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Enzymatic Modification of Soy Proteins: Effects of Functional Properties of Soy Isolate upon Proteolytic Hydrolysis

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大豆蛋白質의 酵素的 変形：分離大豆蛋白質의 機能性에 미치는 蛋白質加水分解의 影響

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Abstract

To study affinity of proteolytic enzymes to soy proteins, the physicochemical and functional properties of enzymatically modified protein products, kinetic parameters and degree of hydrolysis were measured using trypsin, alcalase (serine type protease) and pronase. Bacterial alcalase and pronase showed much greater affinity to soy protein than animal intestinal trypsin. This effect was very significant when unheated soy isolate was used as a substrate. Specific activities of these enzymes decreased with the increment of substrate concentration (over 2.0%, *w/v*) when heat denatured soy protein was used as a substrate. However, the decrease in specific activity was negligible at substrate concentrations lower than 2.0%. Polyacrylamide gel electrophoretic results showed that the pattern of 2S protein band changed distinctly in alcalase hydrolysis as compared with those of trypsin and pronase. Protein solubilities of alcalase and pronase hydrolyzates increased by 25-30%, at their pI (pH 5.0) over the control. Virtually no change was observed in solubility by trypsin hydrolysis. Heat coagulability and calcium-tolerance of the protein increased by enzymatic hydrolysis. No clear tendency, however, was observed for emulsion properties, foam expansion and the amount of free -SH groups. The enzyme treatment considerably decreased foam stability.

Introduction

The food uses of soy proteins are increasing more rapidly than any other proteins. The functional properties of soy proteins are, therefore, very important in food application because they are, at present, primarily used as ingredients. Both chemical and enzymatic modifications have been used to alter functionalities of proteins. Since they are generally considered safer than chemical methods, enzymatic methods are preferred.

The degradation of proteins by proteolytic enzymes has been one of the most commonly utilized enzymatic modification methods. A pepsin-digested soy protein product to make a whippable protein to replace egg white [1,2] is a good example of this method successfully applied. The use of enzyme treatment to increase solubility of heat-denatured soy protein[3] and increase the protein solubility in acidic media[4] are other examples.

Puski[5] measured various functionality changes of soy protein isolates related to the extent of treatment with a neutral protease from *A. oryzae*, and found that soluble nitrogen increased with increment of time of enzyme treatment. A dual enzyme system was also reported as an economical, non-deleterious enzymatic method of hydrolysis and solubilization of soy

proteins.[6] An ultrafiltration (UF) reactor system was evaluated to determine the feasibility of controlling soy protein hydrolysis using various proteases.[7]

The objective of this research was to evaluate various commercial proteases on their abilities to alter soy protein isolates. Functional changes resulting from enzyme actions were measured in appropriate buffer systems, not only to minimize experimental variations but also to determine the functional properties under the effects of salts.

Materials and Methods

Materials

Soybeans, enzymes and caseinate were all from commercial sources. Sound, mature soybeans of the white-hilum variety were purchased from a local farmer (College Station, Texas, U. S. A.).

Protein Extraction

Soybeans were dehulled and ground in a Thomas-Willey Lab. mill (Model III) so as to pass through a 20 mesh screen. The meal was defatted three times with *n*-hexane at room temperature for 48 hr, using a solvent-to-meal ratio of 4:4 (*v/w*). The defatted meal was ground again to pass through a 40 mesh screen before the protein was extracted with a Tris-HCl buffer (pH 8.0) and freeze-dried (Fig. 1).

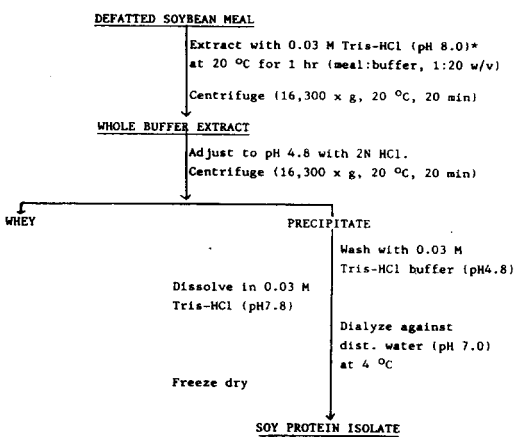

Fig. 1. Extraction of soy protein isolate
*Tris-HCl (Tris hydroxymethyl amino methane -HCl) buffer containing 0.01*M* 2-mercapto-ethanol (2-ME), adjusted to pH 8.0 with 4*N* NaOH.

Table 1. Enzyme reaction conditions on soy isolate for kinetic constants

Substrate Concentration (%, w/v)	0.5, 0.75, 1.0, 1.5, 2.0, 2.5
Enzyme Concentration (*mg/ml*)	0.5
[E] : [S]	1/50, 1/75, 1/100, 1/150, 1/200, 1/250
Reaction Time (min)	0, 1, 3, 5, 8, 10, 15, 20
Incubation Temperature (℃)	40
Buffer	pH 7.0, Sodium phosphatecitric acid

Enzyme Activity Assay

Preliminary experiments showed that trypsin (Sigma T-8253, Type III), alcalase (Novo, 0.6*L*; endoproteinase of serine type; solid form, Denmark) and pronase (Calbiochem, Los angeles, CA) are most suitable for this experiment. One gram casein (Hammerstein-type) was mixed completely with 0.01 *M* sodium phosphate-citric acid buffer (pH 7.0), containing 0.01% sodium azide, and placed in a boiling water bath for 30 min, while stirring. After cooling, the solution was made up to 100 *ml* by adding the buffer. Enzyme activities were then measured by the method of Cunningham[8].

Measurements of Kinetic Parameters

Kinetic parameters of each enzyme were determined on 10% trichloroacetic acid (TCA)-soluble soy proteins with the method[8] discribed in the enzyme activity assay under the reaction conditions summarized in Table 1. *Km* and *Vamx* were then calculated from the Lineweaver-Burke Plots[9].

Protein Hydrolysis

The hydrolysis of protein was carried out in batches. A 2-liter flask containing 1 liter of 1% soy isolate in 0.01 *M* sodium phosphate-citric acid buffer (pH 7.0) containing 0.01% sodium azide was maintained at 40 ℃ with agitation. After equilibration for 10 min, each enzyme was added at an enzyme-to-substrate ratio of 1/100 (*w/w*). After 1 hr hydrolysis, the solution was immediately frozen in an acetone dryice sack (-50 ℃), then lyophilized. The lyophilized soy protein was then used for all functionality tests.

Degree of Hydrolysis (DH)

The TNBS method[10,11] was applied to determine

the degree of hydrolysis at predetermined time intervals from the protein hydrolysis systems. The DH was defined as the following formuls; [(total free amino group in sample minus free amino group in control)/total free amino group] X 100.

PAGE and SDS-PAGE

The PAGE (7% gel) was carried by the method of Green and Moore[12] by applying 60 μl of 1-hr hydrolyzates with upper gel. The SDS-PAGE (10% gel) was carried out by the mehtod of Swank and Munkers[13].

Functional Properties of Protein Hydrolyzates

Selected functional properties, protein solubility profiles, foam expansion, foam stability, emulsification properties[14], heat coagulability and calcium precipitability[15], were measured.

Results and Discussion

Soy Protein Extract

Soy isolate was prepared by the extraction with 0.03 *M* Tris-HCl buffer (pH 8.0), containing 0.01 *M* 2-mercaptoethanol (ME), based on the continuous isolation procedure of 7S and 11S soy proteins described by Thanh and Shibasaki[16]. The isolate contained at least 96% protein (moisture free basis) as measured by the micro-Kjeldahl method. The yield of soy isolate from defatted meal was about 30%, which is similar to the value reported[16].

The isolate was hydrolyzed using proteases (trypsin, alcalase and pronase). These enzymes were chosen from many plant, animal, and microbial proteases surveyed, based on their activities on casein and soy protein. The activities of the three enzymes on heated denatured casein were 199.8 (trypsin), 130.7 (alcalase), and 181.3 (pronase) unit/*mg* enzyme, respectively. The activities of trypsin and pronase were nearly the same, while the alcalase activity was relatively lower than those of the other two proteases. The differences in activities, however, appeared not important for this study since the specificity of an enzyme toward a given substrate is generally considered more important than its relative activity.

Table 2. Kinetic constants for proteinase reaction on the hydrolysis of soy isolate at pH 7.0, 40℃, (E) = *mg/ml*[a]

Enzyme	Constant[b]	Unheated isolate	Preheated isolate[c, d]
Trypsin	*Vmax*	10.02	1.64
	Km	11.44	0.94
Alcalase	*Vmax*	0.12	0.12
	Km	0.52	0.26
pronase	*Vmax*	0.15	0.11
	Km	0.59	0.28

a Calculated from Lineweaver-Burke Plot ($r > 0.95$).
b Units: *Vmax* = absorbance increased at 280*nm*/min
Km = % *w/v*.
c Preheated at 95℃ for 5 min in buffer (pH 7.0) before incubation.
d Data from li near portion of Lineweaver-Burke Plot was considered.

Kinetic Parameters

Kinetic constants (*Km* and *Vmax*) of the hydrolysis of soy isolate are shown in Table 2. The differences in *Km* between unheated and heated soy isolates were about 10 folds for trypsin and two folds for alcalase and pronase, respectively. The results indicate that the proteases appeared to have a greater affinity toward the heated soy protein than the native one. The differences in *Km* values obtained from this study are also similar to the results reported on the pronase-promine D system (0.75% for unheated and 1.15% for heated)[7]. In general, native globular proteins are resistant to enzymatic hydrolysis, and most proteins are more easily hydrolyzed in their denatured forms than in their native ones[17].

Fig. 2 shows the relationships between the initial velocity and the substrate concentration on soy protein hydrolysis by trypsin. Alcalase and pronase hydrolysis showed similar tendency to trypsin, with only slight difference in actual values. These results revealed some significant facts: Preheating of the substrate at low concentrations (lower than 1.5%, *w/v*) accelerated the initial velocity of the reaction, while at higher concentrations (higher than 1.5%, *w/v*) it apparently caused substrate inhibition not only on the initial velocity but also on the whole hydrolysis reaction of soy isolate. The pronase-

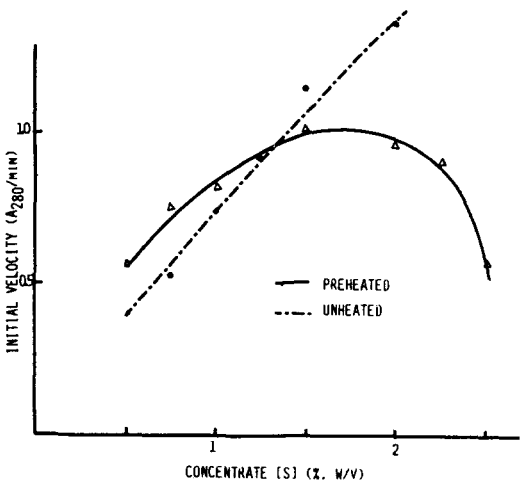

Fig. 2. Initial velocities of trypsin hydrolysis on preheated and unheated isolates

promine D system[7] also reported similar preheating effects of soy proteins on enzymatic hydrolysis. The substrate inhibition seemed a common phenomenon occuring in soy protein hydrolysis regardless of the source of proteases used.

Degree of Hydrolysis (DH)

The DH values of soy protein hydrolysis by three proteases were determined by the TNBS method and the results are shown in Fig. 3. Although the 10% TCA-soluble protein method[18] has been frequently used for the same purpose as the TNBS method, it may not be a suitable one to determine DH values under the reaction conditions used in this study, because the initial rate of

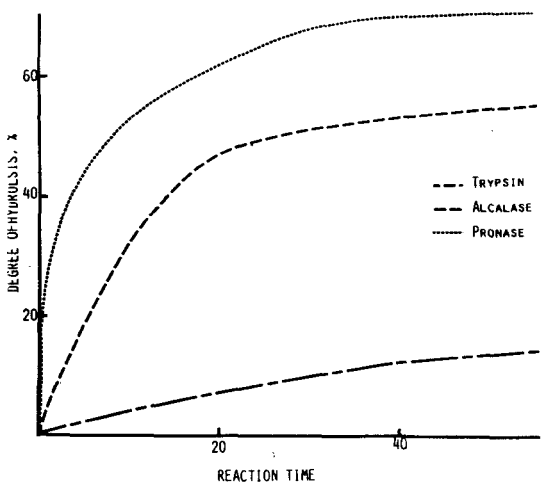

Fig. 3. Degree of hydrolysis (DH) of soy isolate with proteases measured by TNBS method

trypsin hydrolysis as determined by the method was unreasonably high[19]. At present, the exceptionally high initial reaction rate by trypsin was not clear. Various types of pH-stat methods have also been used in recent years as a practical mean of determining DH values, and the results were comparable to those obtained by the TNBS method[11]. According to the results obtained from the TNBS method, the DH value of trypsin was considerably lower than those of alcalase and pronase, indicating that trypsin could not effectively hydrolyze soy isolate under the experimental conditions used. This result was also supported by changes in functional properties which will be discussed later. The maximum DH values of alcalase and pronase reached 67% in 30 min and 50% in 40 min, respectively.

PAGE and SDS-PAGE Analysis

The results of the PAGE and SDS-PAGE analyses of alcalase and pronase hydrolyzates are shown in Fig. 4. The Electrophoretic patterns for trypsin hydrolyzates were almost similar to those of the control as to the location and concentration of protein bands (data not shown)[19]. On the other hands, both alcalase and pronase hydrolysis effected changes in protein bands. Alcalase hydrolysis distinctly changed the location of 2S band as compared with trypsin and pronase hydrolysis, i.e., the position of 2S band was shifted more toward the bottom of the PAGE gel (arrows in Fig. 4), which indirectly indicates that alcalase can change the conformation or peptide chains of the 2S protein fraction. Several trypsin inhibitors and cytochrome C exist in the 2S fraction, as well as other minor proteins[20]. Therefore, it is possible that certain changes have been

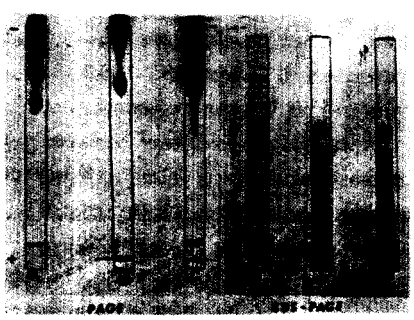

Fig. 4. PAGE and SDS-PAGE of soy isolate and its enzyme hydrolyzates
A, a; soy isolate, B, b; alcalase hydrolyzate, C, c; pronase hydrolyzate.

induced by the alcalase hydrolysis of these compounds. This hypothesis should, of course, be proved by additional experiments in the future.

Solubility Profiles of Hydrolyzates

The pH-dependent solubility profile of soy protein isolate showed decreasing solubility with decreasing pH, reaching the minimum solubility at the isoelectric point (pI), and then increasing as the pH values become more acidic (Fig. 5). The protein solubilities at alkaline pH values were almost the same, about 90% or higher, for all enzyme hydrolyzates over the control. At the pI, enzyme treatment increased the solubility substantially by about 40% for pronase hydrolysis, 30% for alcalase, and 5% for trypsin over the control (5%); however, at pH 6.0, the solubilities decreased by 10% for alcalase, 15% for trypsin, and 2% for pronase hydrolysis, respectively. According to previous work[5], the amount of soluble nitrogen increased at neutral and isoelectric pH values, and in the presence of 0.03 *M* calcium chloride, with increased enzyme treatment. Also, the acid solubility of protein increased with the increase in DH of alcalase hydrolyzates in soy isolate[21]. A review on enzyme modification of proteins[22] showed that the breaking of proteins by proteases resulted in three major modifications: (a) an increase in the number of polar group; (b) a decrease in molecular weight of proteins; (c) a possible alteration in molecular configuration. Likewise, the increase in the number of polar group may have been mainly responsible for the increase in protein solubilities and the slight change in the isoelectric points observed in this study. However, the slight decrease in solubility at pH 6.0 may have been due to changes in molecular configuration.

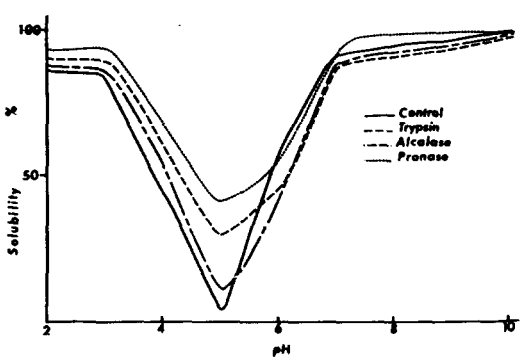

Fig. 5. Solubility profiles soy isolate and its enzyme hydrolyzates

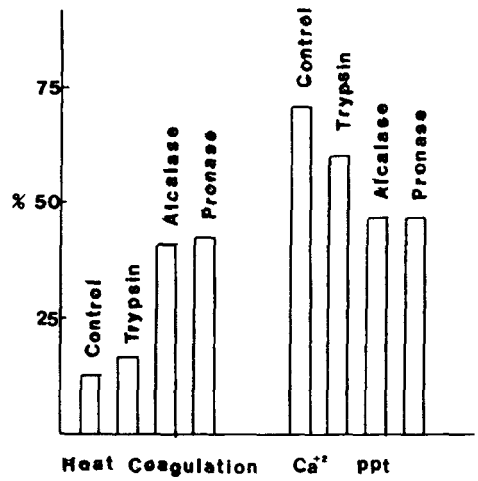

Fig. 6. Properties of calcium precipitation and heat coagulation on soy isolate and its enzyme hydrolyzates
*% = precipitated protein (from 1% soln.)/ total protein × 100.

Heat Coagulation and Calcium Precipitation

As shown in Fig. 6, enzymatic hydrolysis of soy isolate clearly increased, not only the acid solubility (Fig. 5) but also heat coagulability and calcium tolerence (decreasing calcium precipitability) of soy protein. The heat coagulability of soy isolate increased by 5% for trypsin and 30% for alcalase and pronase hydrolysis over the control. Generally, heat treatment, especially moist heat, of soy protein rapidly insolubilizes native soy proteins[23]. However, the control (no enzyme treatment) showed a relatively low value of heat coagulability in this study, due to the use of sodium phosphate-citric acid buffer (pH 7.0) as the solvent. Protein solubilities are influenced by various fators, the nature of samples, analytical conditions, and the nature of the solvent (pH, ionic strength, etc). Also, heat coagulability of chemically modified cottonseed protein had a close relationship with the disulfide bond content of the protein[15]. However, no such relationship was found in this study as shown in Table 3 and Fig. 6. Investigations of covalent binding in heat induced protein gels have focused primarily on disulfide bridging. Heat treatment can result in cleavage of existing disulfide bonds or "activation" of buried sulfhydryl groups through unfolding of the protein[24]. However, disulfides play only a minor role in the progel to gel transformation in soybean globulins, although the protein gelation depends greatly on the formation conditions[25].

Calcium precipitability of soy isolate decreased by about 25% for alcalase and pronase and by 10% for trypsin hydrolysis (Fig. 6). Rackis reported that enzymatic modification of proteins generally increased calcium tolerance, water absorption, and foaming properties[26]. In the discussion on solubility profiles, Puski measured calcium-tolerance which soy isolates were increasingly soluble at pH 4.5 in the presence of 0.03 *M* $CaCl_2$ as treatment of neutral protease progressed[5]. Also, the author pointed out that increased acid solubility would be advantageous in the utilization of soy proteins in acidic foods, whereas calcium tolerance is important when additional calcium is needed for improved nutrition such as in imitation dairy products.

Emulsification and Foaming Properties

Table 4 shows the results of emulsification and heat stability studies. Emulsifying activities of the hydrolyzates decreased by about 29% for alcalase and 10% for pronase over the control. The enzymatic hydrolysis, however, did not appear to have any significant effect on the heat stability of the emulsion. Previous work indicated that limited enzyme treatment of soy protein may not have a detrimental effect on emulsification properties[5]. However, the study showed that enzyme treatments decreased emulsifying activity, and the heat treatment of the emulsions reduced the stability only slightly.

Table 4 also shows the results of foam expansion and stability of the hydrolyzates. The foam expansion showed a slightly increasing tendency, but foam stability decreased significantly by about 25% for trypsin, 35% for alcalase, and 40% for pronase. According to Phillips and Beuchat[22], proteolytic modifications generally produce greater volumes of foam than unmodified proteins, but most proteins, treated with enzymes, have inferior stabilities to the untreated control.

Table 3. Determination of SH- and SS-groups in soy isolate and its enzyme hydrolyzates

	Total(SH+SS) $\mu M/g$	SS $\mu M/g$	SH $\mu M/g$	SH%[a]
Control (no enzyme)	33.19	30.31	2.88	8.6
Trypsin treatment	33.89	31.47	2.42	7.1
Alcalase treatment	29.57	27.66	1.91	6.4
Pronase treatment	29.26	27.31	1.95	6.6

a SH/total (SH+SS) × 100.

요 약

本 硏究는 分離 大豆蛋白質에 蛋白分解 酵素를 作用시킬 때 일어나는 酵素反應性 및 蛋白質 機能性에 미치는 影響을 調査하였다. 使用된 酵素는 動物性인 trypsin과 細菌性인 alcalase 및 pronase였으며 熱 處理되지 않은 大豆蛋白質에 対하여 trypsin보다 細菌性 酵素가 높은 親和力을 나타냈으며 熱 處理된 大豆蛋白質에 対하여서는 酵素種類에 관계없이 基質濃度가 增加함에 따라 反應이 阻害되었다. 加水分解된 大豆蛋白質의 電気泳動 結果, alcalase가 特異的으로 大豆蛋白質 中 2S 蛋白質에 어떤 変化를 가져오는 것이 관찰되었다. 大豆蛋白質의 機能性에 있어서 酵素處理는 等電点에서 25~30%의 可溶性 蛋白質의 增加를 가져왔으며 또한 熱 凝固性의 增加, 칼슘 沈澱性의 減少를 초래하였다. 그리고 에멀젼 特性, 거품 形成能 및 遊離 SH基 등에 対하여서는 큰 変化가 없었으나 거품 安全性은 크게 減少하는 경향을 보였다.

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Table 4. Relative emulsifying and foaming capacities of soy isolate and its enzyme hydrolyzates

	Emulsifying activity (%)[a]	Emulsion heat stability[b] (%)	Foam expansion (*ml*)	Foam stability[c] (*ml*)
Control (no enzyme)	70	68	90	86
Trypsin treatment	68	68	97	65
Alcalase treatment	53	67	96	57
Pronase treatment	61	72	95	52

a % : Emulsion volume/total volume X 100.
b Heated at 80℃ for 30min.
c Measured at 1 hr after foam expasion measurement.

University System, College Station, Texas, U.S.A., where the author was a visiting scientist.

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