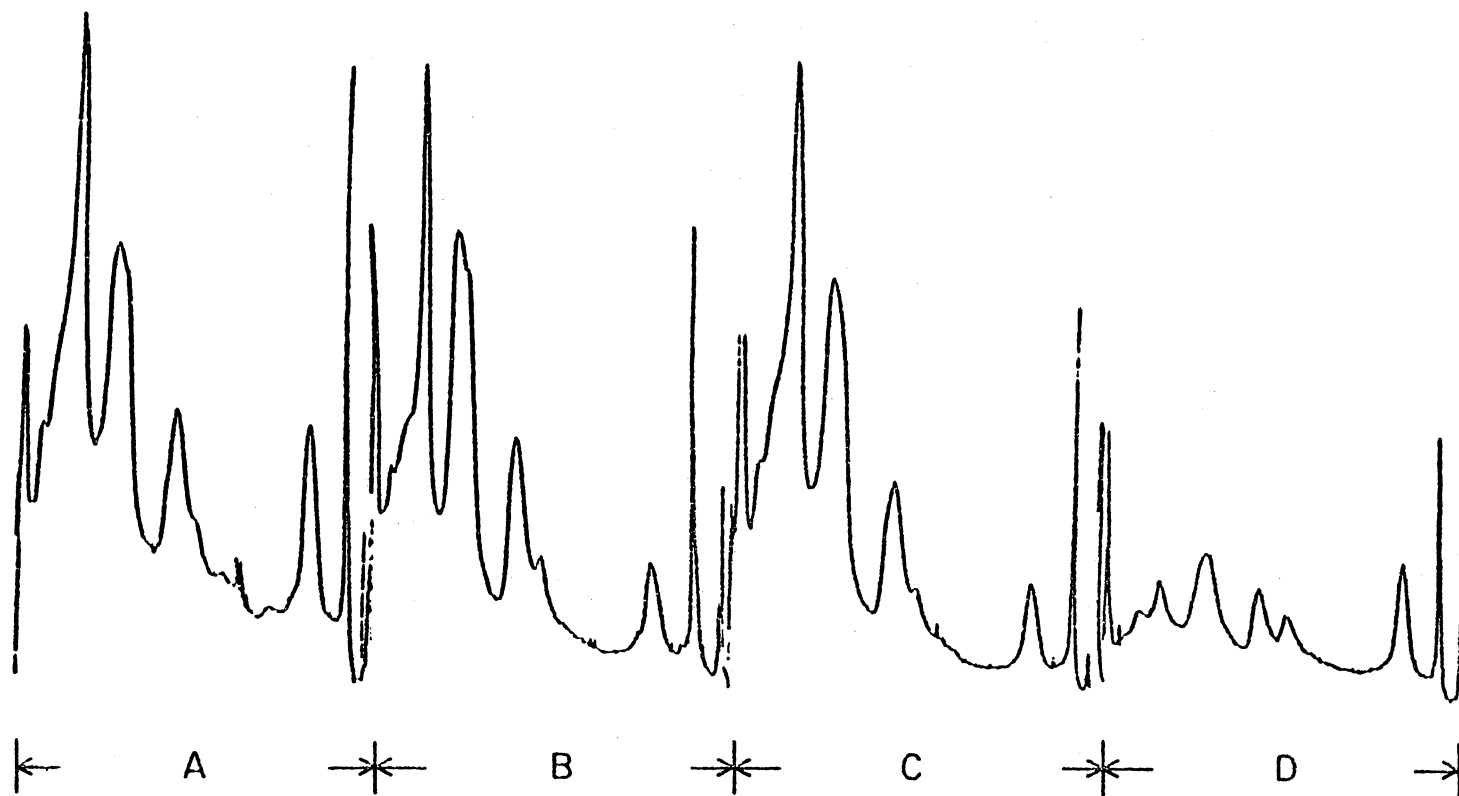
Fig. 38. Densitometer tracings of polyacrylamide gel electrophoretic patterns of soybean proteins soluble in Triton X-100 and 2-mercaptoethanol in 10.5% gels: (A) 7S protein rich fraction; (B) cold insoluble fraction; (C) soybean protein isolate; (D) 11S protein rich fraction.
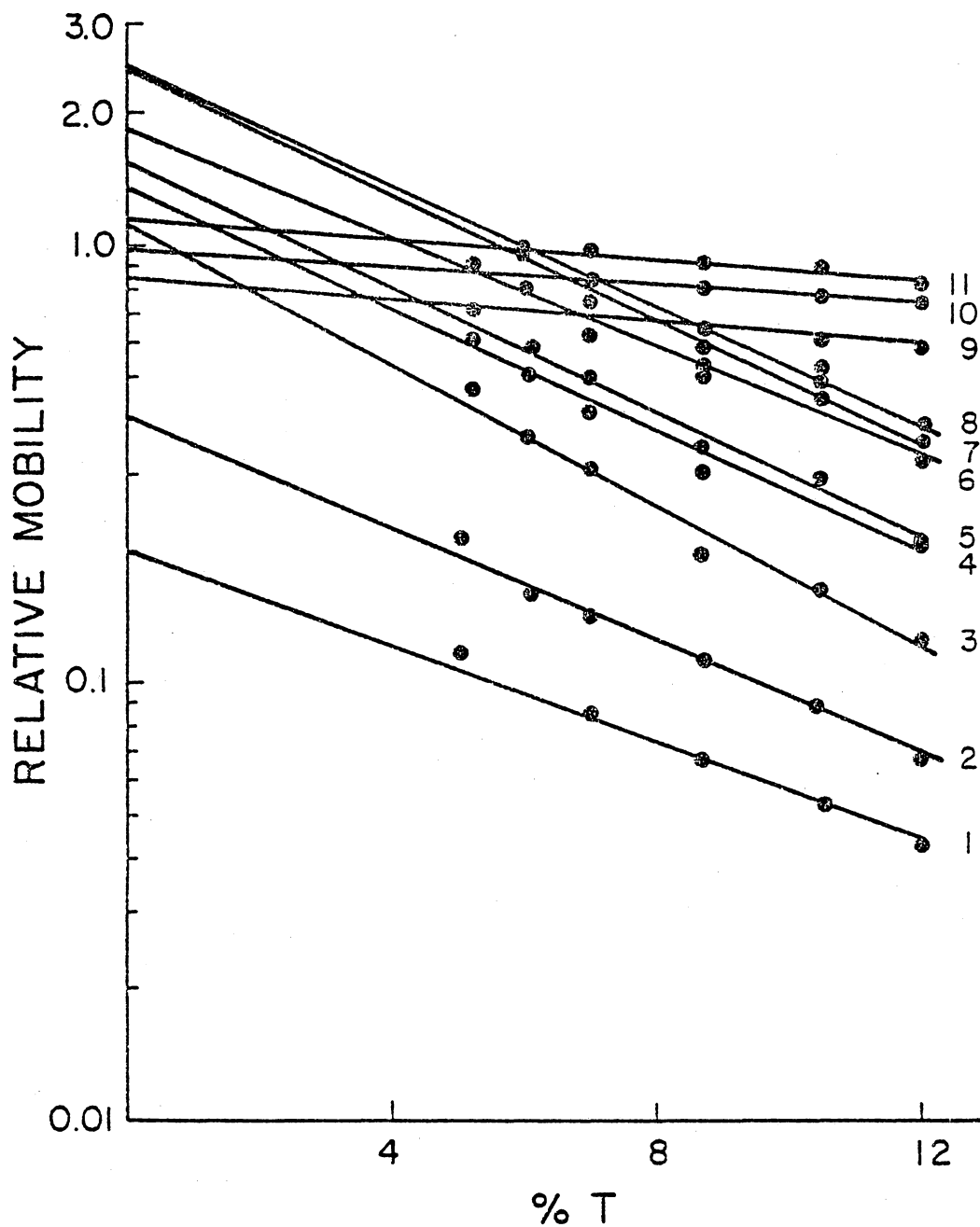
Fig. 39. Ferguson plots of 7S protein rich fraction of soybean proteins. The numbers correspond to bands from the top. The gel concentration, % T, is grams of acrylamide and methylenebisacrylamide in 100 ml solution.

low 2-mercaptoethanol concentrations (0.01M = 0.083%), neutral pH, and ionic strength of more than 0.35, resulting in the formation of sub- units. The intermediate subunits solubilized in the neutral solvents containing 1.0% Triton X-100 and 1.0% 2-mercaptoethanol were presumed to be linked by the electrostatic interactions between acidic and basic subunits. Moreover, these intermediate subunits were dissociated by solubilizing soybean proteins in SDS (anionic detergent) or derivatized anionic groups of the proteins by succinylation (Figs. 26 and 41). Band 8 in the cold insoluble fraction and band 5 in 11S PRF indicate acidic subunits of 11S components as shown by their molecular weights of 39,000 and 27,500, respectively.

4.825   Succinylated soybean proteins

Figs. 40 and 41 present electrophoretic patterns of succinylated 11S PRF and soybean protein isolate, as well as soybean protein isolate succinylated and hydrolyzed by the immobilized trypsin/chymotrypsin. Succinylation of soybean proteins indicated that the acidic and basic subunits of 11S components in 1.0% Triton X-100 and 1.0% 2-mercapto- ethanol might be similar to those of soybean protein isolate in the presence of SDS (bands 9 and 10 in Appendix V and bands 8 and 9 in Appendix VI). Obviously, 11S PRF had higher concentration of the com- ponents with molecular weights of 30,000 and 19,000 expressed as acidic and basic subunits, respectively. Bands 5 and 6 in 11S PRF as well as bands 4 and 5 in soybean protein isolate were presumed to be incom-
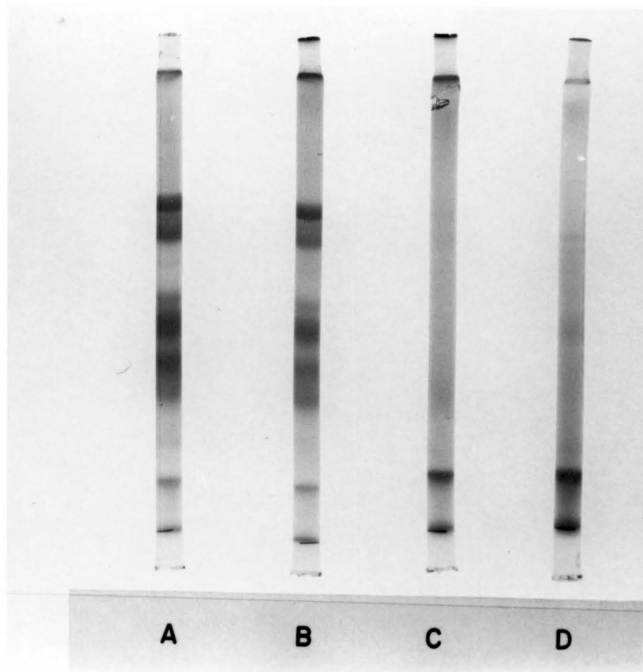
Fig. 40. Polyacrylamide gel electrophoresis disc patterns of
succinylated soybean proteins in 8.7% gels: (A)
succinylated 11S protein rich fraction; (B) succinylated
soybean protein isolate; (C) hydrolyzed and succinylated
soybean protein isolate; (D) succinylated and hydrolyzed
soybean protein isolate.
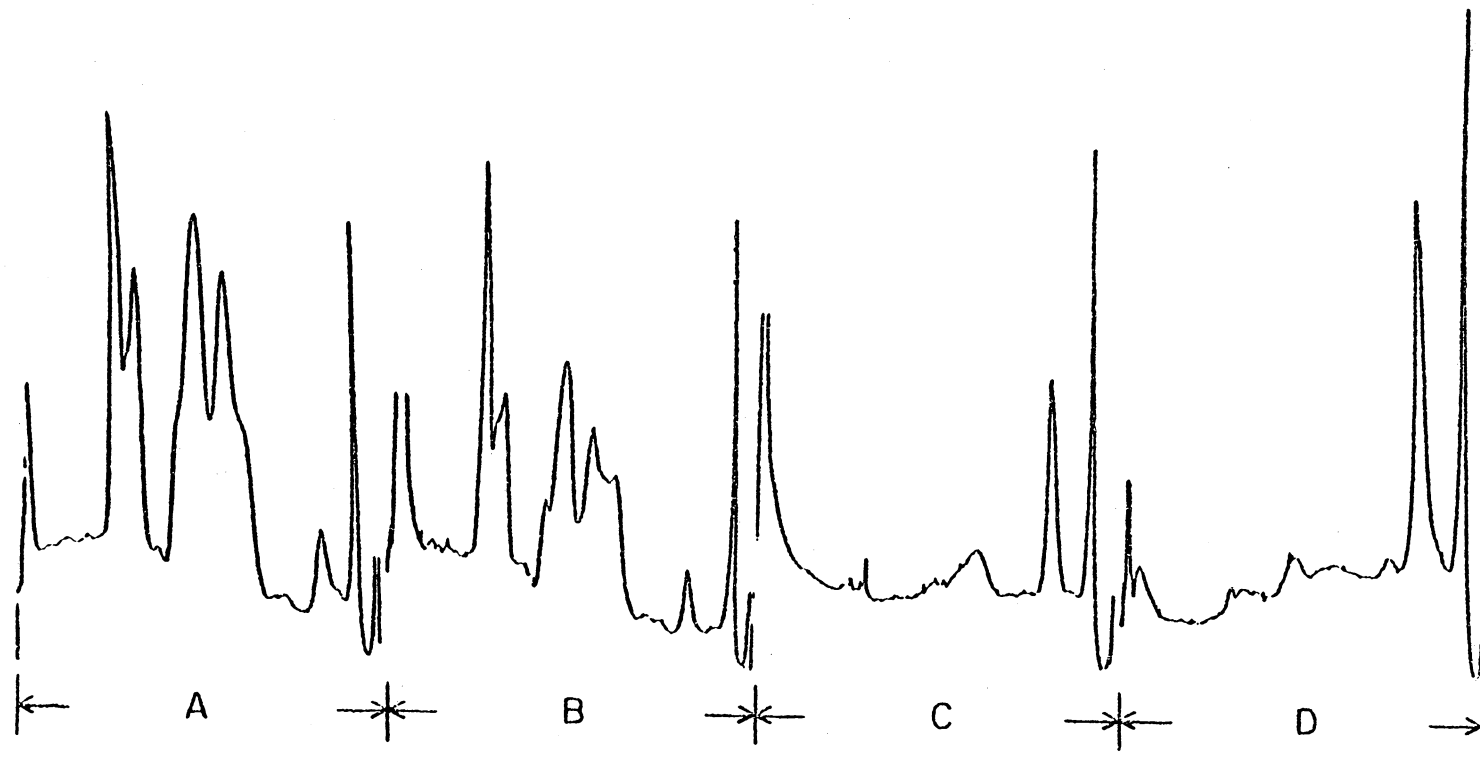
Fig. 41.   Densitometer tracings of polyacrylamide gel electrophoretic patterns of succinylated soybean proteins soluble in Triton X-100 and 2-mercaptoethanol in 10.5% gels: (A) 11S protein rich fraction; (B) soybean protein isolate; (C) hydrolyzed and succinylated isolate; (D) succinylated and hydrolyzed isolate.

pletely succinylated components because of the low net charges as
compared to those of similar size molecules. Overall, the net charges
of succinylated soybean proteins were high except in bands 1, 2, and 3
in succinylated 11S PRF and bands 1 and 2 in succinylated soybean
protein isolate. This is thought to be due to the increase in nega-
tively charged groups of soybean proteins caused by succinylation.
Kabirullah and Wills (1982) showed that after succinylation, sunflower
globulin was dissociated into fractions which moved further into the
gel with increasing acylation. Because of the molecular expansion of
proteins upon succinylation, the molecular weights of the derivatized
proteins can be accurately measured by retardation coefficients rather
than by relative mobilities of SDS-bound proteins on a single gel.

Electrophoretic patterns of soybean proteins isolate modified by
hydrolysis with immobilized trypsin/chymotrypsin and then by succinyla-
tion were different from those of soybean protein isolate modified by
succinylation and then by hydrolysis (gels C and D in Figs. 40 and 41).
The amount of the new fast moving component of soybean proteins near
the tracking dye was greater after two sequential treatments than in
the succinylated soybean protein isolate. Generally, the components
of soybean protein isolate modified by hydrolysis with the immobilized
proteases and then by succinylation, contained higher molecular
weights and molecular charges than those treated by a reverse order of
treatments (Appendices VII and VIII). Hydrolysis without prior
succinylation apparently did not split enough available peptide bonds,

probably due to the conformational resistance and the protease inhibi-
tors. The hydrolyzed proteins were then further dissociated into sub-
units as well as expanded by the subsequent treatment of succinylation
(Kinsella and Shetty, 1979). However, in the reverse treatments, the
succinylation step led to the dissociation of soybean proteins into
subunits, the expansion of their molecular conformation, and the
removal of the active entities like protease inhibitors which enhanced
the subsequent hydrolysis step as the second treatment. The differ-
ences in molecular size and molecular charge of soybean protein
isolates were affected by the order in which hydrolysis and succinyla-
tion were done and caused differences in the functional properties of
modified soybean proteins (Tables 11 and 12). Groninger and Miller
(1975) simply measured foaming properties of fish protein which had
been modified by soluble bromelain and succinylation or by the same
treatments in the reverse order. This is not sufficient to be able to
reliably evaluate molecular sizes and charges of modified proteins and
explain their various functional properties.

4.826   Soybean protein isolate modified by hydrolysis with

         immobilized protease(s)

Polyacrylamide gel electrophoretic profiles of soybean protein
isolate hydrolyzed by the immobilized chymotrypsin, immobilized
trypsin as well as immobilized trypsin/chymotrypsin (1:9) are shown
in Figs. 42 and 43. The hydrolyzed proteins were prepared for electro-
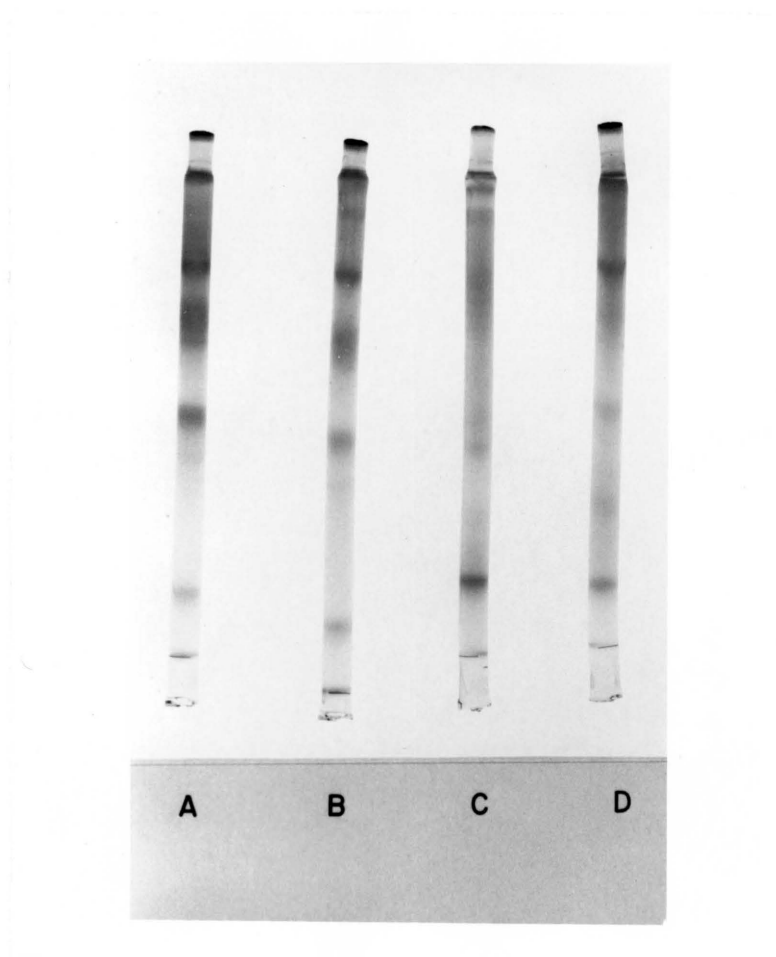
Fig. 42. Polyacrylamide gel electrophoresis disc patterns of soybean protein isolate hydrolyzed by immobilized protease(s) in 8.7% gels: (A) control; (B) chymotrypsin; (C) trypsin; (D) trypsin/chymotrypsin (1:9).
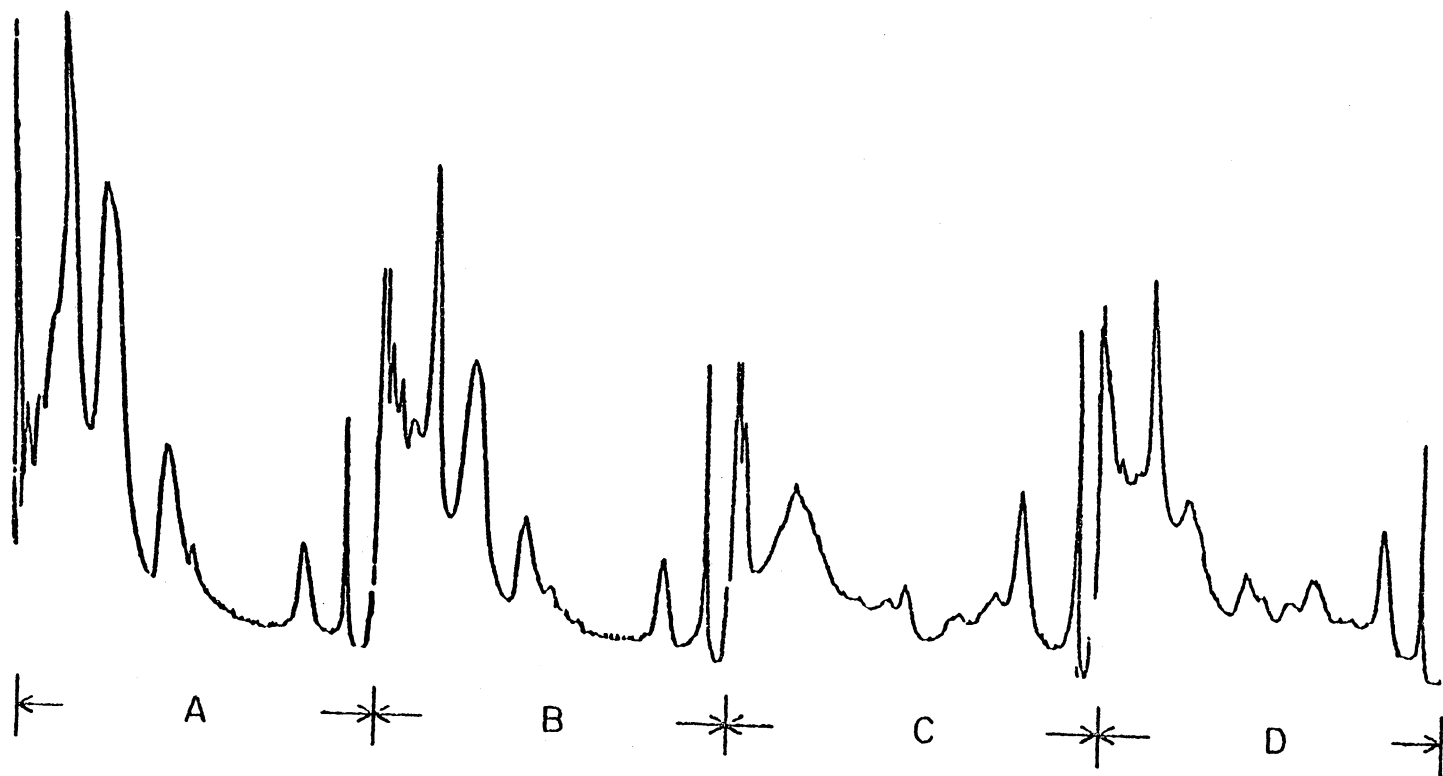
Fig. 43. Densitometer tracings of polyacrylamide gel electrophoretic patterns of soybean protein isolate modified by immobilized protease(s) in 10.5% gels: (A) control; (B) chymotrypsin; (C) trypsin; (D) trypsin/chymotrypsin (1:9).

phoresis by dissolving in 1% Triton X-100 and 1% 2-mercaptoethanol.
Immobilized chymotrypsin reduced major peaks evenly. On the other
hand, immobilized trypsin preferentially hydrolyzed intermediate sub-
units of 11S soybean proteins and produced a high concentration of low
molecular weight components. Preferential hydrolysis of the inter-
mediate subunits was also observed in gel D, Fig. 43, when the ratio
of immobilized trypsin to immobilized chymotrypsin was low in order to
reduce enzymatic activity on the soybean protein. As shown by the
peak differences between B and D in Fig. 43, the intermediate subunits
were preferentially hydrolyzed by immobilized trypsin. Although 11 -
13 bands of hydrolyzed soybean proteins can be distinguished among
various gels ranging from 5.25% to 12.00% T, these bands probably to
not represent a complete resolution. The emphasis of this experiment
was not placed on finding minor heterogeneities in the polypeptides,
but on the evaluation of molecular weight, molecular charge and the
relative amounts of major polypeptides to explain their functional
properties.

A critical analysis of soybean protein disc gel electrophoresis
was achieved by constructing Ferguson plots for as many of the
discernible protein bands as possible. Under non-sieving conditions
of gels, the $\log_{10} Y_0$ of soybean protein isolate modified by the
immobilized chymotrypsin would be expected to decide the migration
with bands 9, 10, and 8 in that order, moving most rapidly, and bands
1, 2, 4, and 3 moving more slowly (Appendix IX). Based upon K_R, bands

1 and 4 had high molecular weights, and band 11 had low molecular weight. Soybean proteins modified by the immobilized trypsin/chymotrypsin (1:9) had different molecular sizes and molecular charges (Appendix XI). Bands 7 and 8 with medium molecular weights had high molecular charges. Band 1 contained low molecular charge and low molecular weight. Band 4, a major band, had medium molecular charge and high molecular weight. With few exceptions, the mobilities of soybean proteins hydrolyzed by the immobilized trypsin/chymotrypsin depended on their molecular weights. After hydrolysis of soybean proteins with immobilized protease(s), gels C and D in Fig. 43 shows new components with molecular weights ranging from 4,000 to 24,000 (Appendices X and XI). The molecular charge distributions of soybean protein isolate hydrolyzed by immobilized trypsin were different from those produced when hydrolyzed by immobilized chymotrypsin. This indicates that, after hydrolysis, the molecular weights and molecular charges of soybean proteins are heterogeneous.

4.827   Soybean protein fractions modified by hydrolysis with
        immobilized trypsin/chymotrypsin

For the purpose of exclusive utilization of immobilized multi-enzymes, immobilized trypsin/chymotrypsin (1:1) was used to modify 7S PRF, 11S PRF, and soybean protein isolate. Electropherograms of modified soybean proteins hydrolyzed by immobilized trypsin/chymotrypsin (1:1) are shown in Figs. 44 and 45. The numerous fast moving
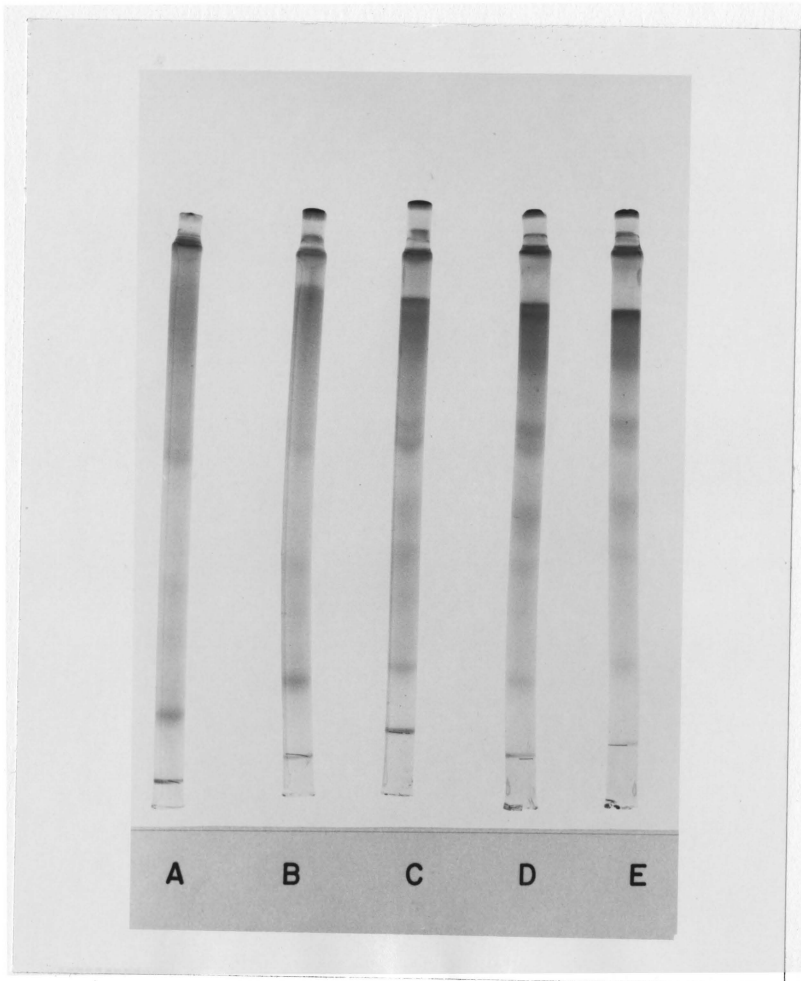
Fig. 44. Polyacrylamide gel electrophoresis disc patterns of soybean proteins hydrolyzed by immobilized trypsin/chymotrypsin (1:1) in 12.0% gels: (A) 7S protein rich fraction; (B) 11S protein rich fraction; (C) soybean protein isolate; (D) and (E) partially hydrolyzed soybean protein isolate.
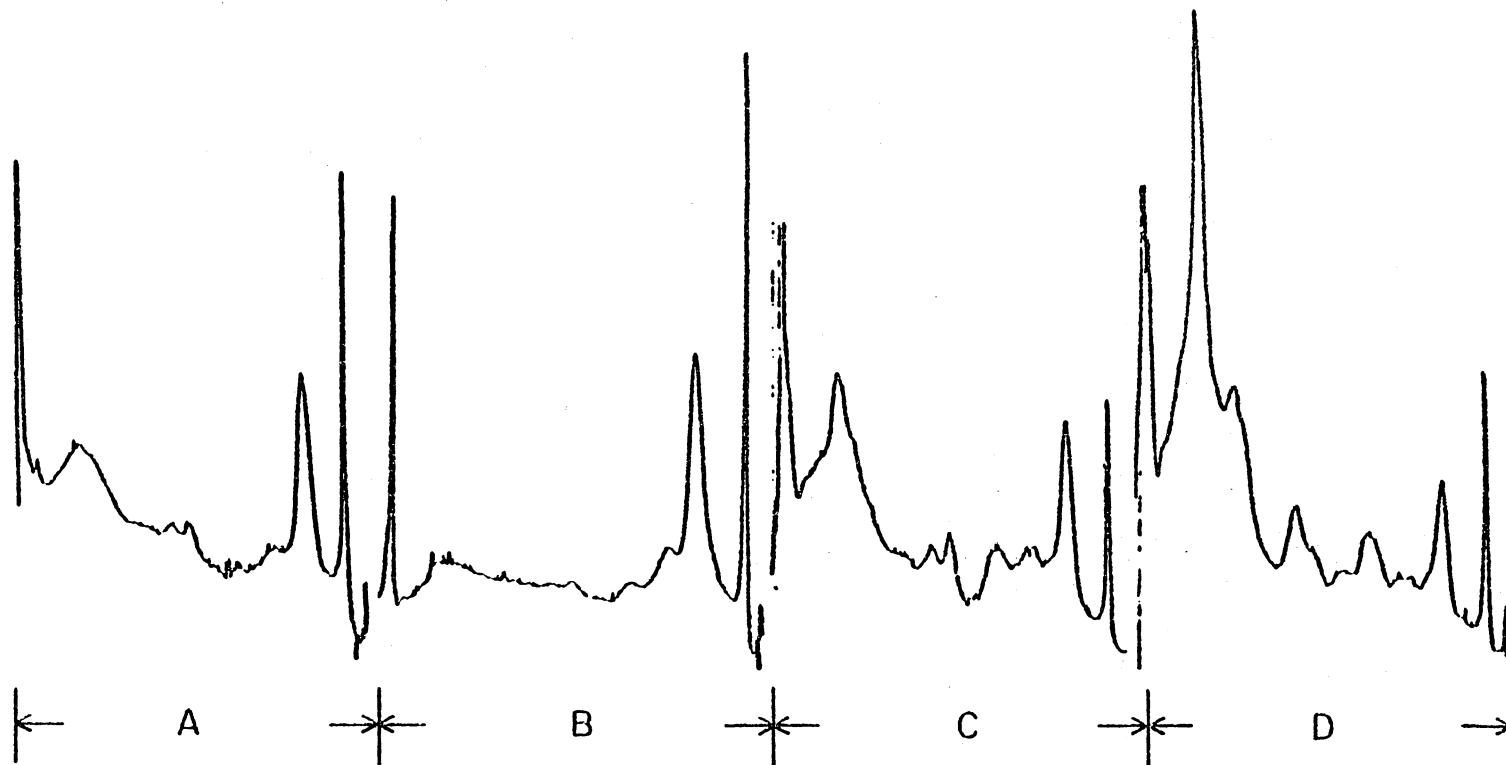
Fig. 45. Densitometer tracings of polyacrylamide gel electrophoretic patterns of soybean proteins modified by immobilized trypsin/chymotrypsin (1:1) in 10.5% gels: (A) 7S protein rich fraction; (B) 11S protein rich fraction; (C) soybean protein isolate; (D) partially hydrolyzed isolate.

components in 7S PRF and 11S PRF appeared when the major bands were hydrolyzed. Fast moving components were formed and major components disappeared from 11S, 7S, and soybean protein isolate in that order. Bands of molecular weights of 2,500 were high in the hydrolyzed 7S PRF and 11S PRF (Appendices XII and XIII). This suggests that the purification procedure for 7S PRF and 11S PRF selectively removed protease inhibitors in the soybean protein isolate. The intermediate subunits of 11S soybean proteins were preferentially hydrolyzed by the immobilized trypsin/chymotrypsin (1:1). Ferguson plots showed that there were various molecular size and molecular charge distribution of modified soybean proteins caused by the immobilized trypsin/chymotrypsin (1:1). The physical constants of 11S PRF hydrolyzed by immobilized trypsin/chymotrypsin (1:1) indicate that band 2 had high molecular charge and high molecular weight (Appendix XIII). Band 1 had low molecular charge and medium molecular weight. In sum, molecular charges and molecular weights strongly affect mobilities of 11S PRF hydrolyzed by immobilized trypsin/chymotrypsin (1:1). The treatment of soybean protein with immobilized trypsin/chymotrypsin gave bands 9 - 15 with high molecular charges and various molecular sizes (Appendix XIV). Partial hydrolysis of soybean protein isolate by immobilized trypsin/chymotrypsin demonstrated the different molecular charge and molecular weight distributions (Appendix XV). Highly charged components such as bands 7 - 13 had medium or low molecular weights. Bands 1 - 3 had high molecular weights and low molecular charges. The

fast moving component, band 14, showed a medium molecular charge and a low molecular weight. This indicates that modified soybean proteins have very different molecular weights and molecular charges.

4.83 Average relative free mobility as a function of average retardation coefficient

To determine the relationships between molecular and functional properties of modified soybean proteins, these proteins were selected to that they would have widely varying molecular sizes and charges as well as functional properties, as shown in Table 13. The modified soybean proteins were obtained by partial purification, succinylation, and hydrolysis with immobilized protease(s). It was, indeed, possible to change the functional properties of soybean proteins to meet specific needs by using proper modification techniques.

The average retardation coefficients and the average relative free mobilities of the modified soybean proteins were calculated by using the weighted average of each component (Catsimpoolas, 1974; Hsieh et al., 1979). Fig. 46 shows the relationship between the average relative free mobility and the average retardation coefficient for the 17 modified soybean proteins under the present experimental conditions. The correlation coefficient is 0.74.

4.84 Average molecular weight to average molecular charge ratio as a function of average molecular weight

Table 13. Modification of soybean proteins for the evaluation of average molecular weight ($\bar{M}$) and average molecular charge ($\log_{10} Y_o$) as well as their functional properties.

Number	Fraction	Modification	Functional property[a]							
			SOL	WHC	OHC	VIS	EA	ES	FA	FS
1	7S protein rich	None (control)	±	±	+	-	±	+	±	±
2	7S protein rich	Hydrolysis (trypsin/chymotrypsin = 1:1)	+	-	-	-	-	+	±	-
3	cold insoluble		±	±	+	-	+	+	±	±
4	protein isolate	None (control)								
5	11S protein rich	None (control)	±	+	+	-	-	+	±	±
6	11S protein rich	Hydrolysis (trypsin/chymotrypsin = 1:1)	+	±	-	-	-	-	±	±
7	11S protein rich	Succinylation	+	-	+	+	-	+	±	+
8	protein isolate	Succinylation	+	-	+	+	-	±	±	±
9	protein isolate	Hydrolysis (trypsin/chymotrypsin = 1:1) -Succinylation	+	-	+	+	±	-	+	±
10	protein isolate	Succinylation-Hydrolysis (trypsin/chymotrypsin = 1:1)	+	-	±	-	-	-	+	-
11	protein isolate	Incubation	±	±	-	±	±	±	±	±
12	protein isolate	Hydrolysis (chymotrypsin)	±	+	-	±	-	-	±	±
13	protein isolate	Hydrolysis (trypsin/chymotrypsin = 1:9)	±	±	-	±	-	-	±	-
14	protein isolate	Hydrolysis (trypsin/chymotrypsin = 1:1)	+	-	-	+	-	-	±	±
15	protein isolate	Hydrolysis (trypsin)	+	-	-	-	-	-	+	-
16	protein isolate	Hydrolysis (trypsin/chymotrypsin = 1:1)[b]	±	-	±	±	-	-	+	-
17	protein isolate	Hydrolysis (trypsin/chymotrypsin = 1:1)[b]	±	-	±	-	±	±	±	-

[a] Functional properties of modified soybean proteins in comparison with those of soybean protein isolate were expressed as + (increased), - (decreased), and ± (not changed). Abbreviations are: SOL, solubility; WHC, water holding capacity; OHC, oil holding capacity; VIS, viscosity; EA, emulsifying ability; ES, emulsion stability; FA, foaming ability; FS, foam stability.

[b] Partially hydrolyzed.
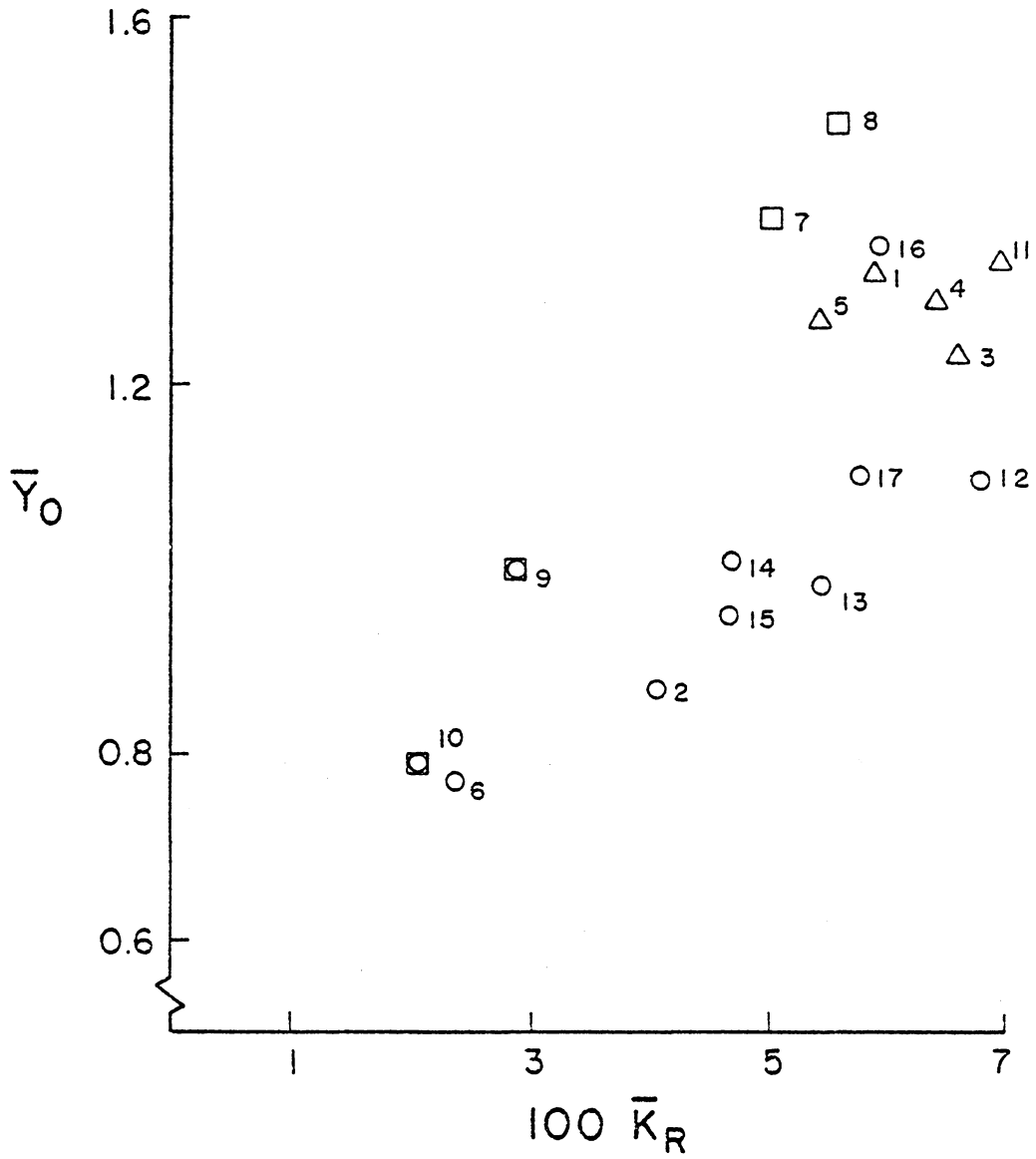
Fig. 46. Relationship between $\bar{K}_R$ and Y_0 estimated from the linear Ferguson plots. Numbers refer to the modified soybean proteins in Table 13.

When the ratio of the average molecular weights to the average
molecular charges of modified soybean proteins are plotted as a
function of the average retardation coefficients or the average molecu-
lar weights, a parabola results as shown in Fig. 47. This indicates
that the parameter, the average molecular weight to the average molecu-
lar charge, could be utilized to describe functional properties of
modified soybean proteins. Hsieh et al. (1979) found that when soybean
proteins were hydrolyzed by soluble trypsin, the nitrogen content
soluble in 5% trichloroacetic acid was increased drastically and
linearly during the initial hydrolysis. This indicates that there is
a linear inverse relationship between the average molecular weight and
solubility. By the introduction of a qualitative model for enzymatic
hydrolysis of partially denatured globular proteins, Adler-Nissen
(1976) suggested that the molecular sizes of the hydrolyzed protein
had an inverse relationship with its solubility.

The solubility-pH relationship for a large number of legumes
showed that the proteins were highly soluble if the pH value was
sufficiently removed from the isoelectric pH (Wolf, 1977a). At the
isoelectric points, proteins have neutral charges, indicating no net
charges. Obviously, protein solubility varies with different salts,
according to their lyotropic effect (Shen, 1981). In an aqueous
solution, the acylation increases net charges on the modified proteins
through the replacement of the positively charged amino groups.
Proteins with high molecular charges have high solubilities under
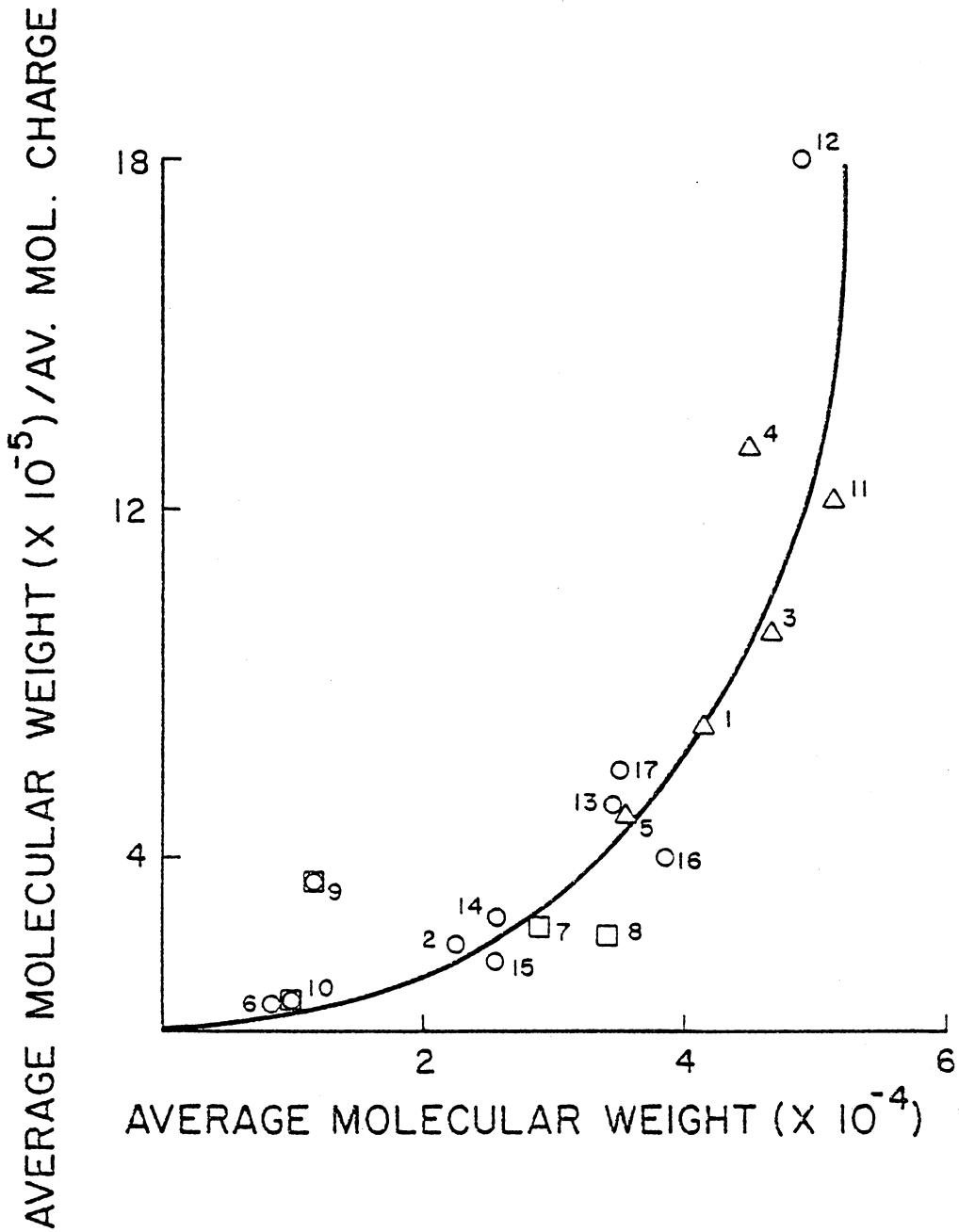
Fig. 47. Relationship between average molecular weight and average
molecular charge estimated from the linear Ferguson plots
of the modified soybean proteins. Numbers refer to the
modified proteins in Table 13.

specific conditions. Since the ionization of prototropic groups of a protein depends on pH (Hutton and Campbell, 1981) and the isoelectric pH is determined by the number and the pK of the ionizing groups, its net charge will be decided by the magnitude of the pH change, the derivation, and other conditions such as the lyotropic effect.

The comments made above indicate that the solubility of a protein is determined by the molecular size and molecular charge of its components. Since the amino acid composition and sequence, as well as the immediate environment govern the conformation of a protein (Lehninger, 1970; Kinsella and Shetty, 1979), the effects of protein conformation on functional properties were mainly decided by its molecular weight and charge. Evidently, the ratio of the average molecular weight to the average molecular charge can be utilized to explain the solubility and other functional properties of a protein under a specific condition.

4.85   Functional properties of modified soybean proteins as a
        function of the ratio of the average molecular weight
        to the average molecular charge

4.851   Solubility

The relationship between alpha-amino groups of modified soybean proteins and the ratio of the average molecular weight to the average molecular charge (the ratio) is given in Fig. 48. Total soluble alpha-

Fig. 48. Solubility of modified soybean protein as a function of average molecular weight/average molecular charge. Numbers refer to the modified proteins in Table 13. Indicated values are means and standard deviations based on four replications.

amino groups of modified soybean proteins in phosphate buffer (pH 6.5)
increased as the ratio was reduced below 5×10^5. The solubility
profiles of the modified soybean proteins leveled off in the range of
$5 \times 10^5 - 20 \times 10^5$ of the ratio. Partially fractionated soybean
proteins had solubilities similar to those of the proteins partially
hydrolyzed by immobilized enzyme(s). At the upper right side of the
average relative free mobility-average retardation coefficient plot
(Fig. 46), the modified soybean protein had low soluble amino groups.
Usually, hydrolysis decreased average molecular weight and increased
net negative molecular charge of soybean proteins. Succinylation
increased the average molecular positive charges moving towards the
anode at above pI, and decreased the average molecular weight of the
soybean proteins. Therefore, two treatments reduced the ratio and
increased solubility. Canella et al. (1979), and Kabirullah and Wills
(1982) observed that the average molecular charge of the succinylated
sunflower protein at above pI seemed to be higher than that of the
control proteins. Thus, it is not surprising to note that these
experiments showed high solubilities of the succinylated sunflower
proteins at above pI. Adler-Nissen and Olsen (1979) reported that
depending on the degree of hydrolysis by soluble proteases the
solubility of enzymatically modified soybean proteins increased more
than that of the non-hydrolyzed control at various pH. Their gel
filtration analyses seemed to show decreased average molecular weight
with increased degree of hydrolysis. Similar results have been

reported by Kabirullah and Wills (1981) who found that the hydrolyzed sunflower protein had a high solubility and a low molecular size distribution.

Since solubility studies provide a good index of the potential applications of proteins, solubility was related to many important functional properties such as emulsifying capacity, gelation, foaming capacity and foam stability, and bitter taste. Moreover, Halling (1981) reviewed the correlation of solubility and emulsifying capacity of modified proteins under different conditions such as succinylation, pH, hydrolysis, salting effect, and heat treatment. Apparently, the relationship between solubility and other functional properties of proteins was not linear; rather, other functional properties decreased as proteolysis continued, suggesting that there is an optimum mean molecular size of proteins that optimizes functional properties (Zakaria and McFeeters, 1978; Phillips and Beuchat, 1981). Indeed, Beuchat et al. (1975) demonstrated that the various functional properties of peanut protein modified by soluble trypsin, pepsin, and bromelain were different during the hydrolysis time ranging from 10 to 50 min, indicating that during protein hydrolysis there were different molecular size and charge distributions of the modified proteins. This was due to the different specificities of the enzymes.

4.852   Water holding capacity

The relationship between water holding capacity and the ratio of
the average molecular weight to the average molecular charge is
expressed in Fig. 49. The reduction of molecular size and the increase
in net molecular charge of soybean proteins hydrolyzed by immobilized
protease(s) reduced their water holding capacity. Moreover, succinyla-
tion with or without hydrolysis remarkably reduced water holding
capacity of the modified soybean proteins. The denatured soybean
proteins hydrolyzed by immobilized chymotrypsin had reduced average
molecular charge and average molecular weight, and high water holding
capacity. Therefore, the physical entrapment of water into the protein
matrix is an important factor for the water holding capacity. Thus, a
highly soluble protein exhibits poor water binding capacity as discussed
by Hermansson et al. (1974). This suggested that water absorption may
be increased by hydrolysis up to a point and then may be decreased.
Peanut flour protein modified by bromelain and trypsin had low water
holding capacity after any time of hydrolysis (Beuchat, 1977b). The
enhancing of water holding capacity by succinylation may be due to the
increase in hydrophilic properties of proteins, as it happens with sun-
flower protein upon succinylation. Sunflower protein is more
structurally hydrophobic than soybean protein (Fleming et al., 1974;
Hutton and Campbell, 1981). On the other hand, water holding capacity
of succinylated sunflower protein was reduced before it reached the
water holding capacity of the control (Canella et al., 1979). To
achieve maximum water holding capacity of modified soybean proteins, it

Fig. 49. Water holding capacity of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified proteins in Table 13.

is necessary to have a large average molecular weight and small net molecular charge.

4.853 <u>Oil holding capacity</u>

Ad demonstrated in Fig. 50, oil holding capacity of modified soybean protein was maximum at 9×10^5, the ratio of the average molecular weight to the average molecular charge. The excessive decrease in molecular weight and/or increase in the net molecular charge of the protein caused by hydrolysis reduced oil holding capacity, as the decrease in molecular weight and the decrease in net molecular charge also reduced oil holding capacity. Succinylated soybean proteins had rather different patterns of oil holding capacity. Soybean proteins modified by succinylation and then by hydrolysis exhibited lower oil holding capacity than that shown by hydrolysis followed by succinylation because of the high net molecular charge of the modified soybean proteins. This result was comparable to those reported by Beuchat (1977a) on succinylated peanut flour and Kabirullah and Wills (1982) on succinylated sunflower protein isolate. They indicated that the enhanced entrapment of oil was due to the unfolding of the protein structure. Small particle, textured soybean flour was reported to absorb more than large particle soybean flour (Hutton and Campbell, 1981). Thus, the oil holding capacity is affected by both the molecular structure and the macrostructure of proteins. Molecular expansion either by succinylation, controlled hydrolysis or physical treatments

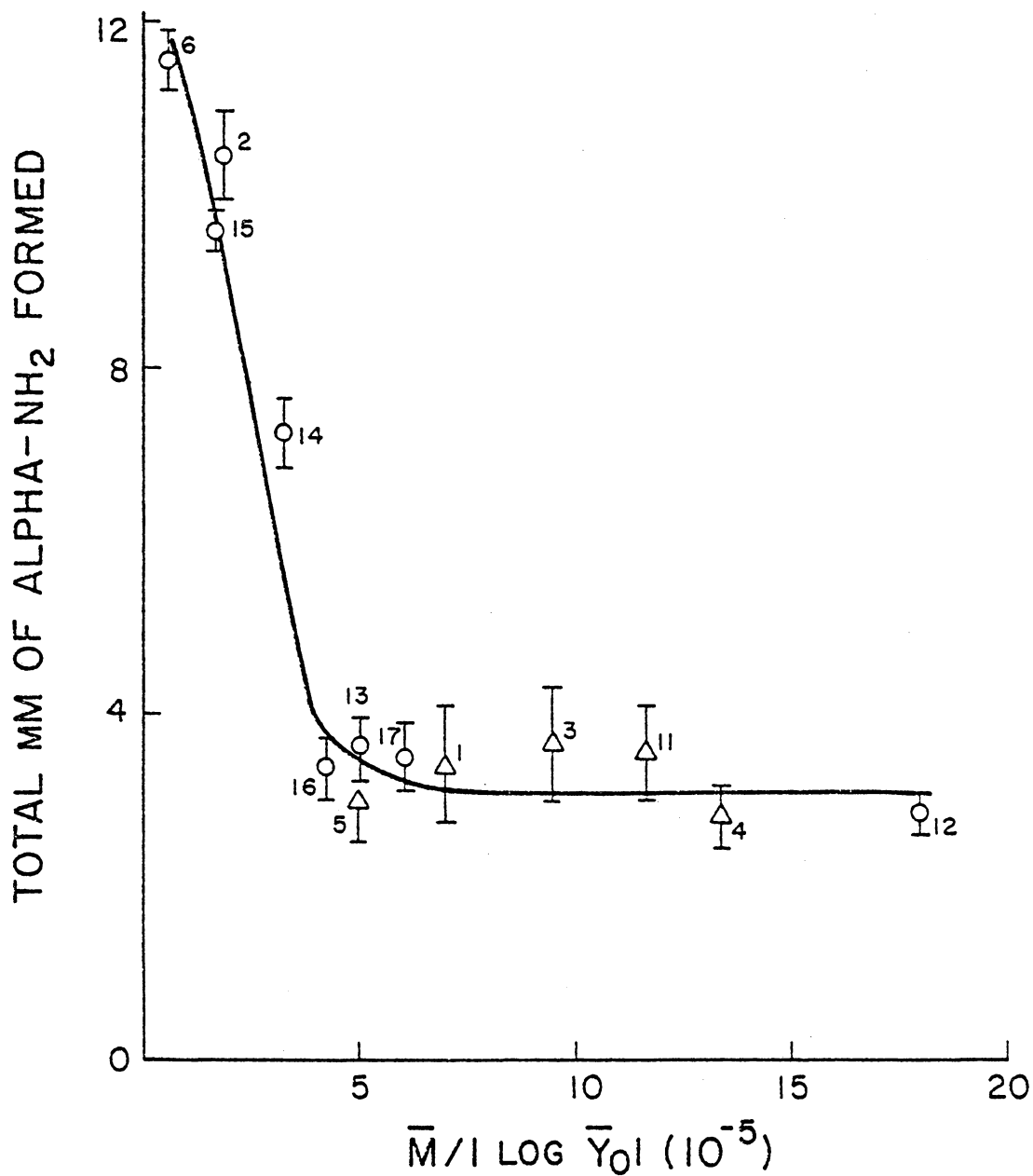Fig. 50. Oil holding capacity of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified soybean proteins in Table 13.

such as heat, increased specific volume, thereby increasing fat absorption. In general, excessive hydrolysis produced small molecules of soybean proteins and weak molecular charges conducive to also weak repulsive forces between protein molecules which helps explain the reduction in fat absorption. In contrast, the introduction of net charges on the surface by succinylation developed repulsive forces strong enough to increase specific molecular volume, which contributes to increasing fat absorption.

4.854   Relative viscosity

As shown in Fig. 51, the relative viscosities of the modified soybean proteins depend upon the molecular sizes and molecular charges of the proteins. Under the experimental conditions, the protein-protein interaction was assumed to occur possibly through hydrophobic interactions of the particular molecular sizes and charges. It is known that soluble protein molecules with exposed hydrophobic surface areas experience greater protein-protein interactions. These intermolecular interactions are affected by protein conformation, hydration, exposure of hydrophobic groups, and charge distribution, and result in increased viscosity. Extensive hydrolysis of soybean proteins by immobilized protease(s), or by succinylation and then hydrolysis, led to decreased viscosity of modified soybean proteins. This suggests that the molecular size of proteins or the increased molecular charges were not enough to induce the protein-protein interactions. On the other hand,

Fig. 51. Relative viscosity of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified soybean proteins in Table 13.

the relative viscosities of modified soybean proteins can reasonably be predicted by using the ratio of the average molecular weight to the average molecular charge, ranging from 5×10^5 to 20×10^5. Evidently, the viscosities of the modified soybean proteins are related to the molecular sizes and molecular charges of the soybean proteins.

4.855   Emulsifying ability

The emulsifying ability of the modified soybean proteins as a function of the ratio of the average molecular weight over the average molecular charge is shown in Fig. 52. The emulsifying capacity of the modified soybean protein was maximum at a ratio of 10×10^5. A decrease in emulsifying ability of modified soybean protein was observed where the proteins had small molecular size and/or high or low molecular charge. Thus, a small molecular size and/or high absolute molecular charge reduced emulsifying ability, and a low net molecular charge decreased emulsifying ability. The simple molecular expansion of soybean protein suspended in water decreased its emulsifying capacity when heated by microwaves or by a conventional method (Armstrong et al., 1979). This reflects the increase in average molecular size with little change in average molecular charge. The emulsifying ability of modified protein could be improved significantly, compared to non-modified control samples, by controlling the extent of hydrolysis. Therefore, the emulsifying ability decreased as proteolysis continued, indicating that there is an optimum molecular size and optimum charge

Fig. 52. Emulsifying ability of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified proteins in Table 13.

of proteins affecting emulsifying ability. Emulsifying ability of
soybean protein hydrolysate prepared by peptic hydrolysis first increased
and then decreased with increasing concentration of free amino groups in
the hydrolysate (Zakaria and McFeeters, 1978). Adler-Nissen and Olsen
(1979) demonstrated that, for optimum emulsification, the hydrolysate
seemed to have equal amounts of soluble and insoluble material. It is,
therefore, important that the molecules not be too small. With peanut
flour protein, Beuchat (1977a) also showed that the emulsifying
capacity of the succinylated protein at a 0.4 ratio of succinic
anhydride to protein increased significantly and then decreased. More-
over, Eisele and Brekke (1981) indicated that the effects of succinyla-
tion on emulsifying capacity of beef heart protein was influenced by
the extent of succinylation and the pH value of the solvent. This
suggested that the introduction of excessive molecular charges by
derivation and ionization reduced emulsifying capacity. Hence, the
initial hydrolysis and succinylation can improve emulsifying capacity
of soybean proteins mainly by means of reduction in molecular size or
by increase in molecular charge of the protein. McWatters and Cherry
(1981) also indicated that in the presence of high salt concentrations,
emulsifying capacity was reduced except at or near the isoelectric
point. This implies that emulsifying capacity of a protein is affected
by its molecular size and molecular charge.

4.856   <u>Emulsion stability</u>

Emulsion stability of soybean proteins modified by molecular size and/or molecular charge changes is shown in Fig. 53. The maximum emulsion stability of the modified soybean proteins reached a maximum when the ratio of the average molecular weight to the average molecular charge was 9×10^5. This indicates that reduction of molecular sizes by hydrolysis was not conducive to emulsion stability. On the other hand, succinylated soybean proteins showed a significant increase in their emulsion stability because of the molecular expansion. To obtain emulsion stability, it is important to have high molecular size or highly expanded protein molecules. Since the peptides after hydrolysis are smaller and less globular compared to those in non-treated soybean protein isolate, they may form a thinner protein layer around the oil droplets, thus, resulting in a less stable emulsion (Zakaria and McFeeters, 1978). Prevention of coalescence of droplets and emulsion stability depend upon increased surface rigidity or elasticity of the proteins. At the pI, the cohesiveness, compactness and rigidity of the protein surface film are usually maximal, whereas electrostatic repulsion between molecules is minimized (Halling, 1981). However, McWatters and Cherry (1981) reported that safflower protein isolate adjusted to pH 7.0 resulted in high emulsion stability as compared to safflower protein isolate adjusted to pH 5.0 or 6.0. Kabirullah and Wills (1982) failed to show high stability of emulsion at near the pI of succinylated sunflower protein or sunflower protein. Soybean protein hydrolyzed by soluble neutral protease (Puski, 1975), and sun-
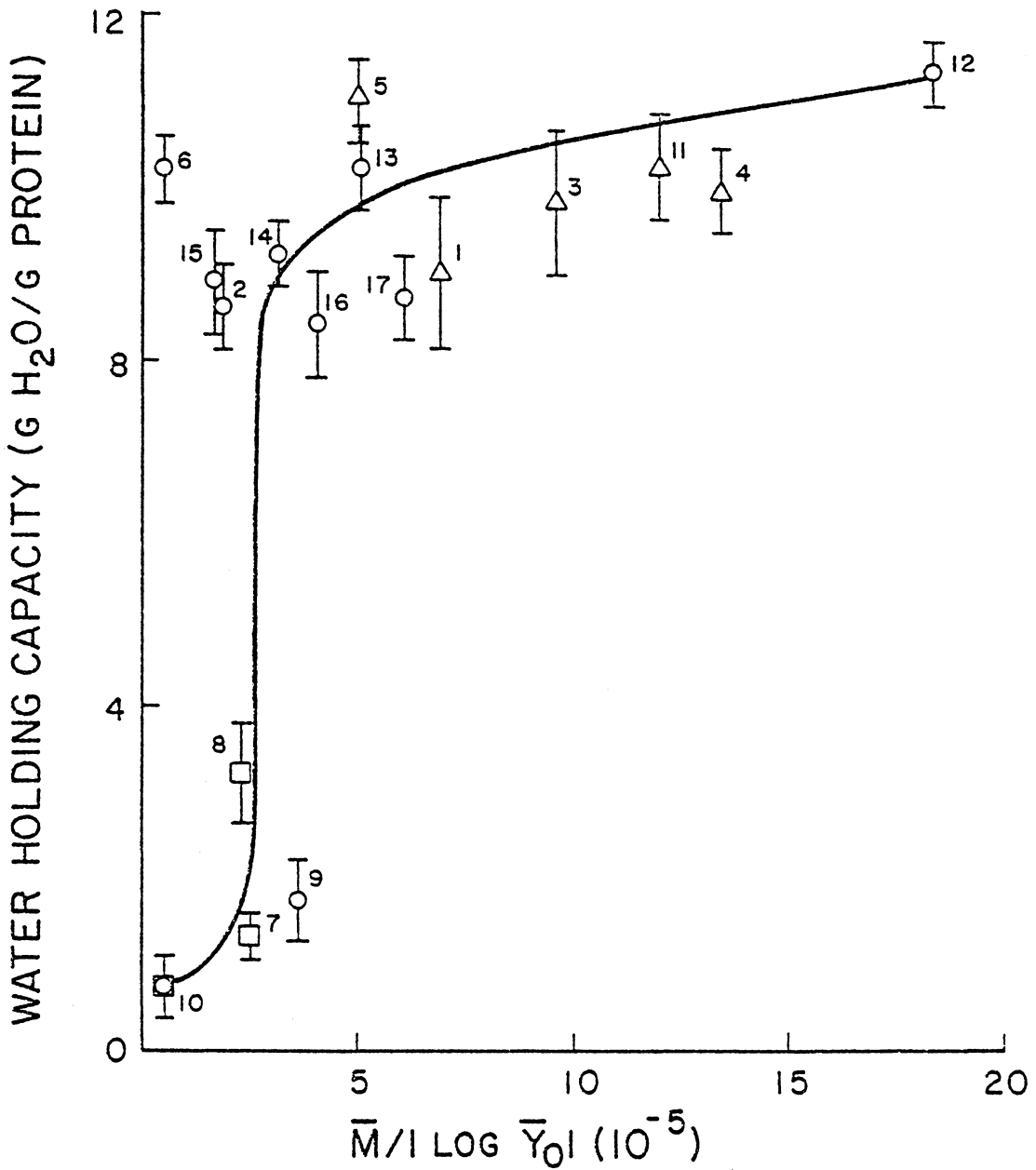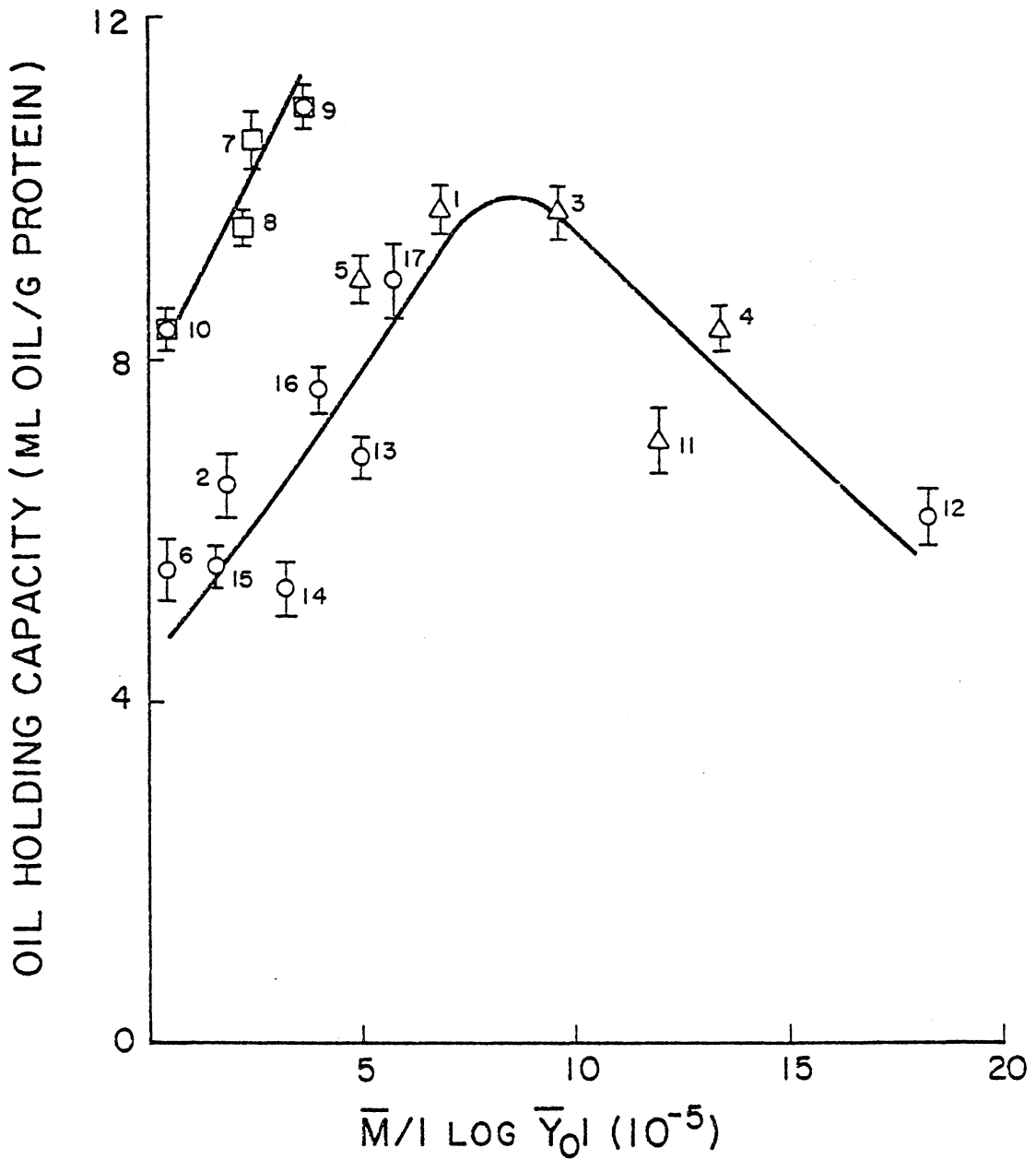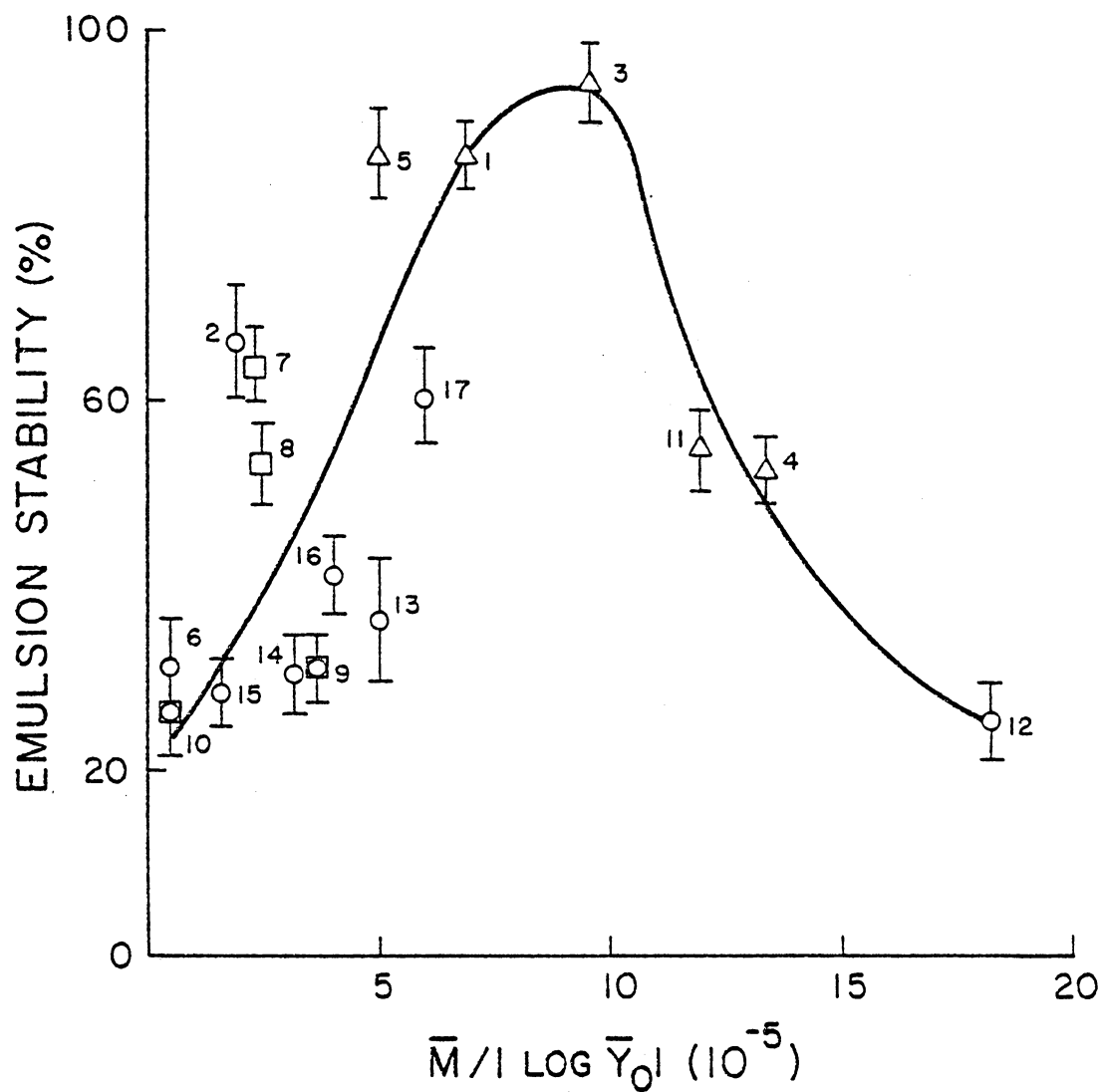
Fig. 53. Emulsion stability of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified soybean proteins in Table 13.

flower protein hydrolyzed by soluble trypsin and pepsin (Kabirullah and Wills, 1981) had low emulsion stability compared to the control protein. In contrast, rapeseed protein isolate hydrolyzed by soluble trypsin or by acid or alkali expressed higher emulsion stability than that of the original sample (Hermansson et al., 1974). Franzen and Kinsella (1976b) demonstrated that leaf proteins succinulated by an equal amount of succinic anhydride and protein had fairly high emulsion stability as compared to the control protein. This indicates the necessity to evaluate the molecular sizes and molecular charges of modified soybean proteins in order to explain the different findings. The results presented here show the optimum ratio of molecular size to molecular charge of modified soybean proteins under specific conditions to obtain high emulsion stability.

4.857   Foaming ability

Fig. 54 illustrates the effect of the average molecular weight to the average molecular charge on foaming ability of the modified soybean proteins. The foaming ability of the proteins did not change when the ratio of the average molecular size to the average molecular charge ranged from 2.5×10^5 to 20×10^5. However, there was a possibility to enhance the foaming ability of the modified soybean proteins below the ratio of 2.5×10^5. Below this desirable ratio of molecular sizes to molecular charges, the increased foaming abilities of the proteins depended on modifications such as succinylation and hydrolysis with
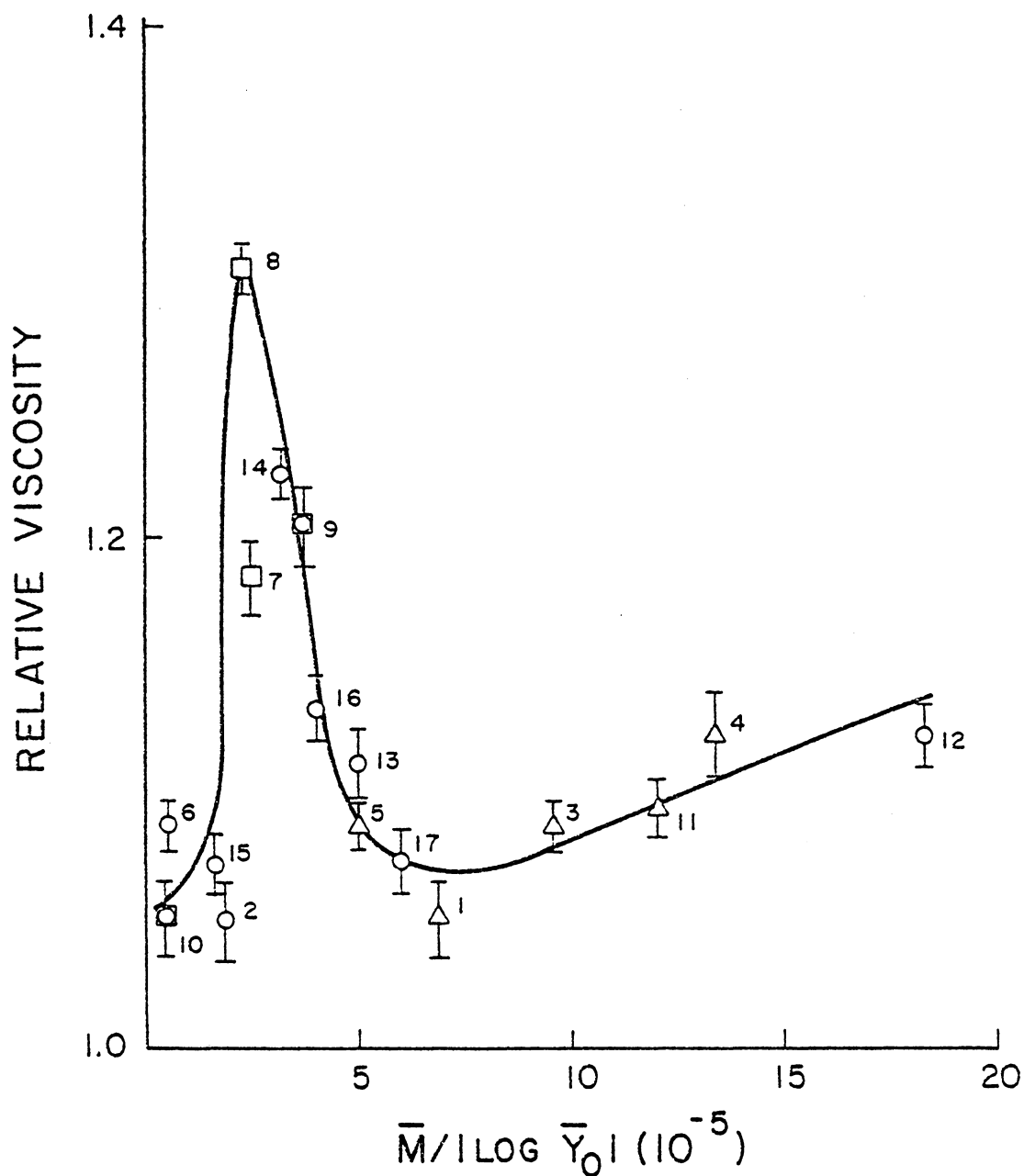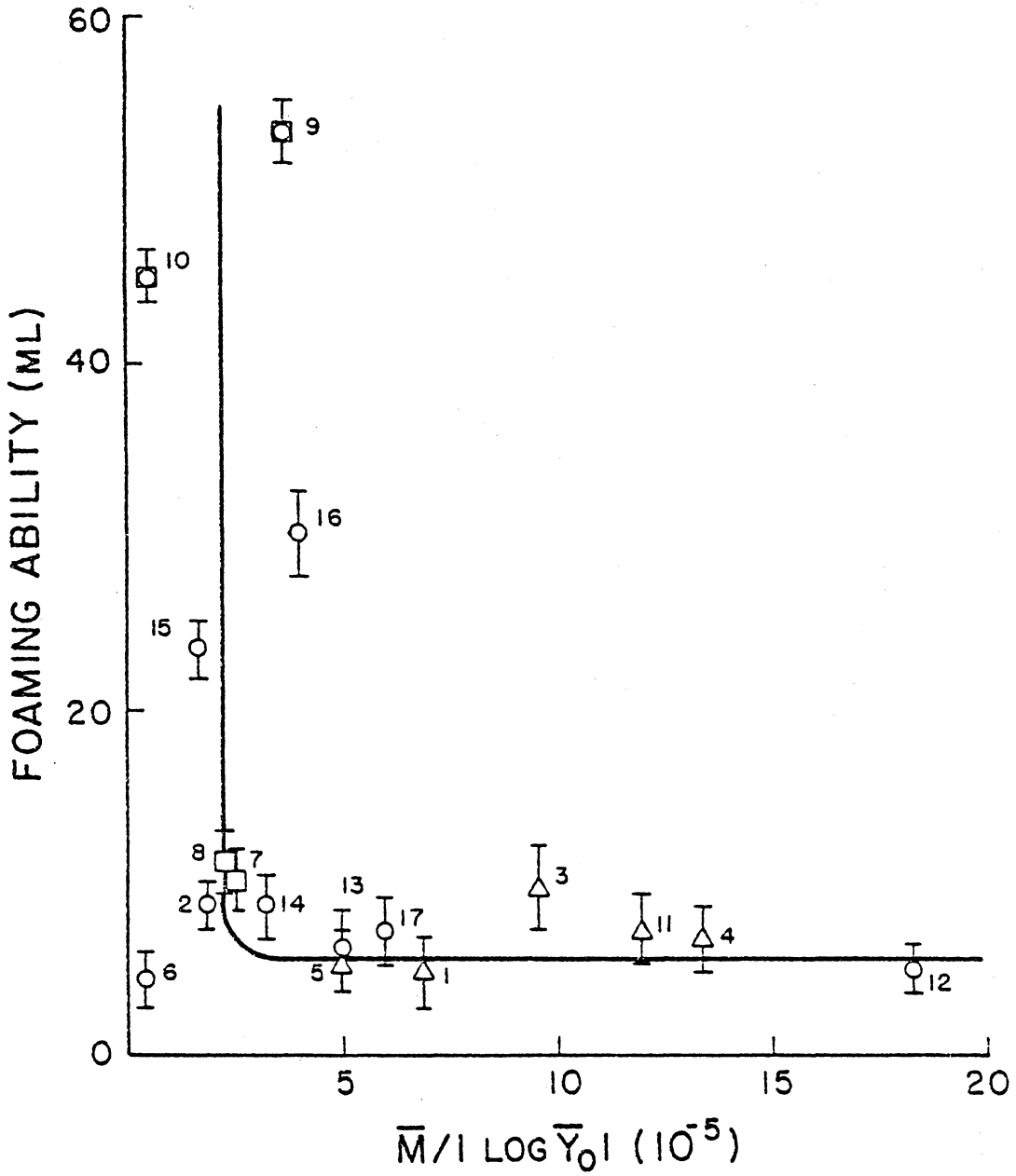
Fig. 54. Foaming ability of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified proteins in Table 13.

immobilized enzymes. This suggested that other factor(s), besides molecular size and charge, are involved in the foaming ability of the modified soybean proteins. A limited amount of hydrolysis appears to be desirable to increase foam volume of the proteins. This is probably due to an increase in the polypeptide content, which acting as emulsifiers, allow air to be incorporated. However, the polypeptides do not have the strength required to maintain a stable foam, and further hydrolysis likely results in forming peptides which lack any capacity to stabilize the air cells of the foam. Kabirullah and Wills (1981) reported that the inactivation methods for soluble trypsin affected the foaming capacity of hydrolyzed sunflower protein. They also demonstrated that when enzyme inactivation was conducted by heating instead of by pH change, the optimum degree of peptic hydrolysis of sunflower protein required a high degree of hydrolysis. Succinylated and hydrolyzed fish protein had maximum foaming capacity when the acylated epsilon amino groups were 54% of the total available amino groups (Groninger and Miller, 1975). Likewise, the largest foam volume was obtained with 50% a succinylated sunflower sample followed by 100% and 10% succinylated products (Canella et al., 1979). However, Kabirullah and Wills (1982) showed that succinylated sunflower protein had high foaming capacity with increasing succinylation. This indicates that size, charge, and conformation of protein molecules play important roles in increasing foaming ability, perhaps in addition to other factors not considered here. Halling (1981) reviewed the literature on pH dependency of

emulsifying capacity to provide more data for interpretation of the foaming mechanisms in a manner consistent with other views and to explain certain anomalous effects observed by several investigators. Furthermore, the absolute and apparent foaming properties of proteins are influenced by numerous factors such as protein source, protein concentration, pH, temperature, denaturation by heating, presence of salts, sugars, and lipids, and method of measurement (Kinsella, 1976).

4.858   Foam stability

As shown in Fig. 55, the foam stability of modified soybean proteins is a function of the ratio of the average molecular weight to the average molecular charge. The foam stabilities of modified soybean protein with a ratio above 5×10^5 leveled off. Nevertheless, at values lower than the ratio 2.5×10^5, the foam stability of soybean proteins was reduced. Clearly, the small protein size with or without highly charged protein molecules had low foam stability. Phillips and Beuchat (1981) discussed that all enzyme treatments, in general, led to inferior foam stability as compared to that of the control. This is due to the low surface rigidity of the hydrolyzed protein below that required to maintain a stable foam. Since surface elasticity and viscosity was maximal near the pI, foam stability was at a maximum near the pI of proteins (Halling, 1981). The films are deformable but compact and tough because of maximum electrostatic adhesion. On the other hand, although film strength is maximum at pI because of extensive
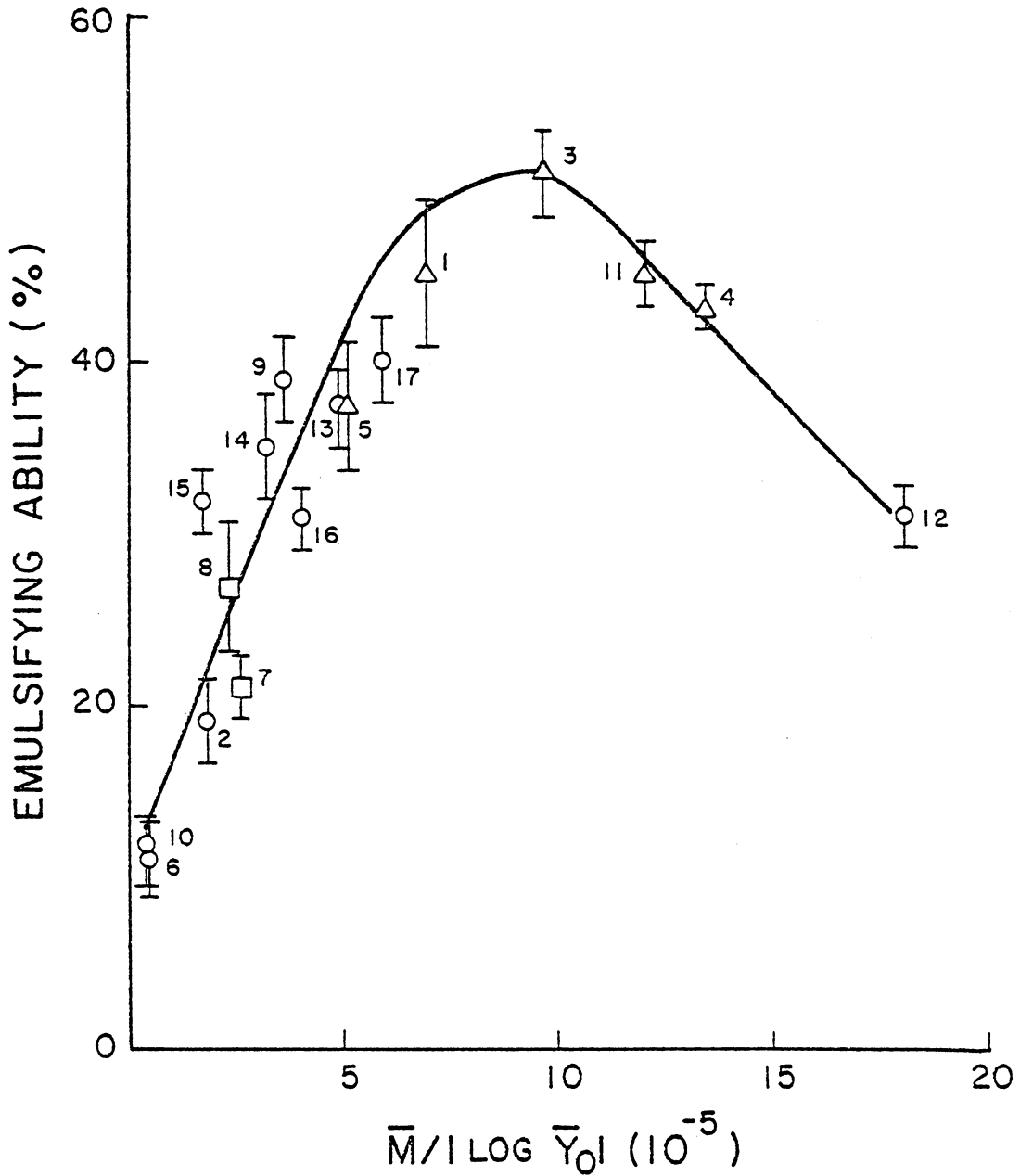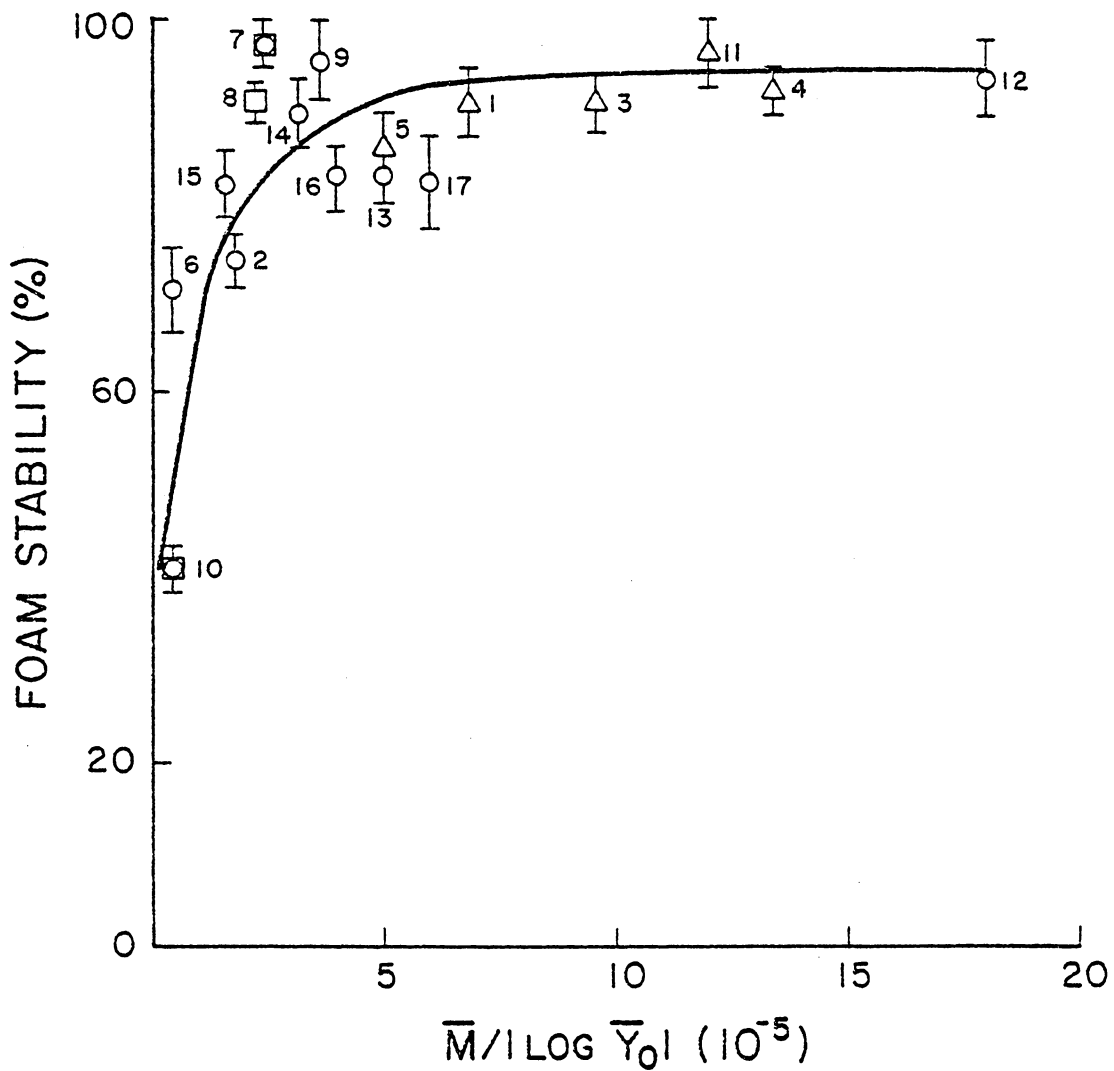
Fig. 55. Foam stability of modified soybean proteins as a function of average molecular weight/average molecular charge. Numbers refer to the modified soybean proteins in Table 13.

interactions, concurrent coagulation may reduce foam stability. Cherry et al. (1979) reported that the foaming stability of peanut protein was maximized by heating at 50°C for 50 min. The hydrolysate of rapeseed protein concentrate by acids, alkali, and pepsin showed low foam drainage compared to the untreated control (Hermansson et al., 1974). Thus, partial denaturation without loss of solubility by enzymes, acid, alkali, sulfite, and heat could be beneficial to foam stability. These processes are related to the unfolding of peptides at the interface, facilitating hydrophobic interactions, increasing film thickness and viscosity, as well as improving foam stability. Moreover, the partial denaturation of certain proteins increased their hydrophobicity and enhanced their surface activity (Kinsella, 1981). The increasing value of foam stability of succinylated sunflower was high initially and then moderate, and then high again with increasing succinylation (Kabirullah and Wills, 1982).

4.9 Basic concepts of modifications to maximize the functional properties of soybean proteins

The desired functional properties of soybean proteins can be obtained by the proper selection of type of modification, and by controlling the extent of modification (Table 14). Solubility and foaming ability of soybean proteins were enhanced by hydrolysis and/or succinylation. Limited hydrolysis with immobilized protease(s) and/or limited succinylation improved water holding capacity, oil holding

Table 14. Basic concepts of modifications to maximize the
functional properties of soybean proteins.

Functional property	Basic modification	Reference section
Increased solubility	Hydrolysis and/or succinylation	4.31, 4.5, 4.7, 4.851
Increased water holding capacity	Limited hydrolysis and /or limited succinylation	4.32, 4.6, 4.852
Increased oil holding capacity	Limited hydrolysis and /or limited succinylation	4.33, 4.6, 4.7, 4.853
Increased viscosity	Limited hydrolysis and /or limited succinylation	4.34, 4.6, 4.7, 4.854
Increased emulsifying ability	Limited hydrolysis, limited succinylation, and/or fractionation	4.35, 4.5, 4.7, 4.855
Increased emulsion stability	Limited succinylation and/or fractionation	4.36, 4.6, 4.7, 4.856
Increased foaming ability	Hydrolysis and/or succinylation	4.37, 4.5, 4.7, 4.857
Increased foam stability	Limited succinylation and/or fractionation	4.38, 4.6, 4.7, 4.858

capacity, and relative viscosity of the proteins. To obtain maximum emulsifying ability of soybean proteins, limited hydrolysis, limited succinylation, and/or fractionation were required. The surface stabilities of soybean proteins could be increased by limited succinylation and/or fractionation. Therefore, to apply specifically soybean proteins to food systems, the method and the extent of modification are important so as to have desired molecular properties having optimum molecular weights and molecular charges.

5.0   SUMMARY AND CONCLUSIONS

Conditions necessary to produce size and charge modification of

soybean proteins by immobilized trypsin and alpha-chymotrypsin were

studied. Nylon pellets and porous glass were selected as supporting

materials for the covalent bonding of the enzymes through aldehyde, acid

azide and isothiocyanate reactions. For maximum immobilization, the

effects of glutaraldehyde, enzyme concentration and chemical treatments

of the nylon support were investigated. Physical constants, optimum

conditions and stability of the immobilized enzymes were evaluated. A

multi-enzyme system with immobilized trypsin and chymotrypsin was found

to be an efficient hydrolytic system as well as to produce various de-

sirable products of hydrolysis.

The molecular charges of soybean protein were modified by succinyl-

ation. Various functional properties of modified soybean proteins were

measured. Molecular weights and molecular charges were evaluated by

polyacrylamide gel electrophoresis with or without sodium dodecyl

sulfate (SDS). Relationships between functional properties and molecu-

lar properties of modified proteins were determined by using the

constant relationship of average molecular weight to average molecular

charge.

The following are the main findings:

1. The amount of immobilized trypsin bound on nylon pellets by

glutaraldehyde was maximized by treating pellets with a methanolic

solution and 6 - 8% glutaraldehyde as well as by having high concentrations of available soluble trypsin.

2. In comparison with the soluble enzyme, immobilized trypsin had broad maximum activity over pH, and higher temperature stability.

3. Immobilized chymotrypsin had optimum activity at pH 8.5, while for soluble chymotrypsin the optimum pH was 8.0.

4. At critical pH values, immobilized trypsin hydrolyzed soybean protein isolate more efficiently than immobilized chymotrypsin.

5. A multi-immobilized enzyme system made up of trypsin and chymotrypsin showed a higher rate of hydrolysis of soybean proteins than single immobilized trypsin and chymotrypsin. The rate of hydrolysis was highest when there was a high proportion of immobilized chymotrypsin in the multi-enzyme system.

6. Immobilized protease(s) increased the number of alpha-amino groups of soybean proteins. Generally, by controlling the degree of hydrolysis of the proteins, it was possible to increase their water holding capacity, oil holding capacity, and relative viscosity, and to improve their emulsifying and foaming characteristics. When hydrolysis was carried further, emulsion and foam stability were reduced.

7. Succinylation reduced amino and lysyl groups of soybean protein. When soybean proteins were modified by hydrolysis and then succinylation, the available amino and lysyl groups were reduced considerably more.

8. Partial fractionation of soybean proteins improved specific

functional properties. Comparing soybean protein isolate with 7S and 11S protein rich fraction (PRF), the following conclusions were reached.

8.1 The 7S PRF had the highest oil holding capacity and foam stability.

8.2 The 11S PRF had the highest water holding capacity.

8.3 The cold insoluble fraction (CIF) had the highest soluble amino groups, emulsifying ability, emulsion stability, and foaming ability.

9. When 7S PRF, 11S PRF and CIF were further hydrolyzed by immobilized trypsin and chymotrypsin, the solubility, relative viscosity and foaming ability of the protein fractions increased; however, the hydrolyzed fractions had lower liquid holding capacities, emulsifying ability, emulsion stability, and foam stability.

10. Increased succinylation of soybean proteins enhanced their oil holding capacity, increased viscosity, and improved emulsifying ability, emulsion stability, foaming ability, and foam stability, but reduced their water holding capacity.

11. Except for emulsifying ability and emulsion stability, hydrolyzed and then progressively succinylated soybean proteins showed similar trends in functional properties when compared with proteins that had been only succinylated.

12. Progressively succinylated and then hydrolyzed soybean proteins had lower liquid holding capacities, lower relative viscosity, and lower emulsifying ability and foam stability than those that had been

only succinylated. However, hydrolyzed and then succinylated proteins had higher emulsifying ability.

13. SDS polyacrylamide gel electrophoresis used for the evaluation of molecular size of soybean proteins indicated that the relative protein peak heights were different from one modified protein to another.

13.1 Succinylation increased the molecular weight of the proteins due to the molecular expansion.

13.2 Succinylated and then hydrolyzed proteins had lower molecular weight components than hydrolyzed and then succinylated proteins due to the molecular expansion and the modification of protease inhibitors.

13.3 Immobilized chymotrypsin hydrolyzed all components of soybean proteins equally. However, immobilized trypsin preferentially hydrolyzed acidic subunits of proteins.

14. When SDS was not used to evaluate molecular size and molecular charge, gels containing a 7M urea concentration produced better band separation in a soybean protein isolate with 1% Triton X-100 and 1% 2-mercaptoethanol.

14.1 Partially separated fractions had distinctive peak heights.

14.2 Slow moving components were separated better on low gel concentrations, while fast moving components were separated better on high gel concentrations.

14.3 Soybean proteins succinylated with succinic anhydride indicated that subunits of soybean proteins soluble in

neutral detergents had been separated.

14.4 High absorbance of components with molecular weights of

29,500 and 19,000 of 11S PRF showed acidic and basic

subunits, respectively. Succinylation increased net

molecular charges.

14.5 Immobilized trypsin preferentially hydrolyzed intermediate

subunits.

15. The ratio of average molecular weights to average molecular

charges is a constant and is an indication of the extent of the

functional properties of modified soybean proteins.

15.1 The correlation coefficient between average molecular

weight and average free mobility was 0.74.

15.2 Modified soybean proteins with a constant lower than

5×10^5 for the molecular weight to molecular charge

ratio had higher soluble amino groups, higher foaming

ability, lower water holding capacity, and lower foam

stability.

15.3 Oil holding capacity, emulsifying ability, and emulsion

stability were maximum at the constant 9×10^5.

15.4 Relative viscosity was maximum at the constant 2.5×10^5.

In short, trypsin and chymotrypsin immobilized by glutaraldehyde

on nylon pellets can be utilized to change molecular sizes of soybean

proteins in order to produce desirable modifications in their functional

properties. Furthermore, molecular size and charge modification have

been achieved by partial fractionation and succinylation, respectively, as well as by combined treatments of immobilized enzyme(s) and succinylation. Electrophoresis analysis showed that there were various molecular size and charge components in each modified protein. The ratio of average molecular weight to average molecular charge can explain various functional properties of diversely modified soybean proteins.

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7.0   APPENDICES

Appendix I. Physical constants determined by PAGE[a] for 7S protein rich fraction of soybean proteins.

Band	K_R	$\log Y_o$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0556	-0.6893	30	2.89
2	0.0638	-0.3897	40	5.23
3	0.0808	0.0489	63.5	14.70
4	0.0687	0.1300	47	11.29
5	0.0741	0.2201	53.5	6.86
6	0.0617	0.2714	37.5	2.53
7	0.0728	0.4189	52	5.25
8	0.0694	0.4218	47.5	2.93
9	0.0134	-0.0662	3.5	4.32
10	0.0093	-0.0104	2.5	1.75
11	0.0109	0.0613	3	6.93

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix II. Physical constants determined by PAGE[a] for cold insoluble fraction of soybean proteins.

Band	K_R	log Y_0	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0867	-0.3587	73.5	2.59
2	0.0605	-0.4437	36	4.00
3	0.0804	0.0239	62.5	9.61
4	0.0684	0.1192	46.5	8.84
5	0.0721	0.2102	51	6.27
6	0.0643	0.2946	41	1.89
7	0.0653	0.3272	42	4.08
8	0.0631	0.0346	39	4.01
9	0.0122	0.0691	3.5	3.40

[a] PAGE, K_R and Y_0 are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00 % acrylamide.

Appendix III. Physical constants determined by PAGE[a] soybean protein isolate.

Band	K_R	$\log Y_o$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0027	-1.4257	1	2.44
2	0.0900	-0.2993	79.5	2.89
3	0.0806	-0.1585	63	4.49
4	0.0820	0.0518	65	14.83
5	0.0697	0.1076	47.5	9.42
6	0.0756	0.2165	55.5	8.80
7	0.0634	0.2825	40	2.91
8	0.0696	0.3761	48	2.93
9	0.0704	0.4327	49	4.15
10	0.0073	0.0103	2	2.69

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix IV. Physical constants determined by PAGE[a] for 11S
protein rich fraction of soybean proteins.

Band	K_R	log Y_0	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0634	-0.4196	39.5	2.45
2	0.0822	0.0352	65	3.17
3	0.0660	0.1222	43.5	5.91
4	0.0615	0.2621	37	2.67
5	0.0525	0.2420	27.5	4.08
6	0.0072	0.0181	2	3.80

[a] PAGE, K_R and Y_0 are polyacrylamide gel electrophoresis, retarda-
tion coefficient and relative free mobility, respectively.
Samples were solubilized in 1% Triton X-100 and 2-mercapto-
ethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix V. Physical constants determined by PAGE[a] for succinylated 11S protein rich fraction of soybean proteins.

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0572	-0.5461	32	1.83
2	0.0723	-0.2106	51.5	2.57
3	0.0327	-0.4309	11.5	1.94
4	0.0697	0.1204	48	9.37
5	0.0617	0.0836	37.5	2.12
6	0.0623	0.1288	38	5.73
7	0.0616	0.1955	37	1.43
8	0.0568	0.2226	32	3.04
9	0.0543	0.2442	29.5	11.36
10	0.0444	0.2011	19	9.13
11	0.0413	0.2092	17.5	4.39
12	0.0279	0.1358	9	0.76
13	0.0136	0.2805	4	1.19
14	0.0086	0.0265	2.5	3.33

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix VI. Physical constants determined by PAGE[a] for succinylated soybean protein isolate.

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0895	0.0287	79.5	2.35
2	0.0537	-0.2315	28.5	2.72
3	0.0741	0.1721	53.5	6.65
4	0.0648	0.1242	41.5	1.92
5	0.0647	0.1591	41.5	3.44
6	0.0646	0.2316	41	1.77
7	0.0593	0.2483	34.5	1.99
8	0.0535	0.2465	28.5	6.75
9	0.0475	0.2334	22	5.93
10	0.0396	0.1959	15.5	4.09
11	0.0402	0.2592	15.5	0.89
12	0.0413	0.2920	16.5	0.80
13	0.0080	0.0196	2.5	1.97

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix VII. Physical constants determined by PAGE[a] for soybean
protein isolate hydrolyzed by immobilized trypsin/
chymotrypsin (1:1) and succinylated.

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0229	-0.3429	7	0.73
2	0.0472	-0.0349	21.5	2.97
3	0.0471	0.0780	21.5	2.19
4	0.0428	0.1423	17.5	2.80
5	0.0418	0.1797	17	1.48
6	0.0420	0.2246	17	4.36
7	0.0239	0.1024	7.5	1.07
8	0.0153	0.0584	4.5	1.37
9	0.0047	-0.0135	1.5	5.69

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retarda-
tion coefficient and relative free mobility, respectively.
Samples were solubilized in 1% Triton X-100 and 1% 2-mercapto-
ethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix VIII. Physical constants determined by PAGE[a] for
soybean protein isolate succinylated and
hydrolyzed by immobilized trypsin/chymo-
trypsin (1:1).

Band	K_R	$\log Y_o$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0688	-0.9918	47	4.55
2	0.0260	-0.2369	8	3.16
3	0.0286	-0.1775	9	0.77
4	0.0279	-0.1599	9	0.84
5	0.0331	-0.0825	11.5	1.49
6	0.0195	-0.0902	5.5	4.67
7	0.0170	-0.0549	4.5	1.95
8	0.0150	-0.0348	4	5.23
9	0.0105	0.0064	3	3.33
10	0.0049	-0.0086	1.5	10.56

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retar-
dation coefficient and relative free mobility, respectively.
Samples were solubilized in 1% Triton X-100 and 1% 2-mercapto-
ethanol. Concentrations of gels were 5.25 to 12.00% acryl-
amide.

Appendix IX. Physical constants determined by PAGE[a] for soybean protein isolate hydrolyzed by immobilized chymotrypsin.

Band	K_R	$\log Y_0$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0855	-0.9713	71.5	1.28
2	0.0672	-0.5736	45	2.59
3	0.0744	-0.3405	54	2.91
4	0.0951	-0.3495	92.5	3.11
5	0.0774	-0.0090	58.5	9.28
6	0.0608	0.0470	36	6.05
7	0.0630	0.0821	38.5	5.00
8	0.0639	0.2781	40.5	1.03
9	0.0697	0.3676	48	2.49
10	0.0583	0.2968	33.5	3.19
11	0.0088	0.0183	2.5	2.12

[a] PAGE, K_R and Y_0 are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix X. Physical constants determined by PAGE[a] for soybean protein isolate hydrolyzed by immobilized trypsin.

Band	K_R	$\log Y_0$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0638	-0.9955	40	3.36
2	0.0627	-0.3163	38.5	2.72
3	0.0690	0.0624	47	3.92
4	0.0550	-0.1305	30	2.05
5	0.0418	-0.2091	17	2.68
6	0.0467	-0.1058	21.5	0.84
7	0.0336	-0.0801	11.5	1.33
8	0.0493	0.1413	24	1.67
9	0.0489	0.1876	23.5	1.81
10	0.0192	0.0170	5.5	1.87
11	0.0413	0.0219	4	2.39
12	0.0102	0.0362	2.5	4.40

[a] PAGE, K_R and Y_0 are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix XI. Physical constants determined by PAGE[a] for soybean protein isolate hydrolyzed by immobilized trypsin/ chymotrypsin (1:9).

Band	K_R	$\log Y_o$	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0438	-0.8344	18.5	4.01
2	0.0783	-0.3236	60	2.79
3	0.0762	-0.2240	56	2.88
4	0.0761	-0.0175	56.5	8.69
5	0.0644	0.0561	43	4.09
6	0.0666	0.1391	47	3.19
7	0.0516	0.1643	27.5	2.52
8	0.0485	0.1773	25	1.56
9	0.0360	0.1058	13.5	1.95
10	0.0346	0.1702	12	1.61
11	0.0258	0.0932	8	1.65
12	0.0211	0.0969	6	1.68
13	0.0090	0.0211	2.5	3.79

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercapto-ethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix XII. Physical constants determined by PAGE[a] for 7S
protein rich fraction hydrolyzed by immobilized
trypsin/chymotrypsin (1:1).

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0619	-1.1831	37	1.63
2	0.0647	-0.7854	41	2.43
3	0.0686	-0.0764	46.5	7.28
4	0.0604	-0.0579	36.5	6.43
5	0.0383	-0.0632	14.5	2.21
6	0.0387	-0.0174	14.5	1.49
7	0.0288	-0.0349	9	2.52
8	0.0268	-0.0078	8.5	3.92
9	0.0264	0.0941	8	1.87
10	0.0120	0.0227	3.5	2.58
11	0.0091	0.0402	2.5	6.84

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis,
retardation coefficient and relative free mobility, respect-
ively. Samples were solubilized in 1% Triton X-100 and 1%
2-mercaptoethanol. Concentrations of gels were 5.25 to
12.00% acrylamide.

Appendix XIII. Physical constants determined by PAGE[a] for 11S
protein rich fraction hydrolyzed by immobilized
trypsin/chymotrypsin (1:1).

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0379	-0.5097	14	10.80
2	0.0471	0.1276	21.5	1.51
3	0.0226	-0.0457	7	3.05
4	0.0110	-0.0448	3	2.09
5	0.0184	0.0836	5	2.87
6	0.0076	0.0197	2.5	8.45

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retarda-
tion coefficient and relative free mobility, respectively.
Samples were solubilized in 1% Triton X-100 and 1% 2-mercapto-
ethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix XIV. Physical constants determined by PAGE[a] for soybean protein isolate hydrolyzed by immobilized trypsin/ chymotrypsin (1:1).

Band	K_R	log Y_o	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0402	-1.1763	15.5	3.04
2	0.0529	-0.6287	28	1.79
3	0.0820	-0.0902	65	2.81
4	0.0742	-0.0645	53.5	4.59
5	0.0632	-0.0941	39.5	3.03
6	0.0509	-0.1350	25.5	3.25
7	0.0369	-0.1701	13.5	2.35
8	0.0454	0.0342	20	2.19
9	0.0460	0.1226	20.5	2.40
10	0.0452	0.1592	20	2.44
11	0.0330	0.1444	11.5	1.91
12	0.0373	0.2011	14	1.95
13	0.0258	0.1257	8	1.52
14	0.0378	0.2706	14	1.41
15	0.0597	0.0577	35.5	1.16
16	0.0059	0.0118	2	4.77

[a] PAGE, K_R and Y_o are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

Appendix XV. Physical constants determined by PAGE[a] for soybean isolate partially hydrolyzed by immobilized trypsin/chymotrypsin (1:1).

Band	K_R	log Y_0	Mol. Wt. (x 1/1,000)	Peak area (cm^2)
1	0.0645	-0.9689	41	3.63
2	0.0801	-0.2673	62.5	1.00
3	0.0759	0.0025	55.5	17.13
4	0.0564	-0.0799	31	3.09
5	0.0468	-0.1039	21	4.72
6	0.0506	0.0776	33.5	4.80
7	0.0500	0.1598	24.5	3.85
8	0.0507	0.2116	30	1.65
9	0.0480	0.2452	22.5	1.77
10	0.0408	0.2195	16	1.81
11	0.0363	0.1921	13	1.85
12	0.0595	0.5021	34.5	1.00
13	0.0325	0.2392	11	1.00
14	0.0137	0.0862	4	4.31

[a] PAGE, K_R and Y_0 are polyacrylamide gel electrophoresis, retardation coefficient and relative free mobility, respectively. Samples were solubilized in 1% Triton X-100 and 1% 2-mercaptoethanol. Concentrations of gels were 5.25 to 12.00% acrylamide.

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MODIFICATION OF SOYBEAN PROTEINS BY IMMOBILIZED PROTEASES

by

Jin Woo Lee

(ABSTRACT)

Trypsin and alpha-chymotrypsin were immobilized on nylon pellets or porous glass by covalent methods to change molecular properties and functional characteristics of soybean proteins. The amount of trypsin immobilized on nylon pellets using the glutaraldehyde method was high when the pellets were treated with methanolic solution and 6 - 8% glutaraldehyde as well as high concentrations of soluble trypsin. Immobilized trypsin and chymotrypsin had uniform pKm and were stable at high temperatures. The optimum pH for activity of immobilized enzymes could be changed by using different supports and different methods of immobilization. A multi-enzyme system with immobilized trypsin and chymotrypsin was designed to produce an efficient hydrolysis and various desirable products of hydrolysis.

Controlled hydrolysis of soybean proteins by immobilized enzyme(s) increased water holding capacity, oil holding capacity, and relative viscosity, and improved emulsifying and foaming characteristics. Hydrolysis by immobilized protease(s) increased solubility, relative viscosity and foaming ability of partially purified fractions. Succinylated soybean proteins had high oil holding capacity, viscosity,

ABSTRACT (CONTINUED)

emulsifying ability, emulsion stability, and foaming ability. The order
in which succinylation and hydrolysis by immobilized enzymes were done,
conferred on soybean proteins various functional properties.

Evaluation of the molecular size of modified soybean proteins
with sodium dodecyl sulfate (SDS) indicated that immobilized trypsin
and chymotrypsin preferentially hydrolyzed specific protein components,
and that succinylation enhanced hydrolysis, expanded protein molecules,
and dissociated subunits. Measurement of molecular size and charge of
the modified soybean proteins without SDS showed that immobilized
trypsin hydrolyzed the intermediate subunits relatively fast, and
succinylation separated the intermediate subunits. Succinylation in-
creased the average molecular charge of soybean proteins, while
hydrolysis decreased their average molecular size and their average
molecular charge. The ratio of the average molecular weight to the
average molecular charge could explain various functional properties.
When the ratio was less than 5.0×10^5, the modified soybean proteins
had high soluble amino groups, high foaming ability, low water holding
capacity and low foam stability. When the ratio was 9.0×10^5, oil
holding capacity, emulsifying ability, and emulsion stability were
maximum. Relative viscosity was high at a constant value of 2.5×10^5.