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Hydrolytic characteristics of chitosan-immobilized As 1.398 neutral proteinase (from *B. subtilis*) to soybean protein

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As 1.398 neutral proteinase (from *B. subtilis* As 1.398) was immobilized on the loose chitosan-precipitate by simultaneous adsorption and covalent bonding. The optimum activity of the chitosan-immobilized proteinase to soybean protein is at pH 8.0. The profiles of proteolytic activity with regard to temperature, as with soybean protein as substrate, show two peaks at 40 and 60°C, respectively. The proteolytic activity toward soybean protein at 60°C is significantly higher than that at 40°C. The maximum extent of hydrolysis of soybean protein by the immobilized proteinase is about 23% which is substantially lower than that of casein. Heating increases the susceptibility of soybean protein to the immobilized proteinase, but not the maximum extent of hydrolysis. In addition, the composition of free amino acids in hydrolyzed soybean protein was analyzed. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

The utilization of soybean protein in the food industry is expanding greatly because of its good nutritional value and relatively low cost. It has been used as an ingredient in human foods in the form of grits, flours, flakes, concentrates and isolates (Traina & Breene, 1994). In the last decade, soybean protein has also begun to be used for coffee whitener, milk-type beverages, mayonnaise, etc. (Ochiai *et al.*, 1982). Recently, soybean protein isolate was used by several investigators as the material of edible films (Gennadios *et al.*, 1993). However, some functional properties of soybean protein, such as solubility, thermal stability, emulsification, foaming or whipping, still limit its utilization in certain food products (Kinsella, 1979; Damodaran & Kinsella, 1982; German *et al.*, 1982). Fortunately, the functional properties of soybean protein can be improved by enzymatic or chemical modification (Kimball *et al.*, 1981; Ochiai *et al.*, 1982; Deeslie & Cheryan, 1988; Hamada & Marshall, 1989; Mathcis, 1991; Guo *et al.*, 1992).

Enzymatic modification is an attractive alternative to chemical treatment because enzyme reactions are generally more specific and generate uniform products under milder conditions, and thus the products maintain nutritional value and digestibility (Feeney & Whitaker, 1985). Amongst the enzymatic modification methods, hydrolysis is the most usual method. The use of immobilized enzymes in protein hydrolysis has the following advantages: (a) automation and continuous processing; (b) convenient separation of products from catalyst; (c) control over the extent of hydrolysis that governs the functional properties of protein hydrolysates; and (d) re-use of enzyme and reduction of autolysis (Deeslie & Cheryan, 1988; Cheryan & Deeslie, 1984; Wilson *et al.*, 1994). As 1.398 neutral proteinase, from *B. subtilis* As 1.398, is one of the proteinases most widely used in the industries of tanning, food, medicine, etc., in China (Zhang, 1984; Shui *et al.*, 1990; Guo *et al.*, 1993). These were the reasons for choosing As 1.398 neutral proteinase as the proteolytic enzyme material for immobilization and manufacturing soybean protein hydrolysates.

The inherent specificity of various proteolytic enzymes and the differences of protein (substrate) structure control the nature and extent of hydrolysis and thus the functional properties of the hydrolysates. This paper reports on a study of hydrolytic conditions for chitosan-immobilized As 1.398 neutral proteinase on soybean protein which can provide fundamental information for the further application of chitosan-immobilized As 1.398 neutral proteinase in hydrolysis of soybean protein.

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MATERIALS

Mature soybean seeds were purchased from the local market (Hehouachi Nongmoshichan) and cleaned manually. As 1.398 neutral proteinase, from *B. subtilis* As 1.398, was a commercial enzyme, of food-grade preparation, obtained from Wuxi Mei Zhiji Chong (Wuxi, China). The enzyme activity was 50 000 casein units per g, where a casein unit is defined as the activity to produce 1 μg of tyrosine equivalents per min at pH 7.0 and 40°C (Lowry *et al.*, 1951). Chitosan was prepared by the conventional method (Roberts, 1990) from prawn shells, white to light yellow, without insoluble substance in 2% acetic acid solution. All other chemicals were analytical grade reagents or bio-reagents.

METHODS

Preparation of soybean protein

Cleaned soybean seeds were ground in a laboratory mill so they passed through a 40 mesh-screen size. The seed meal was defatted twice with hexane at 15°C for 8 h using a solvent meal (v/w) ratio of 1.5:1. The defatted soybean meal was extracted with 0.1 mol/litre NaOH at 5°C for 8 h in a 1:30 (w/v) meal to solvent ratio. After the extraction, the pellet was removed by centrifugation at 1700 **g** for 15 min. The supernatant was adjusted to pH 7.5 with 6 mol/litre HCl diluted to the specified protein concentration, and divided into two equal portions which were contained in two glass-stoppered flasks. One portion was considered to be native soybean protein and stored at 4°C until used as the substrate for hydrolysis of chitosan-immobilized As 1.398 neutral proteinase. Another portion, after heating in a boiling bath for 20 min, was considered to be denatured soybean protein and stored at 4°C until used.

Determination of protein and peptides

The concentration of protein or peptides was assayed according to the method of Lowry *et al.* (1951) with soybean protein as the standard protein. The protein content of the standard soybean protein was calibrated by micro-Kjeldahl method by using the conversion factor of 6.25.

Immobilization of As 1.398 neutral proteinase

Immobilization of proteinase was carried out by the method of Shui *et al.* (1990). Five grammes of chitosan was dissolved in 300 ml of 2% acetic acid solution for 24 h at room temperature. Chitosan was precipitated by adjusting pH to 8.0 using 2 mol/litre NaOH. The precipitate was collected by filtration and washed twice with 0.1 mol/litre, pH 8.0, phosphate buffer. The washed chitosan precipitate was suspended in 300 ml of phosphate buffer, and 1.5 g of enzyme powder were added to the suspension. After stirring for 30 min with a magnetic stirrer, the suspension was maintained in vacuum for 1 h and 3.6 ml of 25% glutaraldehyde was added to the suspension. The suspension was well stirred for 10 min at room temperature and allowed to stand for 8 h at 30°C. The chitosan-immobilized enzyme was collected by filtration, washed four times with distilled water, dried for 12 h in a vacuum at room temperature and kept at 4°C.

Quantitative determination of proteolytic activity of chitosan-immobilized As 1.398 neutral proteinase toward soybean protein

To 0.5 g of immobilized proteinase in a glass-stoppered tube was added 1.5 ml of buffer solution at the indicated pH. After preincubation at the indicated temperature for 20 min, the reaction of proteolysis was started by the addition of 1.5 ml of native or denatured soybean protein solution and allowed to stand for 30 min at the same temperature. During the period of proteolysis the tube was shaken occasionally. The enzymic process was stopped by the addition of 3.0 ml of 20% (w/v) trichloroacetic acid (TCA). The reaction mixture was filtered and 1 ml of the filtrate used for the determination of peptide content by the Lowry method. A blank was prepared under the same conditions, but 3 ml of 20% (w/v) TCA was added before the addition of 1.5 ml of soybean protein solution, and blanks were not incubated for 30 min with the sample. The proteolytic activity was expressed in μg of peptides soluble in 10% TCA produced by 1 g of immobilized proteinase (wet weight) per min.

Hydrolysis of soybean protein

To 5 g of chitosan-immobilized As 1.398 neutral proteinase in a glass-stoppered flask was added 30 ml of pH 8.0 soybean protein (or casein) solution. The hydrolytic process took place at 60 or 40°C. Three millilitres of hydrolysed protein solution were removed from the hydrolytic system at specified intervals for the determination of 10% TCA-soluble peptides and total protein concentration. The degree of hydrolysis (DH) of soybean protein (or casein) hydrolyzed by chitosan-immobilized As 1.398 neutral proteinase was expressed as the ratio of the peptides soluble in 10% TCA to the total protein (Kimball *et al.*, 1981).

$$\mathrm{DH}\% = \frac{\begin{array}{c}\text{peptides concentration in}\\ \text{hydrolyzed protein solution}\end{array}}{\begin{array}{c}\text{total protein concentration in}\\ \text{hydrolyzed protein solution}\end{array}} \times 100\%.$$

Analysis of free amino acids

Free amino acid contents of soybean protein solutions before and after hydrolysis by chitosan-immobilized As 1.398 neutral proteinase, were determined directly with an automatic amino acid analyzer (Hitachi 835, Japan).

Statistics

All data were the means of triplicates. Statistical analysis was not performed because the differences of tri-determination were not significant.

RESULTS AND DISCUSSION

Effect of pH on proteolytic activity

pH activity curves of chitosan-immobilized As 1.398 neutral proteinase with both native and denatured soybean protein were performed using a broad pH range buffer, a mixture of citrate, phosphate and barbiturate. Figure 1 shows the pH activity profiles. The proteolytic activity of chitosan-immobilized As 1.398 neutral proteinase towards denatured soybean protein was significantly higher than that to native soybean protein at all pHs tested. It is clear that the conformational destruction of soybean protein make it more susceptible to enzymic hydrolysis. The second reason that increases the susceptibility of denatured (heated) soybean protein to enzymic hydrolysis is likely to be the denaturation of some substances that inhibit the proteolytic activity. The optimum pH of the immobilized proteinase with soybean protein as substrate is around pH 8.0. It is similar to that reported by Shui *et al.* (1990), with casein as the substrate shifting a pH unit towards the basic side, compared to the free enzyme.

Temperature–activity relationship

The effect of temperature on the activity of the immobilized proteinase was also investigated with both denatured and native soybean protein as the substrate. The results are illustrated in Fig. 2. The temperature–activity profiles show two peaks. One optimum temperature is at 40°C similar to the free enzyme (Zhang, 1984; Shui *et al.*, 1990). Another optimum temperature is at 60°C which is higher than the free enzyme. It was suggested that the optimum temperature of 40°C was not due to the free enzyme, because the immobilized enzyme was washed four times with distilled water to remove the free enzyme. These results indicate that the proteinase was immobilized onto the loose chitosan precipitate by two different means. In other words, there are two types of immobilized proteinase on chitosan. The type of immobilized proteinase which gives an optimum temperature of 40°C is most likely to be immobilized by adsorption, whereas that giving an optimum temperature of 60°C is likely to be immobilized by covalent bonding. In addition, the proteolytic activity at 60°C is significantly higher than that at 40°C.

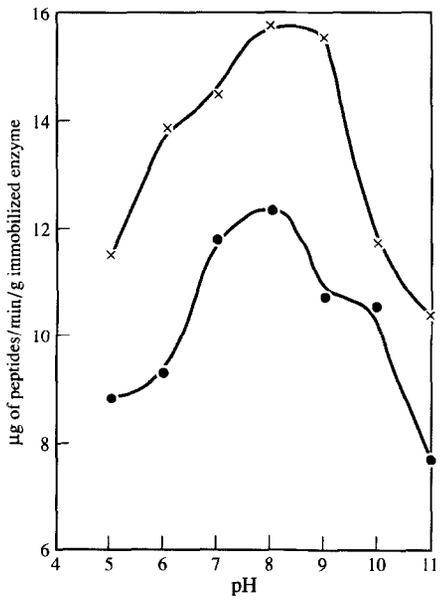

Fig. 1. Effect of pH on the proteolytic activity of the immobilized proteinase to native (•) and denatured (×) soybean protein at 40°C.

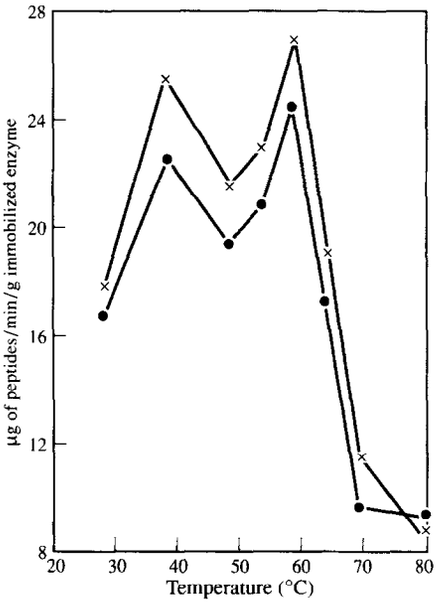

Fig. 2. Effect of temperature on the proteolytic activity of the immobilized proteinase to native (•) and denatured (×) soybean protein in pH 8.0 phosphate buffer.

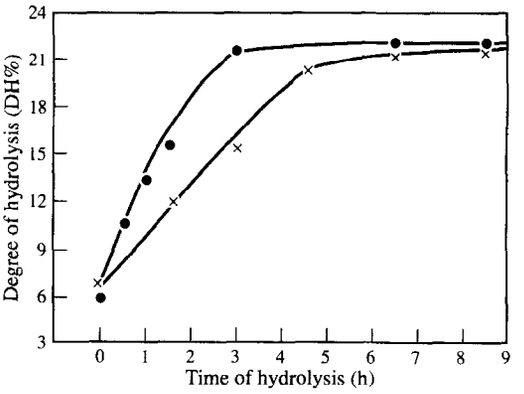

Fig. 3. Time course of hydrolysis of native soybean protein by the immobilized proteinase at pH 8.0 and two different temperature: 40°C (×) and 60°C (•).

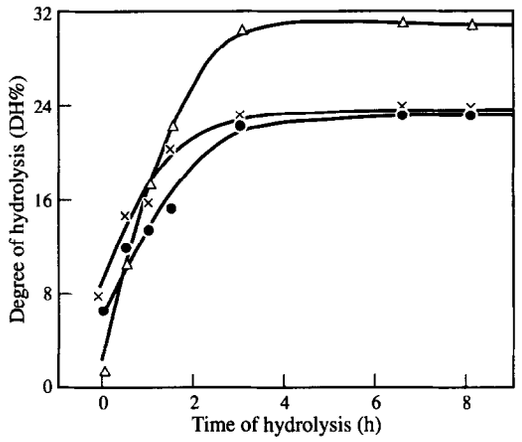

Fig. 4. Time course of hydrolysis of native soybean protein (•), denatured soybean protein (×) and casein (△) by the immobilized proteinase at pH 8.0 and 60°C.

Progress of soybean protein hydrolysis

Figure 3 shows the time course of native soybean protein hydrolysed by immobilized proteinase at 40 and 60°C. The maximum extent of hydrolysis at both 40 and 60°C was about 23%, but the rate of hydrolysis at 60°C was higher than that at 40°C because the initial slope of the reaction at 60°C was steeper than that of the reaction at 40°C. The reaction at 60°C reached a plateau within 3 h, whereas the reaction at 40°C reached a plateau in 6.5 h. Figure 4 compares the progress of hydrolysis of native soybean protein, denatured soybean protein and casein under the same conditions. The rate and extent of hydrolysis towards casein was higher than those towards soybean protein. The rate of hydrolysis of denatured soybean protein is slightly higher than that of native soybean protein.

Composition of free amino acids in hydrolyzed soybean protein

The changes in free amino acids of soybean protein solutions before and after hydrolysis are shown in Table 1. After the soybean protein was hydrolyzed by the immobilized proteinase to the maximum extent the contents of all the free amino acids, which can be separated and determined, increased significantly, except for histidine which increased only slightly and arginine which decreased slightly. The peaks of aspartic acid, threonine, serine, glutamic acid and proline in chromatograms given by the amino acid analyzer could not be separated. It is suggested that these amino acids occur in peptides not in the free state. The results showed that phenylalanine, leucine, lysine and valine, most of them hydrophobic amino acids, increased dramatically, reflecting the selective hydrolysis of peptide bonds.

Table 1. The changes of free amino acid content of soybean protein solution before and after hydrolysis by the immobilized proteinase

Amino acid	Content before hydrolysis (μg/ml)	Content after hydrolysis (μg/ml)
Aspartic acid	NS*	NS
Threonine	NS	NS
Serine	NS	NS
Glutamic acid	NS	NS
Proline	NS	NS
Glycine	NS	1.53
Alanine	2.02	4.51
Cystine	9.73	14.3
Valine	1.07	9.16
Methionine	1.05	4.14
Isoleucine	1.20	5.79
Leucine	0.75	11.5
Tyrosine	1.43	12.6
Phenylalanine	0.95	26.9
Lysine	0.80	12.5
Histidine	1.57	1.66
Tryptophan	2.18	6.05
Arginine	42.8	39.7
Total	65.6	150

NS = cannot be separated.

REFERENCES

Cheryan, M. & Deeslie, W. D. (1984). Protein hydrolysis. US Patent 4 443 540.

Damodaran, S. & Kinsella, J. E. (1982). Effect of conglycinin on the thermal aggregation of glycinin. *J. Agric. Food Chem.*, **30**, 812–17.

Deeslie, W. D. & Cheryan, M. (1988). Functional properties of soy protein hydrolysates from a continuous ultrafiltration reactor. *J. Agric. Food Chem.*, **36**, 26–31.

Feeney, R. E. & Whitaker, J. R. (1985). Chemical and enzymatic modification of plant proteins. *New Protein Foods*, **5**, 181–219.

Gennadios, A., Brandenburg, A. H., Weller, C. L. & Testin, R. F. (1993). Effect of pH on properties of wheat gluten and soybean protein isolate films. *J. Agric. Food Chem.*, **41**, 1835–9.

German, B., Damodaran, S. & Kinsella, J. E. (1982). Thermal dissociation and association behavior of soy proteins. *J. Agric. Food Chem.*, **30**, 807–11.

Guo, M.-L., Chen, J., Jiang, Y.-M. & Zheng, J.-J. (1992). Production of acid-solubility peptides beverage with defatted soya-bean cake. *Shipin Kexue*, **1992**, (10), 1–3.

Guo, M.-L., Jiang, Y.-M., Sun, X.-Y. & Xia, J.-Y. (1993). Hydrolysis of defatted soya-bean cake by As 1.398 neutral proteinase (from *B. subtilis* As 1.398). *Shipin Kexue*, **1993**, (7), 10–13.

Hamada, J. S. & Marshall, W. E. (1989). Preparation and functional properties of enzymatically deamidated soy proteins. *J. Food Sci.*, **54**, 598–601.

Kimball, M. E., Hsieh, D. & Rha, C. (1981). Chymotrypsin hydrolysis of soybean protein. *J. Agric. Food Chem.*, **29**, 872–4.

Kinsella, J. E. (1979). Functional properties of soy proteins. *J. Am. Oil Chem. Soc.*, **56**, 242–58.

Lowry, O. H., Rosebrough, J., Farr, A. L. & Randall, R. J. (1951). Protein measurements with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–75.

Matheis, G. (1991). Phosphorylation of food proteins with phosphorus oxychloride—improvement of functional and nutritional properties: a review. *Food Chem.*, **39**, 13–26.

Ochiai, K., Kamata, Y. & Shibasaki, K. (1982). Effect of tryptic digestion on emulsifying properties of soy protein. *Agric. Biol. Chem.*, **46**, 91–6.

Roberts, G. A. F. (1990). Method for producing chitosan. WO 90/04608.

Shui, D.-X. Jiang, Y.-M., Zhao, G.-J., Ma, Y.-L., Shi, W., Jiang, J.-X. & Yan, J.-W. (1990). Study on the immobilization of As 1.398 neutral proteinase (from *B. subtilis* As 1.398). *Shengwu Wuli Yu Sheng. Huax. Jinzh.*, **17**, (4), 311–13

Traina, M. S. & Breene, W. M. (1994). Composition, functionality and some chemical and physical properties of eight commercial full-fat soy flours. *J. Food Proc. Pres.*, **18**, 229–52.

Wilson, S. A., Peek, K. & Daniel, R. M. (1994). Immobilization of a proteinase from the extremely thermophilic organism *Thermus Rt 41A. Biotechnol. Bioengng*, **43**, 225–31.

Zhang, S.-Z. (1984). *Meizhiji Gongye*. Science Publishing House, Beijing, China, pp. 430–51.