J. Microbiol. Biotechnol. (1999), 9(4), 509–513

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Molecular Characterization and Bitter Taste Formation of Tryptic Hydrolysis of 11S Glycinin

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Received: May 12, 1999

Abstract The molecular size reduction and the formation of bitterness during a tryptic hydrolysis of soybean 11S glycinin were determined by using quantitative analysis and organoleptic evaluation. The 11S glycinin of 90% purity was prepared by cryoprecipitation and Con A Sepharose 4B affinity chromatography, and hydrolyzed with trypsin in a pH-stat reactor for 4 h. Bitterness was formed within 1 h of hydrolysis, and then slowly increased up to 3.5×10^{-5} M quinine-HCl equivalent. The extent of hydrolysis (DH) was 7% at 1 h and increased up to 12% by the end of the reaction. The α-amino nitrogen content increased from an initial 0.7 mM to 7 mM at the end of the period. The SDS-PAGE analysis showed that the acidic subunit of 11S glycinin was mostly hydrolyzed. The GP-HPLC analysis indicated that the bitterness was mainly contributed by the peptide fractions of molecular weights of 360-2,100 Da.

Key words: Soybean protein, enzymatic hydrolysis, bitterness, peptides

Enzymatic hydrolysis of food proteins improves digestibility and nutritional value along with food functionality and preference [1]. The recent findings on the physiological function of peptides stimulated the interests of researchers on the subject of protein hydrolysates [7]. However, the formation of bitterness during the hydrolysis of some proteins, especially casein and soybean protein, limits the utilization of these proteins. Bitterness formation during the hydrolysis of casein [15], soybean protein [4], and corn zein [20] have been studied widely. The hydrophobicity of protein, the type of enzyme used, and the extent of hydrolysis are known to be the major factors controlling the bitterness of protein hydrolysates. Several debittering methods have also been suggested, such as the solvent extraction with 2-butanol [8], addition of cyclodextrin [19], plastein reaction [3], hydrophobic chromatography [14], ultrafiltration [8], exopeptidase application [13], and fermentation [12]. Adler-Nissen proposed a method to control a degree of hydrolysis (DH) [1]. Based on this method, Kim *et al.* [8] was able to reduce 10% of the bitterness of soybean protein hydrolysate by controlling the DH at 10%.

We have investigated the hydrolysis pattern of soybean 11S protein by trypsin in a pH-stat reactor. The peptides of the hydrolyzed protein were initially identified by their molecular weights using SDS-PAGE electrophoresis. The change in the bitterness during the hydrolysis was monitored by using sensory evaluation, and the range of molecular sizes of the corresponding bitter fractions was estimated by gel permeation chromatography.

MATERIALS AND METHODS

Preparation of 11S Glycinin

Soybean 11S glycinin was prepared from a defatted soybean meal using the method of Kitamura *et al.* [9]. Soybean protein was extracted from defatted soybean meal by using 0.03 M Tris-HCl (pH 8.0) buffer containing 0.01 M β-mercaptoethanol. The pH was adjusted to 6.4 with 2 N HCl solution, and precipitate was obtained at 4°C. The precipitate was washed with Tris-HCl buffer and then dispersed in standard potassium phosphate buffer (pH 7.6). The protein dispersion was adjusted to 1 M-NaCl and applied to Con A-Sepharose 4B affinity chromatography. The column size was 1.5x30 cm, and the flow rate of standard buffer elution was 15.2 ml/h. The 11S glycinin fraction was dialyzed against distilled water and confirmed by SDS-PAGE [10] before lyophilization.

Tryptic Hydrolysis

Aliquots of aqueous 1% 11S glycinin was placed in a reaction bottle of pH-stat apparatus (Titerlab 91, Radiometer, Denmark), and heated to 50°C in a water bath [1]. During this process, 1% trypsin solution (crude type II, serine protease from porcine pancreas, optimum pH 7-9, 1130 BAEE units/mg solid) (Sigma, St. Louis, U.S.A.) was

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stirred until the enzyme/substrate ratios became 0.5, 1, 2, and 3%. It was incubated for 4 h with stirring, and 0.1 N NaOH solution was automatically added to maintain the pH level of 7.6. This reaction was terminated by placing the sample tube in boiling water for 10 min. The α-amino nitrogen content of the hydrolysate was determined by using TNBS [16].

Determination of Molecular Size Distribution

Changes of molecular size of the protein during hydrolysis were monitored by SDS-PAGE [10] and gel permeation chromatography using HPLC (Gilson, Middleton, U.S.A.) [11]. For SDS-PAGE, 10% sodium dodecyl sulfate was used and samples of 2-ME (β-mercaptoethanol) treated or non-treated were applied. For GPC, Superdex peptide column (molecular weight range 100-7,000 Da, Pharmacia Biotech, Denmark) was used, and 0.02 M potassium phosphate buffer (pH 7.2) containing 0.25 M NaCl was used to elute the HPLC at a flow rate of 0.25 ml/min. An injection volume was 100 µl, and the absorbence at 214 mm was measured by a UV-detector. Glycine (75 Da), $(\text{glycine})_6$ (360 Da), gastrine (2,125 Da), aprotinin (6,500 Da), and cytochrome c (12,500 Da) were used as molecular size markers. The molecular size distribution was calculated by adding the percentage of each peak area to the total peak area which was detected. The free amino acid compositions of the hydrolysates were analyzed by the OPA (σ-phthaldehyde) method [5].

Sensory Evaluation

Spotting on the tongue-method [11] was used to determine the quinine-HCl equivalent bitterness of the hydrolysates. Three members of the trained panelists were assessed and 0.5 ml of sample was directly spotted on the rear side of the tongue. The degree of bitterness was recognized after 30 sec and this was marked on a line scale in comparison with the standard quinine-HCl solution ($2.4-6.5\times10^{-5}$ M).

Results and Discussion

Trypsin Hydrolysis of 11S Glycinin

The purity and yield of 11S glycinin obtained by the purification process is presented in Table 1. The purity of

Table 1. Purity and yield of 11S glycinin on purification.

Purification step	Total protein (g)	Purity (%)	Soybean 11S glycinin (g)	Yield (%)
Defatted soy flour	20.27	37.8	7.66	100
Cryoprecipitation (pH 6.4)	5.00	89.2	4.46	58.2
Con-A affinity chromatography	2.34	90.3	2.11	27.6

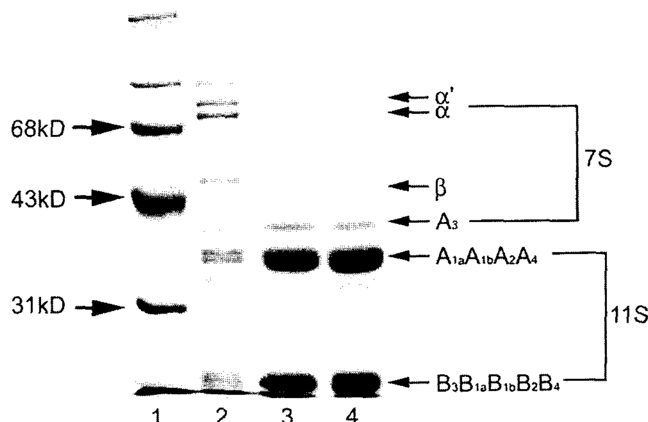

Fig. 1. SDS-PAGE electrophoresis of 11S glycinin purification. Lane 1: Molecular weight marker. Lane 2: Defatted soy flour extract. Lane 3: pH 6.4, precipitated 11S rich fraction. Lane 4: 11S glycinin eluted from affinity chromatography. α, α', and β are identified as 7S conglycinin. A and B indicate the acidic and basic subunits of 11S glycinin, respectively.

the 11S glycinin used in this study was 90.3% and the protein yield was 27.6% of the defatted soybean meal. Figure 1 confirms the purity of 11S glycinin by SDS-PAGE electrophoresis. It is suggested that adding 2-mercaptoethanol (2-ME) breaks the disulfide bonds in 11S glycinin, and results in the separation of acidic (37-42 kDa) and basic (17-20 kDa) subunits as shown in Fig. 1 [17].

The degree of hydrolysis (DH) of 11S glycinin which was hydrolyzed by trypsin at different enzyme/substrate ratios was determined by pH-stat, and this is shown in Fig. 2. The DH increased by using the hydrolysis time and the concentration of enzyme applied. It varied from DH 4% with E/S ratio of 0.5% to DH 12% with E/S ratio of 3% during 4 h of hydrolysis. According to Kang *et al.* [6], trypsin had a relatively weak proteolytic activity as compared to other proteases like Alcalase, Neutrase, papain,

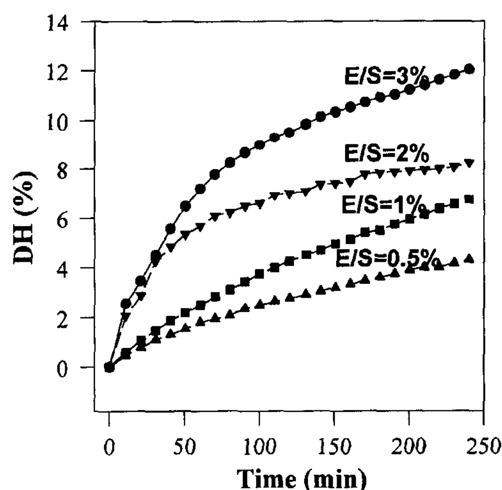

Fig. 2. Degree of hydrolysis (DH) of 11S glycinin hydrolyzed at different enzyme/substrate (E/S) ratios.

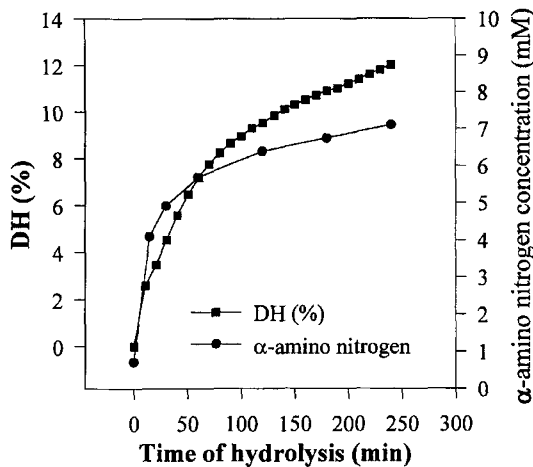

Fig. 3. Degree of hydrolysis (DH) and free α-amino nitrogen concentration of 11S glycinin hydrolysates (3% E/S) at different times.

and bromeline. However, by increasing the E/S ratio, the trypsic proteolysis was sufficient enough to produce a bitter taste. When the E/S ratio was increased more than the suggested level, the bitterness decreased. As shown in Fig. 3, the α-amino nitrogen content increased to 7 mM with 3% E/S ratio for 4 h of hydrolysis.

Changes in SDS-PAGE Pattern by Hydrolysis

Figure 4 shows the changes in the electrophoretogram of 11S glycinin during the hydrolysis (3% E/S) process. Figure 4A represents the electrophoretogram of the 2-mercaptoethanol (ME) treated sample and Fig. 4B of the non-treated ones. From Fig. 4A, the acidic subunit was broken down mostly during the first 15 min of hydrolysis, and disappeared completely after 1 h of hydrolysis, while the basic subunits of 21 kDa molecular weight were slightly hydrolyzed. Also, it persisted mostly during 4 h of hydrolysis. The fractions below

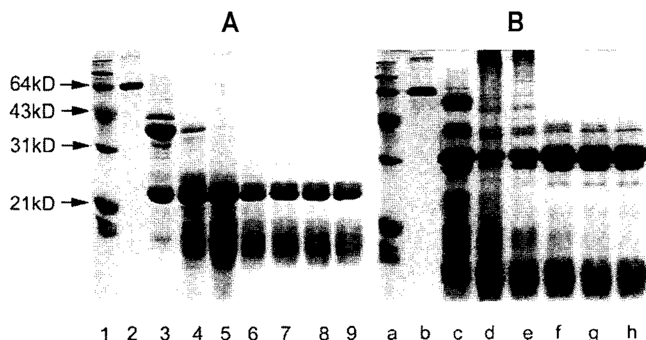

Fig. 4. The molecular weight distribution of 11S glycinin hydrolysates using SDS-PAGE at different hydrolysis times.
A: 2-mercaptoethanol and B: no 2-mercaptoethanol. Lanes 1 and a: Molecular weight marker. Lanes 2 and b: BSA. Lane 3: 11S glycinin. Lanes 4 and c: 15 min hydrolysis. Lanes 5 and d: 30 min hydrolysis. Lanes 6 and e: 1 h hydrolysis. Lanes 7 and f: 2 h hydrolysis. Lanes 8 and g: 3 h hydrolysis. Lanes 9 and h: 4 h hydrolysis.

21 kDa increased rapidly during the first 15 min of hydrolysis but gradually decreased, as they were further broken down.

As for the non-ME treated sample, the major band appeared at 56 kDa, which disintegrated into two fractions, 40 kDa and 30 kDa, respectively, during the first 15 min of hydrolysis. The 56 kDa and 40 kDa fractions disappeared within 1 h of hydrolysis, and 30 kDa and much lower molecular size proteins remained stable. This, indeed, indicated that trypsin reacted mainly on the acidic subunit of soybean 11S glycinin, while basic subunits were relatively stable. The rapid increase in DH and α-amino nitrogen content at an initial stage of trypsin hydrolysis coincided with findings in the SDS-PAGE analysis as shown in Figs. 2 and 3. Kang *et al.* [6] also reported that trypsin was effective for acidic subunits of 11S glycinin, and explained that the hypervariable region (HVR) was vulnerable to the enzyme reaction of the acidic subunit.

Molecular Size Distribution of the Hydrolysate

Table 2 shows the change in molecular size distribution of a soluble fraction of the hydrolysate (3% E/S), as determined by gel permeation HPLC. The large molecular fraction of over 12 kDa decreased rapidly from 19.1% at the first 15 min of hydrolysis to 1.8% at 1 h and further to 0.19% by the end of the hydrolysis. The fraction of 6,500-12,500 Da decreased from 31.8% to 10.4%, while that of 2,100-6,500 Da remained constant during the entire period of hydrolysis. On the other hand, the low molecular size peptides of 360-2,100 Da increased to 33.9%, and the contents of di- and tri-peptides and free amino acids also increased up to 37.6% during 4 h hydrolysis. In general, most of the large molecules of over 6,500 Da were hydrolyzed into small peptides of below 2,100 Da in 1 h. This result was consistent with the findings in SDS-PAGE analysis and DH determination with pH-stat.

Bitterness Formation During the Hydrolysis

Change in bitterness of 11S glycinin hydrolysate during the 4 h tryptic hydrolysis (3% E/S) is presented in Fig. 5. The bitterness was formed during the first 1 h of hydrolysis, reaching to the extent just exceeding the threshold level and then further increased thereafter. Similar

Table 2. Molecular weight distribution of 11S glycinin hydrolysates at different hydrolysis times.

Range of molecular weight (Da)	Hydrolysis time (min)[a]					
	15	30	60	120	180	240
> 12,500	19.13	11.95	1.84	0.66	0.28	0.19
6,500-12,500	31.78	27.88	16.45	14.44	10.96	10.40
2,100-6,500	20.60	22.51	19.48	23.61	19.53	17.97
360-2,100	19.67	24.45	26.64	30.19	32.40	33.86
< 360	8.81	13.21	35.58	31.11	36.83	37.58

[a]ratio of hydrolysis time/distribution (min/%).

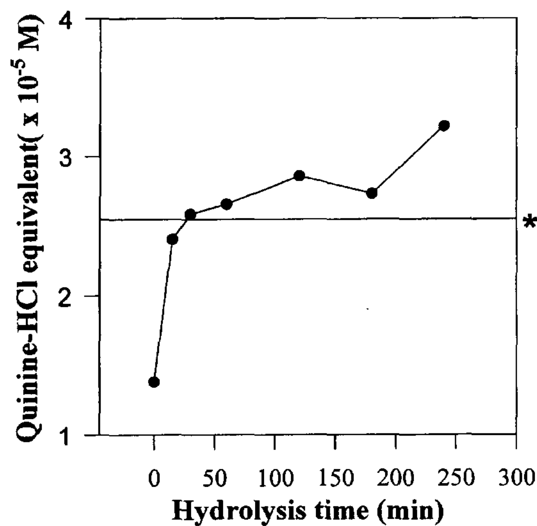

Fig. 5. Bitter intensity of 11S glycinin hydrolysates at different hydrolysis times.
*Threshold value of quinine-HCl solution.

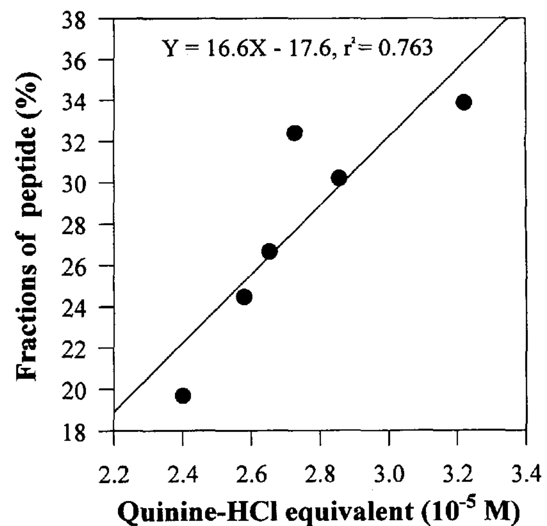

Fig. 6. The relationship between bitter intensity and fraction of M.W. 360-2,100 Da peptide at different hydrolysis times.

pattern was also recognized with DH. Adler-Nissen [1] reported that protein hydrolysates of 5-10% DH generally had a non-bitter bland taste but the bitter taste intensified upon additional hydrolysis. Fujimaki *et al.* [4] claimed that the peptic hydrolysis of the soybean protein developed a weak bitterness after a 1 h hydrolysis, a moderate bitterness after 3 h, and a strong bitterness after 6 h. In our study, the DH after 1 h tryptic hydrolysis (3% E/S) was 7% and, at this point, the bitterness was recognized by the sensory panelists and the intensity increased as the hydrolysis time increased, thus agreeing with the previous researchers.

Table 3. Change of free amino acid contents in 11S glycinin hydrolysates.

Amino acid	Ratio[a]		
	15 min	60 min	240 min
Asp	0.0616	0.0743	0.0716
Glu	0.0493	0.0532	0.0936
Cys	0.0188	0.0530	0.1418
Gly	0.0514	0.0666	0.0468
Arg	2.0906	2.5019	2.4478
Thr	0.0689	0.0957	0.0743
Ala	0.0704	0.0888	0.1288
Tyr	0.7914	0.9015	1.0325
Met	0.0998	0.1897	0.0256
Val	0.1696	0.2723	0.5447
Trp	0.2444	0.3435	0.4309
Phe	0.7026	0.9626	1.1020
Leu	0.1124	0.1716	0.2690
Ile	0.3957	0.6595	0.9521
Lys	-	0.7559	1.8273
Total	14.4061	14.7064	19.0138
Hydrophobic	2.6551	3.6852	4.5599
amino acid (%)	(18.43)	(25.06)	(23.98)

[a]Ratio of time/concentration of amino acid (min/μg/ml).

Table 3 shows the free amino acid composition after hydrolysis for 15 min, 1 h, and 4 h. Free amino acid content increased slightly from 14.4 mg/ml at 15 min to 14.7 mg/ml at 1 h of hydrolysis, but increased significantly up to 19.0 mg/ml at 4 h of hydrolysis. The contents of hydrophobic amino acids, such as phenylalanine, isoleucine, tryptophan, and leucine, contribute to bitterness, which increased with time of the hydrolysis. However, a gradual increase of bitterness after 1 h of hydrolysis appeared to be related to a reduction of large molecular size proteins of 2,100-12,500 Da to the smaller oligopeptides of 360-2,100 Da molecular size. Figure 6 shows the linear relationship between the contents of these oligopeptides and the bitterness intensity of the hydrolysate. Sullivan *et al.* [18] proposed that strong bitter peptides were found in the peptides of 2-23 amino acid residues, and Clegg [2] reported the longest bitter peptides to have 27 amino acid residues. Overall, it can be concluded that bitterness of the tryptic hydrolysate of 11S glycinin is mainly influenced by the oligopeptides of molecular weight between 360 and 2,100 Da.

Acknowledgments

This study was partly supported by a grant (No. KOSEF 96-0402-05-01-3) from Korea Science and Engineering Foundation.

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