MODIFICATION OF FUNCTIONAL PROPERTIES OF SOY PROTEINS BY PROTEOLYTIC ENZYME TREATMENT[1]

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ABSTRACT

Soy protein isolate was treated with various amounts of neutral protease preparation from *Aspergillus oryzae*. The extent of enzyme treatment was determined by measuring the increase in free amino groups. The functional properties of these enzyme-treated soy isolates and untreated controls were examined. As expected, soluble nitrogen was increased, both at neutral pH and at the isoelectric point, and in the presence of 0.03*M* calcium chloride, with increased enzyme treatment. Even limited enzyme treatment significantly reduced the viscosity of concentrated protein solutions and prevented gel formation. Emulsification capacities, as measured by a modified Swift *et al.* method, were increased with enzyme treatment, but emulsion stabilities were decreased. The enzyme-treated proteins had slightly increased water absorption and foaming properties, but foam stability was very low.

Utilization of plant proteins, especially soy proteins, is an old art. Tofu, yuba, and other soybean products have been used in the Orient for hundreds of years. Even in this country a commercial soybean protein isolate has been available since 1959. Owing to improvements in the technology of soy protein production and utilization, coupled with increased costs for meat products, the demand for plant protein has increased tremendously in recent years.

Although commercial soy protein products can fulfill a number of functional properties required of them in today's market, in many cases there is a need to

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modify these properties for specific applications. For example, the various texturization processes opened up a whole new area for utilization of soy protein products.

Enzyme modification of soy proteins could offer a second or third generation of products which perhaps would allow an even broader range of utilization. Actually, in one area this is already achieved by using a pepsin-digested soy protein product to make a whipping protein for egg white replacement (1,2). The utilization of enzyme treatment to increase the solubility of heat-denatured protein (3) and to increase acid solubility (4) was also described recently. However, thus far there is little published information on any systematic study of the effect of enzyme treatment on the various functional properties of soy proteins, such as solubility in both neutral and acid food systems, calcium tolerance, emulsification, gelling properties, water and fat absorption, and foaming. Perhaps one reason for this is that there are no well-accepted methods available to measure some of these functional properties and, also, it is difficult to correlate the results of measurements in a model system with what happens in a complex food system.

In this study, soy protein isolate was hydrolyzed to various degrees with one enzyme, and an attempt was made to measure the change in various functional properties as related to the extent of enzyme treatment.

MATERIALS AND METHODS

Sample Preparation

Soy protein isolate was obtained by aqueous extraction of defatted soybean flakes at pH 8 (ratio of flakes to water 1:20), clarification of the extract by centrifugation, and precipitation of the globulins by acidification to pH 4.5 with HCl. The protein suspension was then washed with water, adjusted to pH 7 with NaOH solution, and freeze-dried.

The enzyme-treated samples were prepared by adding the required amount of enzyme to a 15% aqueous dispersion of the soy protein isolate. The dispersion was stirred for 3 hr at pH 7 and 50°C, then heated at 70°C for 10 min. to inactivate the enzyme, followed by freeze-drying. A neutral protease preparation from *Aspergillus oryzae* was used for these treatments. The following samples were prepared:

1. Untreated control—freeze-dried soybean protein isolate.
2. Heat-treated control—treated as described above except no enzyme was added.
3. Enzyme treatment A—0.030 g enzyme was added per l. of protein dispersion.
4. Enzyme treatment B—0.075 g enzyme was added per l. of protein dispersion.
5. Enzyme treatment C—0.15 g enzyme was added per l. of protein dispersion.
6. Enzyme treatment D—0.30 g enzyme was added per l. of protein dispersion.

Free Amino Group Determination

The method of Habeeb (5) was followed with slight modification. Since some of the products were not completely soluble in water, they were dispersed in 1% sodium dodecyl sulfate (SDS) solution at pH 9.5.

To 1 ml of a 1% SDS solution of protein (0.3–1 mg protein/ml) were added 1 ml of 4% $NaHCO_3$, pH 9.5, and 1 ml of 0.1% trinitrobenzene-sulfuric acid (TNBS). The solution was allowed to react at 40° C for 2 hr; then 1 ml 10% SDS was added to prevent precipitation on further addition of 0.5 ml of 1*N* HCl.

The absorbance of the solution was read at 335 nm against a blank treated as described above but with 1 ml of 1% SDS solution instead of the protein solution. The absorbance reading was converted to free amino groups by preparing a standard curve using glycine solutions of increasing concentrations.

Solubility at Neutral pH

Nitrogen Solubility Index (NSI) was measured according to the AACC Standard Method 46-23 (6).

Solubility at Acid pH

Three grams of sample was dispersed in 100 ml of water for 30 min., and the pH was adjusted to 4.5 with 6*N* HCl, and stirred for 10 min. A sample was taken for nitrogen analysis; then the remainder of the dispersion was centrifuged at 1,300 × *g* for 10 min. The supernatant was also analyzed for nitrogen.

$$\%\text{ Acid sol. protein} = \frac{\%\text{ Nitrogen in supernatant}}{\%\text{ Nitrogen in uncentrifuged dispersion}} \times 100$$

Solubility in 0.03*M* Calcium Chloride Solution

Three grams of sample was dispersed in 90 ml distilled water for 30 min., then 0.33 g of $CaCl_2$ was added. The pH was readjusted to 7.0, stirred further for 30 min., and the solution made up to 100 ml. A portion of the stirred dispersion was analyzed for nitrogen; the remainder was centrifuged at 1300 × *g* for 10 min. The clear supernatant was also analyzed for nitrogen.

$$\%\text{ Protein soluble in } 0.03M\ CaCl_2 = \frac{\%\text{ Nitrogen in supernatant}}{\%\text{ Nitrogen in uncentrifuged dispersion}} \times 100$$

Emulsifying Properties

Various methods have been suggested to measure the emulsification properties of proteins, but none of these has met with general acceptance. Swift *et al.* (7) proposed a measure of emulsion capacity as a means of studying the emulsifying properties of meat proteins. In their test method, oil is added continuously at a given rate to a stirred protein solution until the initially formed emulsion breaks as observed by a sudden drop of viscosity. Emulsion capacity can be defined as the quantity of oil emulsified by a given protein solution under the specified conditions of the test. A large number of experimental factors, such as the type of stirring apparatus, speed of stirring, type of fat, rate of fat addition, temperature, geometry and size of the containers, influences the result. Several modifications of this method have been suggested (8–11), consisting mostly in optimizing some of these experimental conditions. The major disadvantage of this method is the difficulty of reproduction of results since even small variations in technique may give significantly different results. Yasumatsu *et al.* (12) and

Titus *et al.* (13) suggested the emulsification of fixed amounts of oil and protein solution. They differ in the method of emulsification and in the detection of emulsion instability. Yasumatsu *et al.* (12) measure the volume of the emulsified layer after centrifugation whereas Titus *et al.* (13) measure fat depletion in the lower portion of the emulsion after standing. The latter method detects emulsion instability even when no visual separation occurs; however, it requires extensive analytical work for fat analysis rather than a simple reading of volumes. Both of these methods measure creaming as emulsion instability in addition to fat separation. Each of these general methods was used in this study.

1. *Emulsifying Properties by the Method of Yasumatsu et al. (12).* Emulsifying activity: 7 g of soybean product was suspended in 100 ml of water, and 100 ml of soybean salad oil was added to it. The mixture was emulsified with Omni-Mixer (Sorvall, Type OM) at 10,000 rpm for 1 min. The emulsion obtained was divided evenly into four 50-ml centrifuge tubes and centrifuged at 1300 × *g* for 5 min.

$$\text{Emulsifying activity} = \frac{\text{Volume of emulsified layer}}{\text{Volume of whole}} \times 100$$

Emulsion stability: The emulsion prepared by the procedure for measurement of emulsifying activity was heated for 30 min. at 80° C, cooled with tap water for 15 min., divided evenly into four centrifuge tubes, and centrifuged at 1300 × *g* for 5 min.

$$\text{Emulsion stability} = \frac{\text{Volume of emulsified layer}}{\text{Volume of whole}} \times 100$$

2. *Emulsion Capacity by a Modification of Swift et al. (7) Procedure.* The protein samples were dispersed to give 0.5% protein suspension (% N × 6.25). A 35-ml portion of the suspension was pipetted into a 1000-ml beaker suspended in ice bath and stirred with an overhead, propeller-type mixer (Talboy Engineering) with rheostat set at 60 while adding 250 ml soybean salad oil from a graduated cylinder at the rate of about 8 ml/sec. The rheostat setting was then increased to 140, and soybean salad oil was added from the buret at the rate of 2–3 ml/sec until the emulsion which initially formed inverted. The inversion point was determined by the sudden thinning of the oil-water mixture. The test was run in triplicate, and the amount of soybean salad oil emulsified was averaged.

$$\text{Emulsion capacity} = \frac{\text{ml oil emulsified}}{\text{ml protein sol.}} = \frac{\text{ml of soybean oil}}{35}$$

3. *Emulsion Stability Index by a Modified Procedure of Titus et al. (13).* Four hundred milliliters of 1% aqueous protein dispersion and 600 ml of soybean salad oil were prewarmed to 60° C, mixed together, then homogenized with a Manton-Gaulin two-stage homogenizer at 3000 + 500 psi pressure. A portion of the emulsion was taken immediately and analyzed for fat content. The remainder of the emulsion was held undisturbed at 4° C for 24 hr, after which a second sample was taken from the lower quarter portion of the undisturbed emulsion and analyzed again for fat content.

$$\text{Stability index} = \frac{\%\ \text{Fat in lower portion after standing}}{\%\ \text{Fat in total}} \times 100$$

Water Absorption

The method described by Mellon *et al.* (14) was used. Saturated potassium chloride solution was placed in the bottom of the vacuum desiccator to give 84% relative humidity. Samples of approximately 0.75 g were added to weighing bottles of 23 mm diameter and placed, uncovered, in the desiccator. The samples were incubated for 24 hr at 75° F, and the moisture uptake was measured.

Gelation

The gelation properties of soy proteins were described by Circle *et al.* (15) and Catsimpoolas and Meyer (16). The conditions for the test were taken from their publications.

The soy protein was dissolved in distilled water to give a 10% w/v protein dispersion which was then heated to 80° C for 30 min. The samples were immediately cooled in an ice bath and equilibrated to 25° C. The viscosity of the gels was measured with a Brookfield viscometer Model RVT with helipath stand and T-bar spindles.

Whipping Properties

Foam expansion and foam stability have been suggested as the indices of whipping property (12). We found the stirring method for whipping is simpler and more reproducible than shaking.

A 1% aqueous suspension of the soybean product was prepared. One hundred milliliters of this suspension was placed in a 250-ml volume Omni-Mixer cup, attached to the Omni-Mixer (Sorvall, Type OM), and stirred at 10,000 rpm for 1 min. The content was immediately transferred to a 250-ml graduated cylinder and the foam volume noted. This foam volume was defined as "foam expansion." After standing for 30 min., the residual foam volume was measured again, and the result was described as "foam stability."

RESULTS AND DISCUSSION

A critical problem in studying the effect of enzyme treatment on the functional properties of proteins is the elimination or inactivation of the enzyme at the end of the desired treatment. If heat inactivation is used, the proteins may be denatured and insolubilized, and thus one actually measures the combined effect of enzyme and heat treatments. Washing out the enzyme at the isoelectric point of the protein would also wash out a portion of the protein which is solubilized by the enzyme. Depending on the enzyme treatment used, more than half of the protein may be washed out. Inactivation of enzyme by chemical means may also cause significant changes in the protein. Further, such chemicals may not be used in food products.

From a practical standpoint for food applications, one would have to heat the protein solution not only to inactivate the enzyme, but also to reduce microbiological contamination. Hence, in this study, we elected to use heat treatment to inactivate the enzyme and attempted to differentiate between the effects of enzyme and heat treatments by utilizing two controls. The "untreated

control" is a soy protein isolate prepared without any heat treatment. The "treated control" was prepared exactly the same way as the enzyme-treated products but without the addition of enzyme. The difference between the two controls indicates the effect of the heat treatment on the functional properties.

The neutral protease of *A. oryzae* was selected because it requires a relatively low inactivation temperature. The soy protein isolate was hydrolyzed by varying amounts of this enzyme, and the extent of hydrolysis was followed by the determination of an increase in free amino groups as shown in Table I. The results are expressed as the number of free amino groups per 20,000 g protein, which is the approximate molecular weight of the subunits in soy proteins (17,18). Since soy protein isolates contain 5.72 g lysine/16 g nitrogen (19), which represents 0.0453 mol of lysine residue per 100 g protein, such subunit would contain nine lysine residues. Thus, the ten free amino groups in the unhydrolyzed protein may come from the nine ϵ-NH_2 groups of lysine residues plus the one α-NH_2 group from the terminal amino acid residue. The increase in free amino groups is related to the extent of enzyme treatment. However, these results must be viewed with caution since TNBS has been shown to react with the ribose and guanine moieties of nucleic acids (20). Soy protein isolate does contain nucleic acids (21), and during the enzyme treatment these reactive groups may be liberated to react with TNBS. The use of the extinction coefficient suggested by Habeeb (5) did not give the same result as obtained by using the standard curve derived from glycine.

The solubilities of the enzyme-treated proteins are shown in Table II. As expected, heat treatment lowered the NSI, but with the increasing enzyme treatment, less protein was rendered insoluble by the heat treatment.

Neither control showed significant solubility at pH 4.5 or in the presence of 0.03*M* calcium chloride, but the treated proteins showed increased solubility in both cases with increased enzyme treatment. Increased acid solubility would be advantageous in the utilization of soy proteins in acidic foods whereas calcium tolerance is important when calcium addition is needed for improved nutrition, such as in imitation dairy products (milk contains 0.03*M* calcium).

Table III shows the results of the various emulsification tests employed in the present study. Surprisingly, heat denaturization of the proteins did not show much change in emulsification behavior as indicated by the similarities between the two controls. This does not mean that overheating would not lower emulsification properties but rather that NSI may not be the best way to detect heat damage to the proteins. Emulsion capacity increased with enzyme treatment whereas emulsion stability decreased. One may theorize that enzyme digestion of proteins will 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, since these peptides are smaller and less globular, they will form a "thinner" protein layer around the oil droplets resulting in an emulsion with less stability. Enzyme treatment may also uncover buried hydrophobic groups which may improve the hydrophilic-lipophilic balance for better emulsification. Heat treatment of the emulsions reduced emulsion stability only slightly. In all cases the emulsification properties were good, and hence limited enzyme treatment may not be detrimental to emulsification properties of soy proteins.

Mellon *et al.* (14) and Hagenmaier (22) suggested the measurement of moisture

TABLE I
Free Amino Groups Determination

Samples	Free Amino Groups per 20,000 g Protein
Untreated control	10.0
Treated control	10.1
Enzyme treatment A	12.2
Enzyme treatment B	13.6
Enzyme treatment C	15.6
Enzyme treatment D	17.9

TABLE II
Solubilities of Enzyme-Treated
Soybean Proteins under Different Conditions

Samples	NSI[a]	Acid Solubility (at pH 4.5)	Solubility in 0.03*M* $CaCl_2$
Untreated control	89.0	0	0
Treated control	47.2	0	3.2
Enzyme treatment A	63.7	14.5	22.1
Enzyme treatment B	73.1	21.7	26.2
Enzyme treatment C	69.4	25.1	31.1
Enzyme treatment D	80.4	34.5	43.4

[a]Nitrogen Solubility Index.

TABLE III
Emulsifying Properties According to the Various Methods

Samples	Emulsifying Activity[a] %	Emulsifying Stability[a] %	Emulsion Stability Index[c]	Emulsion Capacity[b] ml oil/ml protein
Untreated control	99.0	95.6	97.8	8.77
Treated control	95.6	90.7	98.3	8.71
Enzyme treatment A	91.6	91.3	97.1	8.82
Enzyme treatment B	86.8	89.7	97.5	13.1
Enzyme treatment C	87.5	88.8	94.8	12.8
Enzyme treatment D	84.0	82.5	62.0	14.9

[a]According to the method of Yasumatsu *et al.* (12).
[b]According to the modified method of Swift *et al.* (7).
[c]According to the modified method of Titus *et al.* (13).

uptake at a constant relative humidity as a means of measuring the water absorption of proteins. There is insufficient evidence thus far to demonstrate that such a measurement will correlate with actual performance in food products. According to Hagenmaier (22), casein binds more water at 84% relative humidity than soy protein isolate; however, in processed meat products, the soy protein isolates have been shown to have excellent water retention (23).

Table IV shows the moisture uptake of the proteins at 84% relative humidity and room temperature. Water binding is increased proportional to extent of enzyme treatment. Mellon *et al.* (14) demonstrated that moisture uptake of proteins under these conditions is proportional to the number of ionic groups present, *i.e.*, free amino and carboxyl groups. Since enzyme treatment increases both of these, it is not surprising that moisture uptake increased with enzyme treatment.

It is possible that in the finished food products the enzyme-treated samples may not be able to hold water as well as untreated soy proteins in spite of higher moisture uptake indicated in this study.

Hermansson's (24) method of measuring swelling of proteins in contact with water would be a better method to investigate the contribution of proteins to water-binding properties in food products.

The gelation properties of the treated samples are shown in Table V. The unheated control gave a strong gel; however, the treated control did not. Catsimpoolas and Meyer (16) reported that the progel-gel transformation is reversible upon heating and cooling. However, they did not examine a heating-drying-redissolving step as in the present study. Such treatment as described herein apparently denatured sufficient quantity of protein to prevent good gel formation. None of the enzyme-treated proteins showed gel formation, and the viscosities of their dispersions were very low. Although it is possible that at higher protein concentrations gelation would still occur, enzyme treatment is an effective means to produce a low viscosity, non-gelling protein.

Table VI shows the results of foam expansion measurements. Enzyme treatment increases foam expansion, but foam stability was zero in each instance. Previous work by Turner (1) indicated that to make a stable protein containing foam, partially hydrolyzed protein is needed to increase foam expansion and some larger protein components are needed to stabilize the foam. In the present study, the heat treatment used may have denatured the larger protein components sufficiently so that they could not stabilize the foam. As mentioned

TABLE IV
Water Absorption at 84% Relative Humidity

Samples	g Moisture Absorbed per 100 g Dry Protein
Untreated control	25.4
Treated control	26.6
Enzyme treatment A	28.1
Enzyme treatment B	28.6
Enzyme treatment C	29.6
Enzyme treatment D	32.2

TABLE V
Gelation Properties of Soy Proteins

Samples	Visual Observation	Gel Viscosity cP
Untreated control	Gel	385,000[a]
Treated control	Semi-gel	3,440[b]
Enzyme treatment A	Liquid	148[c]
Enzyme treatment B	Liquid	84[c]
Enzyme treatment C	Liquid	80[c]
Enzyme treatment D	Liquid	68[c]

[a]Brookfield viscometer, RVT model, Helipath stand, T-E spindle, 5 rpm.
[b]Brookfield viscometer, RVT model, Helipath stand, T-B spindle, 5 rpm.
[c]Brookfield viscometer, RVT model, No. 4 spindle, 100 rpm.

TABLE VI
Foaming Properties of Soybean Proteins

Samples	Foam Expansion
Untreated control	46
Treated control	56
Enzyme treatment A	158
Enzyme treatment B	174
Enzyme treatment C	178
Enzyme treatment D	180

in the introduction, enzyme hydrolysis of soy proteins can be used to produce a product with good whipping properties (1,2).

Enzyme treatment is an effective way to modify the functional properties of soy proteins. In general, such enzyme treatment increases solubility, prevents heat coagulation, and increases foaming volume. The extent of enzyme treatment will depend upon the application for which the protein product is designed. Obviously, a protein product designed for supplementation of acidic beverages will require much more extensive treatment than, for example, one in which enzyme treatment is carried out to reduce viscosity sufficiently to ease pasteurization of concentrated protein solutions. Enzyme treatment should be carried far enough to achieve the desired result without destroying other functional properties required for the specific application.

Enzymes vary in their specificity, so the type of enzyme used may affect the final product even if the hydrolyses are carried out in such fashion as to achieve the same number of free amino groups per unit weight of protein.

Evaluation of enzyme-treated proteins in model systems, such as described here, is not sufficient to determine the usefulness of such products. For example, two protein-stabilized emulsions may have the same stability but consistency and appearance may be very different. However, such a study is useful to determine what type of enzyme treatment, if any, is required to achieve the desired end result.

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