

Antigenic activity and epitope analysis of β -conglycinin hydrolyzed by pepsin

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Abstract

BACKGROUND: Soybean is among the 'big eight' allergenic foods, and β -conglycinin, the main antigenic protein of soybean, has high levels of antigenic activity. Why the antigenic activity of soybean β -conglycinin is not eliminated by enzymatic hydrolysis is not clear. In this study, changes in the molecular composition and antigenicity of β -conglycinin hydrolyzed by pepsin were analyzed and it was determined whether complete sequential epitopes exist in the resulting hydrolysates. The nature and antigenic activity of protein subunits obtained after β -conglycinin hydrolysis were also assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and competitive enzyme-linked immunosorbent assay, respectively.

RESULTS: The residual antigenic activity of β -conglycinin was 52%, α' - and α -subunits completely disappeared, the 49 kDa fraction partially disappeared, and peptides measuring 27 and 23 kDa were newly formed after 60 min of enzymatic hydrolysis. Prolonged enzymatic hydrolysis did not result in remarkable changes in these peptides; thus, the peptides show some resistance to enzymatic hydrolysis. The amino acid sequences of the peptide chains were analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and aligned with the related sequences in the corresponding protein and antigen databases. Ten complete sequential epitopes were identified in the residual 49 kDa fraction, of these epitopes, two were from α -subunits and eight were from β -subunits.

CONCLUSION: The presence of complete sequential epitopes in hydrolysates obtained from the enzymatic hydrolysis of soybean is an important reason for the incomplete disappearance of the antigenic activity of β -conglycinin.

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Keywords: β -conglycinin; enzymatic hydrolysis; antigenicity; sequential epitope

INTRODUCTION

Soybean is the most important source of vegetable protein. This crop is characterized by high protein content and a reasonable amino acid composition. Thus, it is extensively used in the food industry and animal feeds.^{1,2} In 2017, the global yield of soybean meal (SBM) exceeded 200 million tons, and 80% of this yield was used to produce animal feeds.³⁻⁵ However, soybean is also recognized as among the 'big eight' allergenic foods,³⁻⁵ and approximately 25% of all food allergies may be traced back to this crop.^{6,7} The cardinal symptoms of soybean allergy include stomach discomfort, allergic dermatitis, increased vascular permeability, and diarrhea, all of which can adversely influence human health.⁸⁻¹³ Reports indicate that 5–8% of all infants and 2% of all adults in the USA and Europe are allergic to soybean protein.¹⁴ In the feed industry, hypersensitivity caused by soybean antigens is fairly prevalent among young mammals.¹⁵⁻¹⁸ Recent research has therefore focused on the antigenic protein components of soybean and methods to eliminate their antigenicity.

Fifteen proteins in soybean are known to demonstrate antigenicity.¹⁹ β -Conglycinin, one of the major antigenic proteins of the crop, is also the main storage protein of soybean.²⁰ β -Conglycinin is a trimer protein composed of α' - (71 kDa), α - (67 kDa), and β - (49 kDa) subunits,⁹ all of which have extensive amino acid homology.²¹ The purified α' -, α -, and β -subunits of

β -conglycinin in soybean can be identified and recognized by IgE antibodies in the serum of soybean allergy sufferers.¹⁹ Conventional food-processing means, such as heating treatment, cannot effectively eliminate or reduce the antigenicity of soy proteins,²² mainly because soybean protein molecules contain not only conformational epitopes but also sequential epitopes.^{23,24} Conventional heating can eliminate conformational epitopes but not sequential epitopes.^{25,26} Enzymatic hydrolysis, which can cleave peptide linkages in protein molecules, is considered an effective method to reduce protein antigenicity. The

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technique can not only change the spatial conformation of antigenic proteins but can also degrade sequential epitopes.^{27–29} Recent reports indicate that enzymatic hydrolysis can rapidly reduce the antigenicity of soybean protein, but is very different for different enzymes. However, new peptide chains produced during hydrolysis may still show some antigenicity and even resistance to further enzymatic degradation.^{30–32}

Soybean protein hydrolysates with molecular weights greater than 10 kDa may retain some of the antigenicity of the original protein.³⁰ Studies on which proteins produce new peptide chains after enzymatic hydrolysis and the relation between the molecular composition of a protein and its antigenicity are scarce.

Pepsin is widely used during protein hydrolysis, and soybean protein can enhance the human immunity due to a decrease in antigenicity after hydrolysis with pepsin.^{33,34} Whether enzyme-resistant fragments and epitopes remain in the hydrolysates of soybean proteins after pepsin hydrolysis is, thus, worthy of further study.

In the present study, we investigated changes in the molecular composition and antigenicity of β -conglycinin hydrolysates obtained from pepsin hydrolysis. The amino acid sequences of newly produced enzymatic hydrolysis-resistant peptide fragments were then analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) and matched with the data in the antigen database and subunit sequences of β -conglycinin. The results may be helpful in explaining antigenic changes in soybean β -conglycinin after enzymatic hydrolysis.

MATERIALS AND METHODS

Reagents

The protein gel mini-extraction kit used in this work was obtained from Beijing Baiaolaibo Technology Co., Ltd (Beijing, China). A competitive β -conglycinin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shanghai Jining Company (Shanghai, China). Pepsin and trypsin were obtained from Sigma-Aldrich Co., Ltd (Shanghai, China). The protein marker was provided by Beijing BioDee Biotechnology Co., Ltd (Beijing, China). All other chemicals used in the experiments were of analytical grade.

Preparation of β -conglycinin

β -Conglycinin was separated from low-temperature defatted SBM using the isoelectric point precipitation method with slight modifications.³⁵ Briefly, 30 g of crushed SBM was suspended in 500 mL of water (pH 8.0). After stirring at 25 °C for 2 h, the suspension was centrifuged at 4000 $\times$ g for 20 min to remove precipitates. The resultant supernatant was added with solid sodium bisulfite to a final concentration of 0.01 mol L⁻¹, adjusted to pH 6.4, stored in a refrigerator at 4 °C overnight and then centrifuged at 4000 $\times$ g for 20 min. The supernatant was adjusted to pH 5.0 with 2 mol L⁻¹ HCl solution, and non-target proteins were removed by centrifugation at 4000 $\times$ g for 20 min. The supernatant was added with NaCl solution to a final concentration of 0.4 mol L⁻¹. After incubation at 55 °C for 10 min, the supernatant was adjusted to pH 4.6 and centrifuged at 4000 $\times$ g for 20 min. The sediments were collected and lyophilized for further use.

Enzymatic hydrolysis of β -conglycinin

β -Conglycinin was mixed with double-distilled water at a 1/10 ratio of protein / water. The pH of the suspension was adjusted

to 2.0 with 2 mol L⁻¹ HCl solution at 37 °C. The ratio of pepsin / β -conglycinin (E/S) was 0.5/100 (w/w). Approximately 1.0 mL of the hydrolysates obtained from the reaction solution at different times of 0, 10, 30, 60, and 120 min was heated in boiling water for 10 min to stop the enzymatic reaction and then lyophilized for further analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was carried out to analyze changes in β -conglycinin components during enzymatic hydrolysis following a previously reported method with a slight modification.³⁶ In brief, lyophilized powders were dissolved in loading buffer (0.5 mol L⁻¹ Tris-HCl, 10% SDS, glycerol, 0.1% bromophenol blue, and β -mercaptoethanol) to a final concentration of 2 μ g μ L⁻¹. Then, the sample was heated in boiling water for 10 min and run in a Mini-Protein tetra-cell (Bio-Rad, Hercules, CA, USA) with each well of 10 μ L sample for approximately 1.5 h. Finally, the gel was stained with 0.25% (w/v) Coomassie brilliant blue (CBB) R-250 at room temperature for further analysis.

Determination of the antigenic activity of enzymatic hydrolysates

The antigenicity of enzymatic hydrolysates was measured with a competitive ELISA kit according to the instructions of the manufacturer. In brief, a 50 μ L mixture of 10 μ L of the enzymolyzed sample with 40 μ L of the dilution solution provided in the kit or 50 μ L of twofold diluted standard samples with concentrations ranging from 4800 μ g mL⁻¹ to 300 μ g mL⁻¹ was added to the wells of 96-well plates that had been pre-coated with mouse anti- β -conglycinin monoclonal antibody. Then, 50 μ L of horseradish peroxidase-labeled β -conglycinin was added to each well, and the plates were incubated at 37 °C for 60 min. The plates were washed five times with 0.01 mol L⁻¹ phosphate-buffered saline solution containing 0.15 mol L⁻¹ NaCl and 0.05% Tween 20 and then tumble dried. Approximately 100 μ L of tetramethyl benzidine (Promega, Madison, WI, USA) was added to each well. The color of the solution was developed for 15 min, and then the reaction was terminated with 2 mol L⁻¹ H₂SO₄ solution. The optical density of each well at 450 nm (OD₄₅₀) was recorded using an iMark™ microplate absorbance reader (Bio-Rad). Each sample was analyzed in triplicate. The concentrations of the samples were calculated on the basis of the standard curve derived from the standard samples as described above. The effect of enzymatic treatment was expressed as the residual ratio of antigenic activity (%) by using the following formula:

$$R = \frac{A}{B} \times 100\%$$

where R is residual ratio of antigenic activity and A and B are the antigenic activities of the enzymatic hydrolysates and unenzymolyzed β -conglycinin, respectively. The data obtained were processed using Origin 8.0 (OriginLab, Los Angeles, CA, USA).

In-gel trypsin digestion

In-gel digestion of newly generated protein bands in the SDS-PAGE gel was conducted following a previously reported method.³⁷ Briefly, protein bands of interest in the SDS-PAGE gel were manually excised and destained by washing with 50% acetonitrile (ACN) (v/v) in 25 mM NH₄HCO₃ and in-gel reduced/

alkylated by incubation first with 10 mM dithiothreitol at 56 °C for 1 h and then with 55 mM iodoacetamide at room temperature for 45 min in 25 mM NH_4HCO_3 solution. The gel pieces were then dehydrated with 100 μL of pure ACN and re-hydrated with 10–20 μL of a 12.5 ng μL^{-1} solution of sequencing grade modified trypsin (Sigma-Aldrich) in an ice bath for 45 min. The proteins were finally immersed in 50 μL of digestion buffer (25 mM NH_4HCO_3 , pH 7.8) and incubated at 37 °C overnight. Tryptic peptides were extracted three times with 40 μL of 50% ACN/5% formic acid (v/v) in an ultrasonic bath for 20 min. The supernatants were pooled and dried in a vacuum centrifuge, and a small aliquot (approximately 10%) of the peptide or glycol-peptide mixture was analyzed using MALDI-TOF-MS.

Sequence analysis of peptides by MALDI-TOF-MS

Peptides in the enzymatic hydrolysates were analyzed by MALDI-TOF-MS following a previously reported method.³⁸ Briefly, 1 μL of the dissolved sample was directly spotted on the sample target and allowed to dry naturally. Subsequently, 0.6 μL of oversaturated α -cyano-4-hydroxycinnamic acid matrix solution (50% cerium ammonium nitrate + 0.1% trifluoroacetic acid) was dropped onto the corresponding target position and dried naturally. The sample target was then blown clear with nitrogen and placed in the intake tank of the MALDI-TOF-MS instrument (SCIEX5800, AB Sciex, Foster, CA, USA). A Nd:YAG laser source with a wavelength of 349 nm and acceleration voltage of 2 kV were used for analysis, and the data were collected automatically in positive ion mode. The scanning area for primary MS analysis was 800–4000 Da, and MS/M analysis was carried out on parent ions with a signal/noise ratio greater than 50. MS/MS was superposed 2500 times, and the CID was closed when the collision energy reached 2 kV. The data map was collected by Flex Control and analyzed using Data Explorer 4.5.

Mass database retrieval

Original mass spectrometric data were retrieved from the corresponding databases using Mascot 2.2 software. The database retrieval parameters are as follows. Database: Uniprotglycine max; Type of search: Combined (MS + MS/MS); Enzyme: Trypsin; Mass values: Monoisotopic; Protein Mass: unrestricted; Peptide Mass Tolerance: +100 ppm; Peptide Mass Tolerance: ± 0.4 Da; Max Missed Cleavages: 1.

Determination of the antigenicity of peptide chains

Protein / peptide bands in the SDS-PAGE gel were recovered to analyze the antigenicity of newly produced peptide components in the enzymatic hydrolysate. Briefly, two pieces of gel were prepared and electrophoresis was conducted under exactly the same conditions. One gel was stained with CBB R-250. In the other gel, the marker lane was cut off and stained with CBB R-250. The band positions of protein components in the two gels were then compared, and newly produced peptide bands in the non-dyed gel were cut off. The resultant proteins / peptides were recovered following the instructions of the protein gel mini-extraction kit, desalinated on a C18 cartridge (Sigma-Aldrich), dried under nitrogen, and stored at -20 °C for further use. The antigenicity of the resulting peptides / proteins was determined using competitive ELISA kits as described above.

RESULTS AND DISCUSSION

Changes in β -conglycinin composition after enzymatic hydrolysis

Changes in the composition of the enzymatic hydrolysates of β -conglycinin hydrolyzed with pepsin for different lengths of time were analyzed by SDS-PAGE. As shown in Fig. 1, the bands of the α' -, α -, and β -subunits of β -conglycinin changed regularly as the enzymatic hydrolysis time was prolonged. Specifically, α' - and α -subunits decreased and nearly completely disappeared after 60 min of enzymatic hydrolysis. By comparison, the degree of enzymatic hydrolysis of β -subunits (49 kDa) was much lower than that of the α' - and α -subunits and the band of the β -subunit remained relatively clear even after 120 min of enzymatic hydrolysis. This finding suggests that the α - and α' -subunits are more sensitive to pepsin than the β -subunit. The hydrophilic peptide chain of α - and α' -subunits is hydrophilic and can therefore easily be degraded by pepsin.³⁹ The band representing the β -subunit (49 kDa) did not completely disappear after enzymatic hydrolysis was completed in that perhaps the 49 kDa band (identical to molecular weight of β -subunit) may contain new degraded components of α' - and α -subunits, which has been reported by Chen et al. (2013).⁴⁰ The amino acid sequence analysis presented later in this paper supports this hypothesis.

Bands at 27 and 23 kDa were newly generated after enzymatic hydrolysis. These low-molecular weight peptide bands are produced by pepsin hydrolysis of the high-molecular weight subunits of β -conglycinin. Moreover, as the enzymatic hydrolysis time increased, the colors of these two newly produced bands gradually deepened, which indicates some resistance to further enzymatic hydrolysis by pepsin. This result is consistent with those of studies reporting on the enzymatic hydrolysis of β -conglobulin with gastric juice.^{20,30,38} Increases in enzyme dosage ($E/S = 1:1$) and prolongation of the enzymatic hydrolysis time were unsuccessful in completely removing the 27 and 23 kDa bands.³² Newly produced bands of 27 and 23 kDa that appear to be resistant to further degradation by protease may also be observed in the hydrolysates of SBM hydrolyzed by alkaline protease.³¹

Antigenic activity of enzymatic hydrolysates

The antigenic epitopes of protein molecules may be categorized as conformational or sequential.²⁸ Conformational antigenic

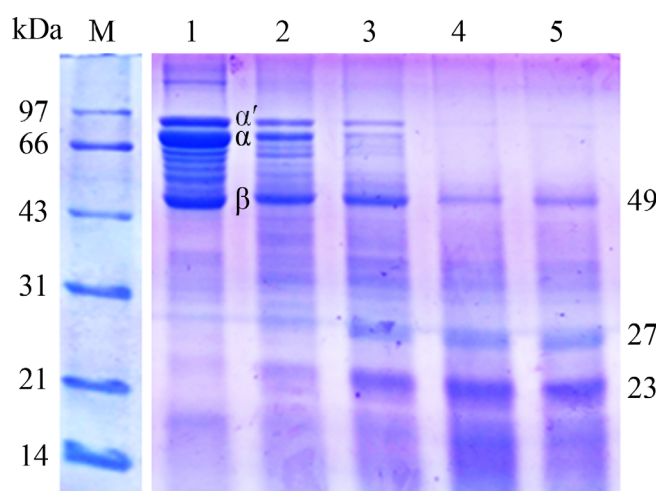


Figure 1. SDS-PAGE pattern of β -conglycinin components at different post-hydrolysis times. M: Protein marker; 1: 0 min; 2: 10 min; 3: 30 min; 4: 60 min; 5: 120 min. Note: α' (71 kDa), α (67 kDa) and β (49 kDa) are representative of three subunits of β -conglycinin.

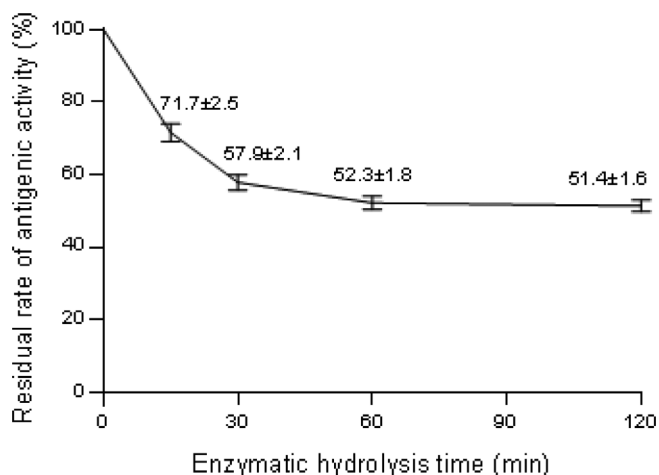


Figure 2. Residual antigenic activities of β -conglycinin at different hydrolysis times by pepsin digestion.

epitopes generally disappear along with changes in the spatial conformation of a protein but sequential epitopes are relatively stable. A protein molecule that is hydrolyzed by enzymes may still show antigenic activity if the peptide of the sequential epitope is not cut off.⁴¹ A competitive β -conglycinin ELISA was used to determine the antigenic activity of β -conglobulin and its

hydrolysates obtained at different enzymatic hydrolysis times, to understand changes in the antigenic activity of β -conglycinin during proteolysis (Fig. 2). This method can be used to determine the antigenicity of macromolecular antigenic proteins and is suitable for measuring the antigenicity of small-molecule proteins with high sensitivity.

As shown in Fig. 2, the antigenic activity of β -conglycinin decreased remarkably after hydrolysis with pepsin for 10 and 30 min (Fig. 2). This phenomenon may be correlated with changes in the α' , α and β -subunits of β -conglycinin. Enzymatic hydrolysis may destroy the spatial conformation of β -conglycinin molecules and degrade the protein into smaller molecules, resulting in a rapid decrease in its antigenic activity. However, when enzymatic hydrolysis was prolonged from 30 to 120 min, the antigenic activity of β -conglycinin decreased slowly and remained at 50% even after 120 min of hydrolysis. Among the three subunits of β -conglycinin, the α' - and α -subunits are known to be the most sensitive to pepsin whereas the β -subunit is acknowledged to be fairly stable.⁴² These characteristics may explain why enzymatic hydrolysis of β -conglobulin cannot completely eliminate its antigenicity.

Antigenic activity of peptide fragment

The peptides, indicated by bands appearing at 49, 27, and 23 kDa in the SDS-PAGE gel after 120 min of enzymatic hydrolysis of β -conglycinin, were recovered by using a protein gel mini-recovery

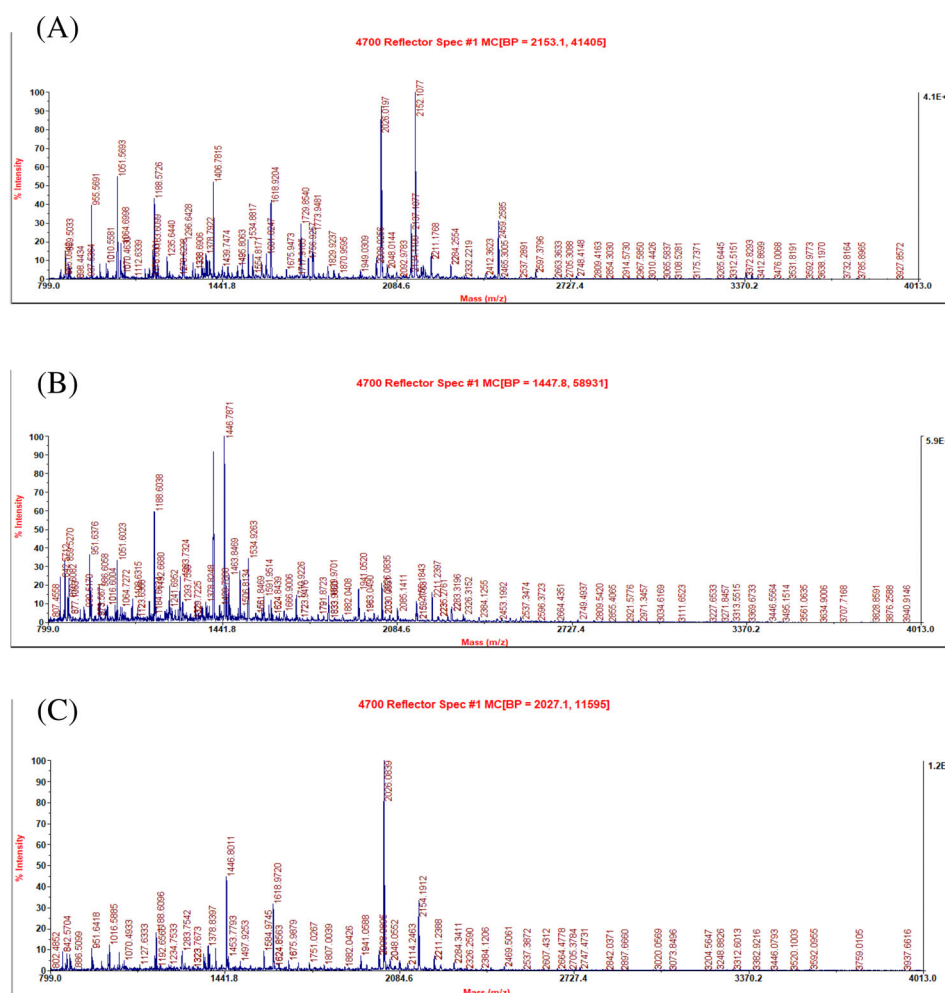


Figure 3. MALDI-TOF-MS spectra of three main components in the enzymatic hydrolysates. (A) 49 kDa. (B) 27 kDa and (C) 23 kDa.

kit, and their antigenic activity was determined by competitive ELISA. The residual antigenic activities of the 49, 27, and 23 kDa fractions relative to the antigenic activity of unhydrolyzed β -conglycinin were 29.6%, 1.5%, and 1.2% respectively. These results reveal that the antigenicity of β -conglycinin hydrolyzed by pepsin for 120 min may originate from an antigenic peptide with a molecular weight of 49 kDa.

The combined antigenic activity of the 49, 27, and 23 kDa peptides was lower than that of the total enzymatic hydrolysate obtained after 120 min of enzymatic hydrolysis. These results are similar to findings reported in the literatures.^{30,38} Previous studies revealed that 27 and 23 kDa peptides could be obtained by hydrolyzing β -conglycinin with pepsin and pancreatin. Western blotting using rabbit anti-soybean protein IgG antibody showed an extensive reaction between IgG and the β -subunit (49 kDa) but only weak reactions between IgG and the 27 and 23 kDa peptides. Moreover, Western blot with IgE antibody from serum of allergenic patients can only react with β -subunit.

MALDI-TOF-MS peptide fragment analysis

Identification of the major components of the enzymatic hydrolysates

We investigated the relation between the antigenic activity and molecular composition of the enzymatic hydrolysates and determined the origins of the 27 and 23 kDa peptides. The peptides indicated by bands appearing at 49, 27, and 23 kDa (Fig. 1) in the SDS-PAGE gel after 120 min of enzymatic hydrolysis were collected and in-gel digested with trypsin. The in-gel enzymatic hydrolysates were then analyzed by MALDI-TOF-MS. The corresponding spectra are shown in Fig. 3.

The amino acid sequences of the three peptide fragments were obtained by matching their MS/MS spectra with the amino acid

sequences of three subunits of β -conglycinin stored in the soybean protein database (<http://www.uniprot.org>) using Mascot software. The results showed that the 49 kDa fraction (Fig. 1) contains not only the amino acid sequences of the β -subunit but also those of α' - and α -subunits. In other words, the 49 kDa peptide fragment is produced by the hydrolysis of α' - and α -subunits with pepsin. This result is similar to the reports of Fu *et al.* (2007)⁴³ and Chen *et al.* (2013).⁴⁰ According to the literature,⁴³ a new electrophoretic band is produced at approximately 49 kDa after the hydrolysis of the α -subunit of β -conglycinin by pepsin. Chen *et al.* (2013) reported that, when soy protein isolate is hydrolyzed with pepsin for 1 h, the color of the β -subunit band (49 kDa) deepens in SDS-PAGE and a new band appears at approximately 53 kDa above the β -subunit band.⁴⁰ This new peptide is speculated to originate from the hydrolysis of some high-molecular weight protein. The partial amino acid sequences of the α' - and α -subunits of β -conglycinin were detected in the newly produced 27 kDa peptide segment, but only the partial amino acid sequence of the α -subunit was detected in the 23 kDa peptide. These findings indicate that the 27 kDa peptide obtained after enzymatic hydrolysis originates from the α' - and α -subunits of β -conglycinin whereas the new 23 kDa peptide originates from the α -subunit only.

Analysis of the antigenic epitopes of the main components of the enzymatic hydrolysates

The hydrolytic site of trypsin is a peptide bond formed by the carboxyl group of lysine (K) or arginine (R). The amino acid sequences of the 49, 27, and 23 kDa peptides were spliced and matched with the amino acid sequences of epitopes in the antigen database (<http://www.immuneepitope.org>) on the basis of this characteristic. Analysis was then conducted to determine whether complete epitope sequences exist in the three peptide

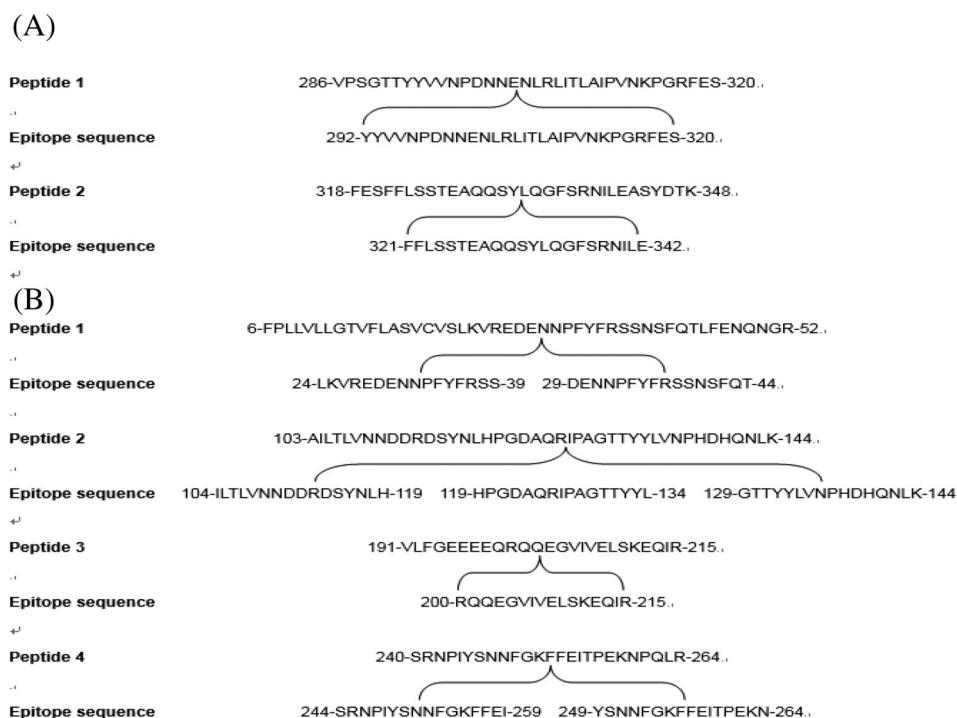


Figure 4. Epitope analysis of the 49 kDa fraction in the enzymatic hydrolysate. (A) 49 kDa originated from α -subunit and (B) 49 kDa originated from β -subunit.

chains and to interpret why antigenic activity still exists in enzymatic hydrolysates.

Ten complete epitopes were detected in the 49 kDa fraction of these epitopes. Two were derived from the α -subunit of β -conglycinin (Fig. 4(A)) and eight were derived from the β -subunit (Fig. 4(B)). The locations of these epitopes are consistent with the speculation of a previous report.⁴² Some amino acid sequences were also detected in the epitopes of the α' -subunit, but no complete epitope was found. No amino acid sequence of complete epitopes was detected in the 27 kDa peptide obtained from α - and α' -subunits or the 23 kDa peptide from the α -subunit of β -conglycinin. If a peptide is not desorbed by the laser during MALDI-TOF-MS, the amino acid sequence of this peptide cannot be identified⁴⁴ and its epitopes cannot be obtained. This issue could be explored by using other mass spectrometric methods.

All three subunits of β -conglycinin are known to be potential food allergens.¹⁹ Although the spatial conformation of these proteins may be destroyed by enzymatic hydrolysis, some antigenicity remains in the enzymatic products.^{30,32,38,43} Moreover, prolonged enzymatic hydrolysis is unable to eliminate some protein components, and the resulting hydrolysates may be able to resist enzymatic hydrolysis.³¹ The existence of linear epitopes within peptide fragments may explain this characteristic. Research on linear epitopes in newly produced peptide chains with enzymatic hydrolytic resistance is limited. In the present study, we found that the 49 kDa peptide chain of β -conglycinin hydrolyzed by pepsin contains not only the linear epitopes of the β -subunit but also those of the degradation components of the α -subunit. Thus, this peptide chain is resistant to enzymatic hydrolysis. Our findings may explain why soybean proteins can cause allergenic responses in the gastrointestinal tract even after digestion by pepsin.

CONCLUSIONS

In this work, changes in the subunits and antigenic activity of β -conglycinin during enzymatic hydrolysis were analyzed. The α' - and α -subunits nearly completely disappeared and β -subunits decreased significantly after 60 min of enzymatic hydrolysis. The residual antigenic activity of β -conglycinin was 52%. However, prolonged enzymatic hydrolysis resulted in minimal changes to the molecular subunits and antigenicity of antigenic proteins.

New peptides measuring 27 and 23 kDa in size produced during enzymatic hydrolysis showed resistance to enzymatic hydrolysis. The 49 kDa fraction retained approximately 29.6% of its antigenic activity after 120 min of enzymatic hydrolysis and this peptide contained not only incompletely degraded β -subunits but also α' - and α -subunits. Moreover, ten complete sequential epitopes were identified in the hydrolysates; two of these epitopes were derived from α -subunits and eight epitopes were derived from β -subunits.

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