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Reduction of the allergenic protein in soybean meal by enzymatic hydrolysis

Zhangcun Wang*, Lejing Li, Daoqiang Yuan, Xuewei Zhao, Shengwen Cui, Jinqiang Hu and Jizhong Wang

School of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou, China

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The effects of Alcalase and Trypsin hydrolysis on the allergenic protein and its solubility of soybean meal (SBM) were investigated by SDS-PAGE and ELISA. After 10 min of hydrolysis with Alcalase, the solubility of the protein was 87.19%, the α' , α and β -subunits of β -conglycinin disappeared and the acidic and basic subunits of glycinin decreased obviously by SDS-PAGE pattern. However, with Trypsin for 10 min, the solubility of the protein was 72.74%. The contents of α' , α subunits of β -conglycinin decreased slightly in SDS-PAGE, and the acidic and basic subunits of glycinin were almost not affected. For the insoluble hydrolysates prepared by Alcalase, the α' , α subunits of β -conglycinin and acidic subunit of glycinin decreased significantly, that by trypsin did not. The content of immune-reactive β -conglycinin obtained by ELISA was consistent with them. These results confirmed that the allergenic proteins of SBM were more sensitive to Alcalase than to Trypsin.

Keywords: soybean meal; soy allergenic protein; proteolysis; protein subunit; ELISA

1. Introduction

Soybean is an important protein in the food industry and animal feeding because of its high protein content and excellent nutritional properties. However, soybean is one of the 'big 8' food allergens (Cordle, 2004; Krishnan, Kim, Jang, & Kerley, 2009). There are over 30 kinds of soy proteins to which children and young animals generate food hypersensitivity reactions (Breiteneder & Ebner, 2000). The β -conglycinin (7S globulin) and glycinin (11S globulin), the major storage proteins (accounting about 75% of the total soy proteins) in soybean, are also major allergens (Adachi et al., 2009; Krishnan et al., 2009). They have good thermal stability that traditional heat treatments cannot remove its antigenicity (Mills et al., 2003).

The β -conglycinin is a trimeric polymers composed of three subunits (α' , α and β), the molecular weights (MWs) of them are about 76, 72 and 53 kDa, respectively (Amigo-Benavent, Clemente, Ferranti, Caira, & Dolores del Castillo, 2011). The allergenicity of β -conglycinin was contributed by its subunits, especially α' and α subunits (Maruyama et al., 1998). Glycinin is a hexamer composed of two trimers, each of the trimer consisting of three non-identical subunits formed by an approximately 40 kD acidic(A) and approximately 20 kD basic(B) chain linked by

*Corresponding author. Email: dwzhc2012@163.com

a single disulfide bond (Christensen, Bruun, & Frøekiær, 2003). The acidic and the basic subunit of glycinin are also important allergens in patients who are susceptible to soybeans (Beardslee, Zeece, Sarath, & Markwell, 2000). The allergenicity of the soy proteins is also related to the conformational epitopes, which is a special domain of the macromolecules (Shreffler, Beyer, Chu, Burks, & Sampson, 2004). Solubility, stability, size and compactness of the overall protein fold are aspects of protein structures related to allergenicity (Aalberse, 2000), so breakage of the protein conformational structure will be of benefit to reducing the allergenicity.

It has been reported that proteolytic hydrolysis could completely eliminate the allergenicity using purified soy proteins as substrates and some of them have been used in infant formulas (Franck et al., 2002). Tsumura, Kugimiya, Bando, Hiemori, and Ogawa (1999) showed that protease Proleather FG-F was effective to hydrolyze α and α' subunits of β -conglycinin in soybean protein isolates, thereby reducing its allergenicity. When soybean glycinin was hydrolyzed by pepsin and chymotrypsin, the overall allergenicity of the protein reduced evidently, and the fragments of less than about 20 kDa in hydrolysates were not immune-reactive (Lee et al., 2007), which correlated well with the data reported by Burks et al. (1992). Franck et al. (2002) reported that soy protein hydrolysates with the fragment of less than 28 kDa have been proved to lack allergenicity for infant. Another study indicated that pepsin and trypsin were of evident specificity to hydrolysis β -conglycinin and glycinin, but the β -subunit of β -conglycinin could not be effectively hydrolyzed by pepsin. The immune-reactivity was positively related to stability of glycinin and β -conglycinin (Zhao, Qin, Sun, Zhang, & Wang, 2010). However, the information about the changes of soy protein molecules was not clear during proteolytic hydrolysis using soybean meal (SBM) as substrates.

In this report, the defatted SBM was hydrolyzed with Alcalase and trypsin. The changes of the soybean proteins subunits were investigated with SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) and the contents of immunoreactive protein in the hydrolyzed SBM were determined with a sandwich enzyme-linked immunosorbent assay (ELISA) method in order to evaluate the effectiveness of the enzymolysis in removing the allergenic protein. The specificity ELISA was extensively used to detect the allergenicity of soybean proteins and soy product (L'Hocine & Boye, 2007; L'Hocine, Boye, & Munyana, 2007; Pedersen et al., 2008).

2. Materials and methods

2.1. Materials

Low-temperature defatted SBM was purchased from Jiahua Oil Co. (Shandong, China). Alcalase (food-grade enzyme, 2.4 AU/g) and Trypsin (food-grade enzyme, 3.3 AU/g) were kindly provided by Novozymes Enzyme Co. All electrophoretic reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Soybean β -conglycinin ELISA kit was purchased from Rapidbio Co., USA. All chemicals were of analytical grade.

2.2. Enzymatic hydrolysis of SBM

SBM was smashed to pieces through 60 mesh sieves and dispersed in distilled water to a final protein concentration of 100 mg/mL (w/v) and adjusted to pH8.0 with 1 mol/L NaOH, which was then incubated and gently stirring at 50°C for 50 min. The hydrolysis was carried out at 50°C for Alcalase or 37°C for trypsin with continuous agitation. The enzyme addition level used was 0.30% protein for Alcalase or trypsin. Then the SBM was hydrolyzed for 0, 10, 30, 60, 90 or 120 min by two proteases, respectively. During hydrolysis, the pH of the solution was constantly adjusted to pH8.0 with 1 mol/L NaOH. The reaction was terminated by heating in boiling water for 10 min to inactivate the enzyme, and subsequently the hydrolysate was cooled to room temperature and centrifuged at $2000 \times g$ for 10 min. The precipitate was washed twice with distilled water. The supernatant was collected together and freeze-dried and the precipitate was also freeze-dried. The amount of NaOH consumed during hydrolysis was used for calculating the degree of hydrolysis (DH) according to pH-Stat method (Adler-Nissen, 1989).

2.3. Determination of protein solubility

The content of protein in supernatant was determined with Biuret method (Gornall, Bardawill, & David, 1949). The per cent of the content to total protein was as protein solubility. All the tests were conducted in duplicate.

2.4. SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for supernatant and precipitate was performed according to the method of Laemmli (1970). The sample was treated with Tris-Glycine buffer (pH8.8, containing 10% SDS and 0.2% β -mercaptoethanol) and separated with a 12% acrylamide separating gel and a 5% acrylamide stacking gel. The electrophoresed proteins were stained with 0.25% (w/v) Coomassie Brilliant blue R-250 at room temperature. MW values of protein fractions were estimated by using Protein Marker (14.4–97.4 kDa) from Sigma Chemical Co. (St. Louis, MO, USA). The protein-stained bands were analysed using the Gel-Pro Analyzer software.

2.5. Sandwich ELISA

2.5.1. Preparation of testing samples

The soluble hydrolysate was dissolved in 0.2 M phosphate buffer (pH7.4). The insoluble hydrolysate was dispersed in 0.01 mol/L NaOH for 2 h firstly and then centrifuged at $2000 \times g$ for 10 min. The supernatant was dialyzed with distilled water and freeze-drying for testing. Each sample was in step dilutions and the assay range for the determination of β -conglycinin was 5–300 ng/L.

2.5.2. Determination of β -conglycinin with ELISA

A commercially available specific sandwich ELISA for β -conglycinin of soybean was used as introduced by the manufacturer. A 96-well antibody immobilised microplate

in the original aluminium pouch was firstly balanced 30 min at room temperature. Five standard wells in replicate were set on the ELISA plates and the standard concentration was 240, 160, 80, 40, 20 ng/L, respectively. Blank well without addition of sample and HRP-conjugate solution (other each step operation is same) and testing sample wells separately were set. A 50 μ l of standard and samples was added to the wells. The solution was lightly agitated and incubated at 37°C for 30 min. After the reaction was completed, the standard and sample were discarded and each well was washed five times with washing buffer. A 50 μ l of the prepared HRP-conjugate antibody solution was added to each well, except for blank well, and incubated at 37°C for 30 min. After the reaction was completed, the HRP-conjugate antibody solution was discarded and the plate was washed five times. Then 50 μ l of Chromogen solution A and 50 μ l of Chromogen solution B were added to each well, evading the light preservation at 37°C for 15 min, the reaction was terminated by adding 50 μ l of stop solution to each well. After that, the optical density (OD) value was measured at the wavelength of 450 nm using automatic ELISA plate reader (TECAN Genios). The standard curve was obtained by plotting the OD values against standard sample concentrations and the equation was as follows: $Y = 0.0007X + 0.0173$, $R^2 = 0.994$, where Y is the OD values at 450 nm, X is the standard sample concentrations (ng/L). The concentration of β -conglycinin was determined by calculating the sample density, multiplied by the dilution factor, with the sample OD value in the equation. Each sample was analysed in triplicate.

3. Results and discussion

3.1. Effects of two proteases on solubility and DH of soybean protein

The solubility and DH profiles of protein in SBM after hydrolysis with Alcalase and trypsin were showed in Figure 1. Within first 10 min of hydrolysis, the solubility of protein treated with Alcalase was increased from 62.3 to 87.19%, and it was only from 62.3 to 72.73% with trypsin. However 30 min later, the solubility of the two hydrolysates did not increase significantly. The DH of SBM proteins treated with Alcalase increased evidently among the hydrolysis, while DH of the proteins treated with trypsin increased a little after 30 min of hydrolysis. The results showed that the protein in SBM was more sensitive to Alcalase than to trypsin, indicating that the

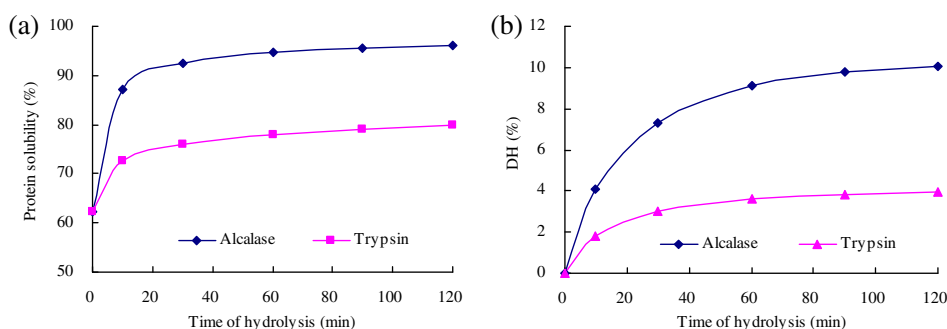


Figure 1. Solubility and DH of SBM protein hydrolyzed by Alcalase and Trypsin.

Alcalase should be more efficient than trypsin for degradation of soy allergenic protein.

3.2. SDS-PAGE of soluble components in the hydrolysates

There were many kinds of water soluble protein or subunits in native SBM, including α , α' and β subunits of β -conglycinin (7S globulin) and acidic and basic subunits of glycinin (11S globulin). After 10 min hydrolysis by Alcalase (Figure 2a), the α' , α and β -subunits disappeared completely, the acidic subunit (38 kDa) and basic subunit (21 kDa) also decreased obviously. Meanwhile, some new components (23 kDa, 17 kDa) and mass fractions below 14 kDa also appeared. After hydrolysis of 60 min, the acidic subunit (38 kDa) of glycinin entirely disappeared. At 90 min of hydrolysis, the basic subunit (21 kDa) of glycinin almost disappeared and 23 kDa fraction decreased. Meanwhile, the content of below 14 kDa fraction obviously increased. However, the content of 15 kDa subunit of the hydrolysates showed no decrease after different hydrolysis time. In the trypsinolysis of SBM (Figure 2b), the contents of α' , α and β subunits of β -conglycinin (7S globulin) were decreased about 40–50% after hydrolysis from 10 to 90 min than that of raw SBM (Table 1). The contents of the acidic and basic subunits of glycinin (11S globulin) were almost no change compared with that of raw SBM, which showed that the glycinin was not sensitive to trypsin.

The results are consistent with the previous reports. Some researches have pointed the difference of β -conglycinin and glycinin of soy protein in sensitivity to various enzymes (Molina Ortiz & Wagner, 2002; Tsumura, Saito, Kugimiya, & Inouye, 2004). The acidic subunits of glycinin are always easier to be hydrolyzed than basic subunits, which probably lie in the amino acid compositions and the existing state of polypeptide. The basic subunits of glycinin have more hydrophobic amino acids than acidic subunits and usually present in the interior of the structure of glycinin (Shutov et al., 1996).

It has been reported that α' , α and β subunits of β -conglycinin were the major allergens in soybean (Krishnan et al., 2009; Maruyama et al., 1998). The fast disappear of these subunits during proteolysis was propitious to reducing or

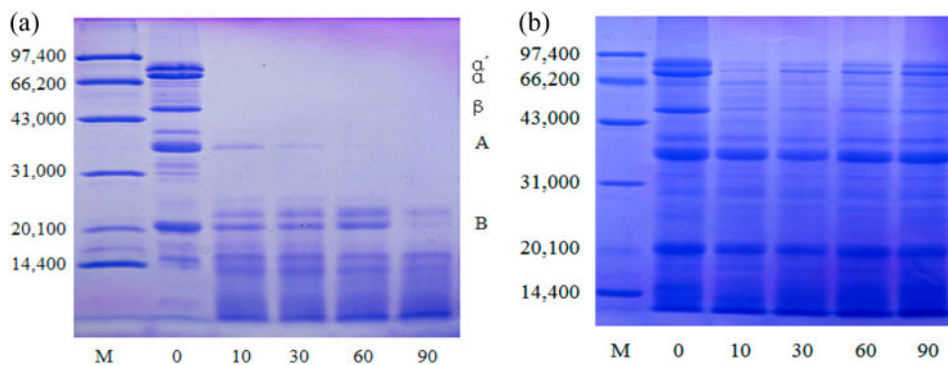


Figure 2. SDS-PAGE patterns of soluble components from SBM after different hydrolysis time (0, 10, 30, 60 and 90 min) by Alcalase (a) and Trypsin (b). Lane M: low MW markers with 14.4, 20.1, 31, 43, 66.2 and 97.4 kDa.

Table 1. Relative content of the subunits in soluble hydrolysates.

MW/kD	Control	Alcalase hydrolysis/min				Trypsin hydrolysis/min			
		10	30	60	90	10	30	60	90
76.0 (α')	13.9	—	—	—	—	12.0	11.1	9.9	9.0
72.0 (α)	14.1	—	—	—	—	13.4	11.9	10.1	8.8
53.0 (β)	12.7	—	—	—	—	6.9	6.5	5.9	5.6
40.0	2.8	—	—	—	—	2.1	1.7	1.7	1.6
38.0	26.0	4.6	1.7	—	—	20.0	20.0	19.0	19.0
23.0	—	5.5	8.6	8.8	3.4	—	—	—	—
21.0	24.5	12.3	12.6	12.3	2.7	19.5	19.5	19.5	19.5
15.0	4.4	6.8	6.0	6.9	7.7	10.2	12.2	13.7	14.7
≤ 14.4	1.6	70.8	71.1	72.0	86.2	15.9	17.1	20.2	21.8
Total	100	100	100	100	100	100	100	100	100

eliminating the allergenicity of SBM. Some researches have showed that the fragments of below 20 kDa peptides were lack of allergenicity and safe for the infants (Franck et al., 2002; Lee et al., 2007; Song, Martinez-Villaluenga, & De Mejia, 2008). So the hydrolysates of SBM by Alcalase could be used as safe ingredient of foodstuff.

3.3. SDS-PAGE of insoluble components in the hydrolysates

The water-insoluble hydrolysates (residues) obtained by centrifugation of the hydrolyzed SBM solution was analysed by SDS-PAGE so as to further understand the whole components of the hydrolysates, which has not often been reported in previous literatures. The results showed that considerable quantity of protein in SBM has not been dissolved in water, but could be extracted by Tris-Glycine buffer (pH8.8) containing 10% SDS and 0.2% β -mercaptoethanol from the residues of the hydrolyzed SBM (Figure 3 and Table 2). But in the residues of SBM hydrolyzed for

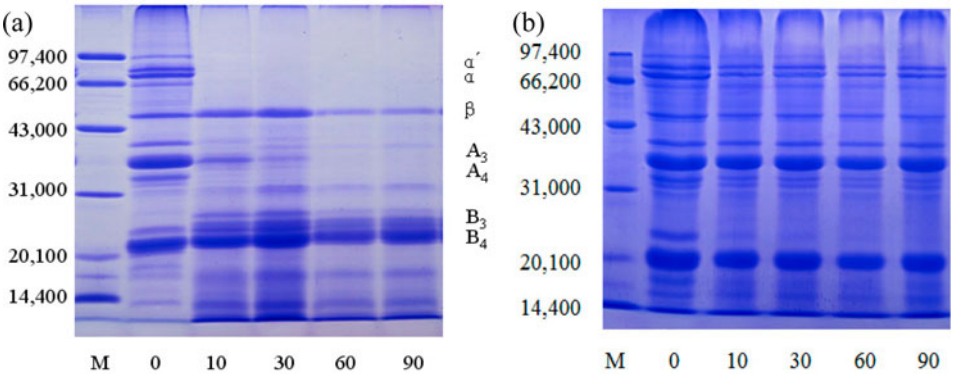


Figure 3. SDS-PAGE patterns of insoluble components from SBM after different hydrolysis time (0, 10, 30, 60 and 90 min) by Alcalase (a) and Trypsin (b). Lane M: low MW markers with 14.4, 20.1, 31, 43, 66.2 and 97.4 kDa.

Table 2. Relative content of the subunits in insoluble hydrolysates.

MW/Kd	Control	Alcalase hydrolysis/min				Trypsin hydrolysis/min			
		10	30	60	90	10	30	60	90
94.0	2.6	—	—	—	—	1.4	1.2	—	—
76.0 (α')	7.4	—	—	—	—	6.8	6.7	6.7	6.6
72.0 (α)	6.9	—	—	—	—	6.6	6.7	6.6	6.5
66.2	2.8	—	—	—	—	2.0	1.9	1.9	1.8
53.0 (β)	7.0	8.9	9.2	9.5	10.3	7.0	6.9	6.9	6.9
40.1	5.8	4.6	1.9	—	—	6.8	6.7	6.7	6.6
38.0	25.0	6.5	5.1	—	—	22.5	21.3	21.1	21.0
35.0	6.6	—	—	—	—	5.4	5.3	5.1	5.0
31.0	1.5	1.6	1.8	1.8	1.9	1.0	0.9	0.8	0.6
25.0	—	2.8	2.9	2.5	2.2	—	—	—	—
23.0	4.7	8.0	9.2	9.3	9.5	0.5	0.3	—	—
21.0	25.0	35.0	36.8	37.4	37.0	26.1	27.3	27.1	26
19.0	1.8	—	—	—	—	5.0	4.3	4.1	4.3
17.0	1.6	7.3	7.5	7.6	7.7	4.6	4.5	4.2	4.2
≤ 14.4	1.3	25.3	25.6	31.9	31.4	4.3	6.0	8.8	10.5
Total	100	100	100	100	100	100	100	100	100

10 min with Alcalase, the Lipoxygenase (97.4 kD), α' and α subunits of β -conglycinin and the subunit of 66.2 kD, and acidic polypeptides of glycinin were completely disappeared. For the hydrolysates prepared by trypsin, most of the components did not display marked changes. Lipoxygenase (97.4 kD) and the subunit of 66.2 kD decreased only a little with the time of hydrolysis, while the subunit of 23 kD decreased significantly by the time of 10 min.

3.4. Determination of β -conglycinin in different hydrolysates with ELISA

ELISA was performed to determine the concentration of immunoreactive β -conglycinin in the soybean samples. It was well known that ELISA process bases on the reaction between antigen and its relative antibody. Only the immunoreactive β -conglycinin (here as antigen) could be measured in the process (L'Hocine et al., 2007; Pedersen et al., 2008). The present work showed that the allergenicity of β -conglycinin was reduced by Alcalase and trypsin hydrolysis according to the ELISA results for soybean hydrolysates (Table 3). Unhydrolyzed SBM had the highest β -conglycinin concentration at 111.68 mg/g, while β -conglycinin in soy protein hydrolysates showed much lower concentrations. Especially for that prepared by Alcalase the β -conglycinin concentration in soluble protein hydrolysates was 2.71 mg/g and that in insoluble protein hydrolysates was 2.89 mg/g at the hydrolysis time of 120 min. However, for the hydrolysates prepared by trypsin, the β -conglycinin concentration had higher concentrations of 35.86 mg/g and 8.53 mg/g, respectively.

The above samples were also tested with SDS-PAGE. For the soluble hydrolysates prepared by Alcalase, SDS-PAGE showed no band from any subunit of β -conglycinin (Figure 2a), while ELISA can still detect trace amount of β -conglycinin in these samples (Table 3). Probably comparing with SDS-PAGE,

Table 3. Average concentrations of β -conglycinin in soybean meal hydrolysed by different enzymes ($\bar{x} \pm s, n = 3$).

Sample	Time/min	Content of β -conglycinin (mg/g, total protein)	
		by Alcalase	by Trypsin
Soluble hydrolysates	0	111.68 $\pm$ 0.47	111.68 $\pm$ 0.47
	10	4.50 $\pm$ 0.25	45.50 $\pm$ 0.37
	30	3.48 $\pm$ 0.36	38.45 $\pm$ 0.49
	60	3.11 $\pm$ 0.51	37.35 $\pm$ 0.49
	90	2.89 $\pm$ 0.25	36.84 $\pm$ 0.37
	120	2.71 $\pm$ 0.33	35.86 $\pm$ 0.36
Insoluble hydrolysates	0	19.66 $\pm$ 0.36	19.66 $\pm$ 0.36
	10	2.96 $\pm$ 0.43	8.97 $\pm$ 0.46
	30	3.02 $\pm$ 0.38	8.79 $\pm$ 0.15
	60	2.91 $\pm$ 0.50	8.64 $\pm$ 0.43
	90	2.93 $\pm$ 0.20	8.60 $\pm$ 0.28
	120	2.89 $\pm$ 0.32	8.53 $\pm$ 0.45

Note: Average β -conglycinin concentrations were obtained from the mean of three replications.

the ELISA assay had better sensitivity for trace amount of β -conglycinin. The SDS-PAGE results also showed that the hydrolysates prepared by Alcalase contained more fragments with lower MW. Some literatures have reported these fragments had no effects on allergenicity and it is a significant property of the allergen (Keum, Lee, & Oh, 2006). So it demonstrated that β -conglycinin was hydrolyzed by Alcalase significantly than trypsin, which was in agreement with SDS-PAGE results. This may have occurred because the conformational epitopes of β -conglycinin are easily destroyed by Alcalase. These results are qualitatively consistent with previous literature reports (Keum, Lee, & Oh, 2006; Zhao, Qin, Sun, Zhang, & Wang, 2010) and indicate that enzymatic hydrolysis with Alcalase are effective to remove β -conglycinin from SBM.

4. Conclusions

The antigenicity of soybean proteins was related to the macromolecular structural characteristics and conformational epitopes. This research has showed that β -conglycinin and glycinin, the major antigens of SBM, could be hydrolyzed by enzymes and the allergenic proteins reduced or disappeared. The proteins were more sensitive to Alcalase than to Trypsin. The α' , α and β subunits of β -conglycinin were easier to hydrolysis than acidic and basic subunits of glycinin, which was similar to that of purified soy protein. According to this research, the hydrolysates of SBM, treated with Alcalase for 10 min, will be hypoallergenic.

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