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Process Development for the Enzymatic Hydrolysis of Food Protein: Effects of Pre-treatment and Post-treatments on Degree of Hydrolysis and Other Product Characteristics

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An enzymatic process was developed to produce protein hydrolysate from defatted soya protein. Various unit operations were tried, and the effects of pre- and post-treatments on the product characteristics such as degree of hydrolysis (DH), free amino acid content (%FAA) and average molecular weight (MW) were investigated. The use of acid washes showed no difference in %DH. Increasing pH during pre-cooking gave lower %DH. Alkaline cooking made too much insoluble protein, thus the protein yield was too small. A better hydrolysis with more acceptable taste was obtained when the combination of Neutrase/Alcalase/Flavourzyme was used in place of Alcalase/Flavourzyme combination. Untoasted defatted soya was more effective on the proteolysis than toasted one. The MW of the evaporated and spray dried product was higher than that of undried product, due to precipitation of low-solubility components. When the product separation was carried out by ultrafiltration and the product concentration by reverse osmosis, the solubility and the taste of the product were improved. The difference between enzyme hydrolysate and acid hydrolysate was significant in free amino acid composition, especially in tyrosine, phenylalanine, glutamine and asparagine.

Key words: enzymatic hydrolysis, protein hydrolysate, degree of hydrolysis, free amino acid, molecular weight distribution

INTRODUCTION

Protein hydrolysates are widely utilized in food production, particularly in soups, sauces, medical food and processed foods [1-3]. The industrial production of protein hydrolysate is usually carried out by hydrolysis of protein by acids or alkalis. Biological method using proteases has several advantages: mild reaction conditions and avoidance of undesirable byproduct. Enzymatic hydrolysis also yields products that are lighter in color, contain lower salt, and have a milder savory taste. The other use of proteases in the food industry includes the development of new functional properties of proteins and the production of peptides [4-5]. However, the use of the enzymatic method in the production of protein hydrolysates has been limited because of relatively high enzyme cost. Recently the enzymatic hydrolysis has become attractive because the potentially toxic substances existing in acid-hydrolyzed vegetable protein were reported [6-7]. Many attempts to develop cost-effective enzymatic processes were tried [8-9].

The enzymatic modification of food proteins is often required to produce protein hydrolysates with more desirable properties such as good solubility and desirable molecular weight distribution. However, uncontrolled degradation of the peptide chains causes the formation of bitter peptides, which makes the hydrolysate unsuitable for use as food supplements [10]. It was generally known that the control of the hydrolysis process is deterministic to degree of hydrolysis (DH), functional properties and bitterness of peptides [11].

In the present study, the enzymatic process using protease treatment combined with various unit operations (pre- and post-treatments) was developed to produce soy protein hydrolysate. Different process parameters have been evaluated, such as process with carbon treatment, without acid washes, with acid or alkaline pre-cooking, with ultrafiltration, and with different raw materials.

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MATERIALS AND METHODS

Materials

Defatted soya flakes were obtained from Dongbang Oil (Korea) and Cargill (USA) as raw materials for protein hydrolysis. Three types of protease manufactured by Novo Nordisk A/S (Bagsvaerd, Denmark) were used in the protein hydrolysis: Alcalase™ 2.4L from *Bacillus licheniformis*, Neutrase™ from *Bacillus subtilis* and Flavourzyme™ 1000 from *Aspergillus oryzae*. Activated carbon was obtained from Jeil Carbon (Korea). Other reagents were of analytical grade.

Pre-treatment

A diagram of the whole process is summarized in Fig. 1. Defatted soya flake (Dongbang) was dry-milled using Alpine 250 miller (Alpine Augsburg, Germany) through 1 mm screen. 15 kg of the milled soya was suspended with 135 kg of tap water and then pre-cooked at 120°C for 10 min. The pH during the cooking was varied: most of pH was natural (around 6.5), but a few

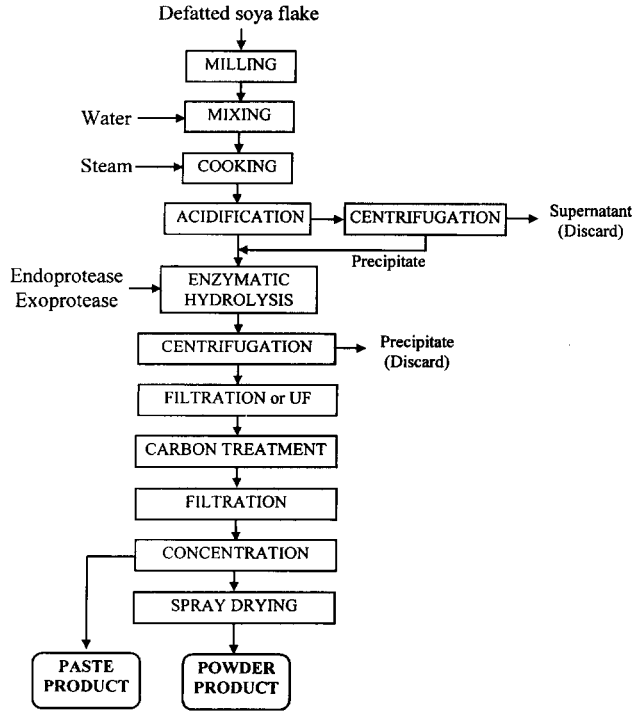

Fig. 1. Process diagram for the enzymatic hydrolysis of soy protein.

alkaline cookings (Trial no. 5, 7 and 8 in Table 1) were performed at pH 8.5, and acid cooking was carried out at pH 4.2. After the cooking, pH was adjusted to 4.2-4.5 by the use of HCl, which was followed by centrifugal separation, and the centrifuge was discharged. Most centrifugations were carried out using SC35 centrifuge (Westfalia, Germany). In a few trials, the precipitate was acid washed at pH 4.2-4.5 additional two times (Trial no. 1, 6 and 9-11). The precipitate was re-suspended with tap water, and the pH was adjusted to 7.5 for hydrolysis using NaOH.

Protein Hydrolysis

Enzyme hydrolysis was divided into two steps. In the first step, one of the following enzyme combinations was applied: 1) Neutrase 0.5L (2.0%), Alcalase 2.4L (0.1%) and Flavourzyme 1000 (1.0%); 2) Alcalase 2.4L (0.5%) and Flavourzyme 1000 (1.0%). In all cases, the enzyme dosage was measured according to the expected protein content in the reaction mixture. The hydrolysis was carried out at 55°C for 4 h. The reaction mixture was stirred by a four-blade impeller at a speed of 200 rpm.

In the second step, the pH was reduced to 5.0 by the use of 30% HCl, after which Flavourzyme 1000 was added to a concentration of 1.0% (w/w), based on protein content. Reaction temperature was held at 55°C. The hydrolysis continued for another 4 h, in a few cases (Trial no. 9 and 11) it was carried out for 16 h. During the hydrolysis the pH was not adjusted. The enzyme reaction was usually terminated by heating the reaction mixture up to 90°C for 10 min.

Post-treatment

Soluble protein fraction was separated from the reaction mixture by centrifugation (6000 g, 15 min). In some cases, the centrifugate was ultrafiltered using PCI ultrafiltration module (PCI Membrane Systems, England) with FP100 membrane (MWCO=100,000) or ENTA01 membrane (MWCO=1,000). The carbon treatment was carried out using 1.0% activated carbon based on protein content at 55°C for 30 min. After the carbon treatment, the solution was filtered on Orion filter (Seitz-Werke GMBH, Germany) with 250/EKS plate. In most cases, the products was concentrated to 30 Brix using LUWA L110 evaporator (LUWA AG, Switzerland) at 50-60°C under vacuum. In Trial no. 12, the concentration of the product was carried out by reverse osmosis (RO) using PCI module AFC30. In case the hydrolysate was produced as a spray dried form, the concentrated product was dried on Niro Atomizer P-6.3 spray drier (Niro Atomizer A/S, Denmark). Tin and Tout were 220°C and 80°C, respectively.

Analytical Methods

Degree of hydrolysis (DH) was assayed by analytical means, as the free amino groups in the peptides were analyzed according to TNBS method [12]. Molecular weight (MW) distribution and FAA composition were

Table 1. Various treatment methods for the production of protein hydrolysates

No. Trial	Raw material[1)]	Method of cooking[2)]	No. of acid wash	Enzyme used in the first hydrolysis[3)]	UF (MWCO[4)])	Carbon treatment	Concentration method[5)]	Spray drying
1	T	N	2	N/A/F	-	+	EVAP	+
2	T	N	0	N/A/F	-	+	EVAP	+
3	T	N	0	N/A/F	-	+	EVAP	-
4	T	ACID	0	N/A/F	-	+	EVAP	-
5	T	ALK	0	N/A/F	-	+	EVAP	-
6	T	N	2	A/F	-	+	EVAP	+
7	U	ALK	0	N/A/F	-	+	EVAP	+
8	U	ALK	0	N/A/F	(1,000)	+	EVAP	+
9	T	N	2	N/A/F	(1,000)	+	EVAP	+
10	T	N	2	N/A/F	-	-	EVAP	+
11	T	N	2	N/A/F	(1,000)	+	EVAP	+
12	T	N	0	N/A/F	(100,000)	+	RO	+

[1)]Raw material: T, toasted (Dongbang); U, untoasted (Cargill)
[2)]Method of cooking: N, natural cooking; ALK, alkaline cooking; ACID, acid cooking
[3)]Enzyme used in the first hydrolysis: N/A/F, Neutrase/Alcalase/Flavourzyme; A/F, Alcalase/Flavourzyme
[4)]MWCO: MW cut off
[5)]Concentration method: EVAP, evaporation; RO, reverse osmosis.

determined by gel permeation chromatography [13] and reverse phase chromatography [14], respectively. Total protein content was estimated from total nitrogen (TN) measured by Kjeldahl method [15].

RESULTS AND DISCUSSION

It was previously found that an exoprotease was required if an acceptable taste should be achieved (data not shown). Thus, enzymatic hydrolysis was carried out in two steps, first with a hydrolysis with combination of endoproteases (Neutrase and Alcalase) and exoprotease (Flavourzyme), followed by a second hydrolysis with an exoprotease. When used in conjunction with endoproteases, exoproteases were reported to produce non-bitter hydrolysates [16, 17]. Various unit operations were tried as shown in Table 1. All products prepared with the experimental conditions and some commercially available protein hydrolysates were analyzed to determine the MW distribution, free amino acid content (%FAA) and protein content (%Protein), and %DH, which were summarized in Table 2.

Pre-treatment

The use of acid washes (Trial no. 1 and 2) showed no significant difference in %FAA, %Protein and average MW, as shown in Table 2. The number of acid washes has been reported to have a clear influence on product purity, as the protein content was increasing with increasing number of washes [11, 17]. However, %DH did show no significant effect of the number of acid washes. The use of two acid washes seemed to reduce the final yield, and taste was not affected positively by the acid washes. The taste was changing somehow, thus the best taste was observed without acid washes. On the other hand, for some applications such as in protein-enriched beverages and clinical use, acid washing two or three times was adequate to increase protein purity and remove trypsin inhibitor [11].

The purpose of pre-cooking was to decompose the cell structure of soya flake, which would result in unfolding of the peptide chains and a final breakdown of the starch contained in soy [18]. Another point was to sterilize the reaction mixture. Several trials (Trial no. 3-5) were carried out to investigate the influence of pH during pre-cooking on %DH. Three levels were tested, i.e. pH 4.2 (acid cooking), 6.5 (natural cooking), and 8.5 (alkaline cooking). Increasing pH resulted in lower % DH, probably by the difference of insoluble protein content due to the heat treatment.

Table 2. Degree of hydrolysis, %FAA and %Protein of protein hydrolysates

Sample	%DH	%FAA	%Protein	average MW
Trial no. 1	48.2	25	73.2	552
2	42.0	26	66.1	521
3	48.5	18	39.3	632
4	52.6	20	34.8	594
5	45.0	16	32.8	520
6	47.4	28	75.2	500
7	48.4	31	63.6	418
8	49.3	32	64.0	382
9	51.8	30	67.9	331
10	46.6	25	73.6	570
11	43.7	28	75.7	497
12	48.3	15	67.7	634
(aH1)[1)]	85.1	14	19.4	220
(aH2)	62.0	30	50.7	252
(aH3)	54.5	24	42.4	303
(aH4)	71.7	27	41.5	282
(3H3/K)	57.5	31	23.5	372
(254/K)	57.2	27	24.5	436

[1)]( ): Commercial products
*aH1, aH2, aH3 and aH4 are acid hydrolysates.
*3H3/K and 254/K are enzyme hydrolysates of Nestle (Switzerland).

Enzyme and Raw Material

There seemed to be a little difference between Alcalase/Flavourzyme and Neutrase/Alcalase/Flavourzyme treatments when %DH and MW were considered (Trial no. 1 and 6). The taste, however, was better when Neutrase/Alcalase/Flavourzyme were used in place of Alcalase/Flavourzyme, in addition the %DH analysis indicated a better hydrolysis (higher % DH) with Neutrase/Alcalase/Flavourzyme.

It appeared that something in the raw material inhibited the action of protease, as there was an influence on %DH in the preliminary experiments (data not shown). Defatted soya flake obtained from Dongbang was toasted one. The purpose of toasting was to remove trypsin inhibitor [19]. However, the solubility of the toasted raw material was relatively low [PDI (protein dispersibility index) was 46%]. It seemed necessary to use highly purified and soluble raw material. In Trial no. 7 and 8, "protein isolate" produced from Cargill flour 200/90 with a PDI of 90% was hydrolyzed. In these trials, soya isolate was produced by an alkaline pre-cooking followed by an acid precipitation: 1) First, the protein was suspended and cooked at alkaline pH 8.5, followed by alkaline centrifugation, whereby the proteins were isolated from the fiber, 2) Second, the centrifugate was acidified to pH 4.5 and a new separation was performed, in order to isolate the protein from soluble sugars and ions. The isolate was then suspended in water and the hydrolysis was then carried out as usual, followed by ultrafiltration with a membrane (MWCO=1,000). It appeared that there was very little impact of the ultrafiltration on %DH, %Protein and MW, but in both cases %FAA were relatively high (Trial no. 7 and 8). Unfortunately, the yields were too small (28.2% in Trial no. 7 and 22.2% in Trial no. 8) since the alkaline cooking made too much insoluble protein.

In Trial no. 5 and 7, the two raw materials (toasted and untoasted) were compared each other. Untoasted soya obtained from Cargill with higher solubility was more effective on the proteolysis of soy protein (higher %DH and lower MW). However, the toasted soya obtained from Dongbang was shown to be more cost-effective than the untoasted one.

Post-treatment

The %DH of protein might be improved by ultrafiltration with a suitable membrane, thus only short peptides could penetrate through the membrane. The permeate might then have desirable high %DH. In Trial no. 9, the first hydrolysis was carried out as usual, but the second was carried out over night for 16 h. Ultrafiltration was carried out simultaneously during the second hydrolysis. The molecular weight of the UF-permeate was quite low (MW=283), whereas it became

Table 3. Comparison of free amino acid composition in protein hydrolysates

Amino acid	Acid hydrolysate, aH2	Enzyme hydrolysate (Trial no. 9)	
		UF-permeate	Spray dried
Arg	6.51	8.07	8.21
His	2.27	2.11	2.21
Lys	6.71	6.45	6.89
Tyr	0.27	4.05	2.92
Trp	-	-	-
Phe	3.27	6.55	6.17
Cys	0.22	0.96	0.74
Met	0.26	1.88	1.98
Ser	6.98	5.45	5.81
Thr	4.96	3.95	4.13
Leu	3.88	9.07	10.25
Ile	2.18	5.31	6.03
Val	4.79	5.83	6.64
Glu	25.24	12.26	12.58
Gln	-	7.59	3.95
Asp	11.16	3.86	4.06
Asn	-	10.57	11.1
Gly	5.17	2.01	2.15
Ala	7.94	4.02	4.19
Pro	7.45	1.12	0.45
Total	99.26	101.11	100.46

-: Not detected.

331 after spray drying. This might be due to that some precipitation occurred during evaporation of the product, as the precipitate could be free amino acids like tyrosine, which had very low solubility. The precipitate was also causing difficulties when the spray-dried product was resolubilized. In the case ultrafiltration was carried out after the second step hydrolysis (Trial no. 11), it appeared that the precipitation was less significant than the case ultrafiltration was performed simultaneously with the second hydrolysis (Trial no. 9).

Activated carbon was employed on the hydrolysates to remove off-flavor including beany flavor. Carbon treatment had only minor effect on the analytical results (Trial no. 1 and 10).

Experiments were carried out to investigate the influence of water hardness and heat treatment on the precipitation properties of the products (data not shown). From the experiments with either deionized water or tap water, the hydrolysis seemed independent of water hardness. The paste products, not spray dried but just concentrated by evaporation, developed haziness during cold storage. The precipitate could not be dissolved unless the solution was heated.

Trial no. 12 was performed using a different UF membrane (MWCO=100,000). The concentration was carried out using a RO module, followed by spray drying at very mild conditions, i.e. 170°C in and 70°C out. The solubility of the powder was improved. However, when the solution was boiled, some precipitate occurred. Again this could be overcome by adding citric acid to lower pH. It was reported that the haze formation was observed at higher pH values and caused by the presence of calcium ion when tap water was used for dilution [11].

Amino Acid Composition

The difference between an enzymatically hydrolyzed product (Trial no. 9) and a commercial acid hydrolysate (aH2) was significant in amino acid composition as shown in Table 3. Comparing the amino acids such as arginine, tyrosine, phenylalanine, leucine, isoleucine, glutamine, glutamic acid, asparagine and aspartic acid, the difference of amino acid content was evident. The lackness of glutamine and asparagine in a commercial acid hydrolysate was probably due to the deamination of them by acid hydrolysis. The acid-hydrolyzed product (aH2) appeared to be originated from wheat gluten, since the contents of glutamic acid, aspartic acid and proline were relatively higher than enzyme hydrolysates. The content of tyrosine might be a main reason for precipitation during storage as the solubility of this amino acid was very low. The low content of tyrosine in the commercial products indicates also that this amino acid has been removed, for instance by an intermediate storage and filtering before final packaging. This coincided with the report that the solubility of a protein is related to the content of hydrophobic amino acids like tyrosine, phenylalanine and leucine [20].

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REFERENCES

[1] Lahl, W. J. and S. D. Braun (1994) Enzymatic production of protein hydrolysates for food use. *Food Technol.* 48: 68-71.
[2] Frokjaer, B. (1994) Use of hydrolysates for protein supplementation. *Food Technol.* 48: 86-88.
[3] Schmidl, M. K., S. L. Taylor, and J. A. Nordlee (1994) Use of hydrolysate-based products in special medical diets. *Food Technol.* 48: 77-85.
[4] Mahmoud, M. I., W. T. Malone, and C. T. Cordle (1992) Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. *J. Food Sci.* 57: 1223-1229.
[5] Herbert, A. B. and P. Dunnill (1988) Limited modifications of soya proteins by immobilized subtilisin: comparison of products from different reactor types. *Biotechnol. Bioeng.* 32: 475-481.
[6] Ericsson, R. J. and G. A. Youngdale (1970) Male antifertility compounds: structure and activity relationships of U-5897, U-15,646 and related substances. *J. Reprod. Fert.* 21: 263-266.
[7] IHPC board (1995) Position paper on 3-monochloropropanediol (3-MCPD). IHPC Report 3-6-95. Washington.
[8] Pommer, K. (1995) New proteolytic enzymes for the production of savory ingredients. *Cereal Foods World* 40: 745-748.
[9] Olsen, H. S. and J. Adler-Nissen (1979) Industrial production and applications of a soluble enzymatic hydrolyzate of soya protein. *Process Biochem.* 14: 6-11.
[10] Quaglia, G. B. and E. Orban (1987) Influence of the degree of hydrolysis on the solubility of the protein hydrolysates from sardine (sardine pilchardus). *J. Sci. Food Agric.* 38: 271-276.
[11] Adler-Nissen, J. (1986) ISSPH-the production and properties of an enzymic food protein hydrolysate. p. 263-313 In: J. Adler-Nissen (ed.) *Enzymatic Hydrolysis of Food Proteins.* Elsevier Applied Science Publisher. New York.
[12] Adler-Nissen, J. (1979) Determination of the degree of hydrolysis of food protein hydrolyzates by

trinitrobenzenesulfonic acid. *J. Agric. Food Chem.* 27: 1256-1262.
[13] Richter, W. O., B. Jacob, and P. Schwandt (1983) Molecular weight determination of peptides by high-performance gel permeation chromatography. *Anal. Biochem.* 133: 288-291.
[14] Waters Associate (1990) p.24-32 In: *Waters amino acid analysis system.* Youngin Scientific Co., Ltd. Seoul.
[15] Hjalmarsson, S. and R. Akesson (1983) Modern Kjeldahl procedure. *Int. Laboratory.* 3: 70-76.
[16] Howell, N. K. (1996) Chemical and enzymatic modifications. p. 262-263 In: S. Nakai and H. W. Modler (ed.) *Food Proteins: Properties and Characterization.* VCH Publishers, Inc. New York.
[17] Chae, H. J., M.-J. In, and M.-H. Kim (1997) Optimization of enzyme treatment for the enzymatic production of hydrolyzed vegetable protein. *Kor. J. Food Sci. Technol.* 29: 1125-1130.
[18] Chae, H. J., M.-J. In, and J. D. Lee (1998) Production of a flavoring material from soymilk residues by combined use of enzymes and microorganisms. *Agric. Chem. Biotechnol.* 41: 73-77.
[19] Friedman, M., D. L. Brandon, A. H. Bates, and T. Hymowitz (1991) Comparison of commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: composition, nutritional value, and effect of heating. *J. Agric. Food Chem.* 39: 327-335.
[20] Bigelow, C. C. (1967) On the average hydrophobicity of proteins and the relation between it and protein structure. *J. Theor. Biol.* 16: 187-211.