

FULL ARTICLE

The separation and identification of the residual antigenic fragments in soy protein hydrolysates

Guanhao Bu | Ting Huang | Tanghao Li

College of Food Science and Technology,
Henan University of Technology,
Zhengzhou, China

Correspondence

Guanhao Bu, College of Food Science
and Technology, Henan University of
Technology, Zhengzhou 450001, China.
Email: buguanhao2008@126.com

Funding information

National Natural Science Foundation of
China, Grant/Award Number: 31871748
and 31201293; Foundation of Henan
Educational Committee, Grant/Award
Number: 19A550005; Science and
Technology Project of Henan Province,
Grant/Award Number: 182102110299;
The Young-aged Backbone Teacher Funds
of Henan Province of China, Grant/Award
Number: 2019GGJS084; The Project of
Henan University of Technology Excellent
Young Teachers

Abstract

Soybean is one of the major food allergens. In this study, soy protein isolate was hydrolyzed by Neutrase and Flavourzyme. The hydrolysates were separated by ultrafiltration and ion-exchange chromatography. The antigenicity of proteins was determined by indirect competitive ELISA. The molecular weight distribution was characterized by SDS-PAGE. The amino acid sequence of chromatography fractions was analyzed by LC-MS. The results showed that proteins with >50 kDa in hydrolysates had the highest antigenicity and were further separated into F₁–F₅ fragments by ion-exchange chromatography. Fragment F₄, which was the most antigenic, was analyzed by LC-MS. The results of mass spectrometry showed that most of the peptides that contained antigen epitopes in chromatography fraction F₄ belonged to glycinin subunits. The antigenicity of soy protein was reduced by enzymatic hydrolysis, but glycinin showed resistance to enzymatic hydrolysis.

Practical applications

The identification of residual antigenicity in soy protein hydrolysates by LC-MS provides important information on the resistance mechanism of enzymatic hydrolysis of soybean protein allergens. In addition, the efficient separation of soy protein hydrolysates could be beneficial for developing low-allergenic soybean products.

KEYWORDS

antigenicity, enzymatic hydrolysis, separation, soy protein

1 | INTRODUCTION

Soybean is one of the most important economic crops and has a wide geographic area of cultivation. It is rich in protein and has eight kinds of amino acids that are necessary for humans (Fang, Yu, & Badger, 2004). However, soybean has been classified as one of the “big 8” most allergenic foods, in which glycinin and β -conglycinin are the primary sensitizing factors (You et al., 2008). Patients who are allergic can suffer from nausea, glossopharyngeal spasms, and swelling, and the reaction can even lead to shock or death (Wang & Sampson, 2007). Soybeans provide most of the proteins eaten by vegetarians and are widely added to infant formula (Verma, Kumar, Das, & Dwivedi, 2012), noodles, ham, and other processed foods (Businco,

Bruno, & Giampietro, 1998). It is difficult for an allergic population to prevent allergies by avoiding the intake of legumes because of the variety of leguminous products. Therefore, it is urgent to find effective methods to reduce and to eliminate soy protein allergens, which would improve the health of allergic groups.

Enzymatic hydrolysis is an efficient method for modifying proteins and soy peptide is also a rich source of bioactive peptides with the function of ACE inhibitory and antihypertensive functions (Chiang, Tsou, Tsai, & Tsai, 2006; Friedman & Brandon, 2001). It has a significant effect on the linear epitopes of protein allergens. Proteases break chemical bonds in proteins and degrade them into small molecule peptides to attenuate or to eliminate their sensitization (Pinto, Neves, & Medeiros, 2009). Lee et al. (2007) found

that enzymatic hydrolysis could reduce the sensitivities of soybean 11S globulin, although peptides with approximately 20 kDa of hydrolysates were still allergenic. Fraczek, Kostyra, and Kostyra (2008) used trypsin to reduce the sensitivities of pea protein. The N-terminal amino acid sequence of the antigenic protein was determined and the predecessor of the antigen protein was hypothesized to be pea protein lectin. Li, Zhu, Zhou, Peng, and Guo (2013) reported that enzymatic hydrolysis was a valid method to decrease the immunoreactivity of the soy protein isolate (SPI) added to infant formula. In addition, Kasera, Singh, Lavasa, Prasad, and Arora (2015) used Alcalase and Flavourzyme to hydrolyze three legumes sequentially and reported that the allergenicity of legumes was significantly attenuated. Taken together, enzymatic hydrolysis is an effective method to decrease the antigenicity of soybean. However, the differences in proteases and degree of enzymatic reaction will lead to unequal antigenicity of the hydrolysates, and little research has focused on the composition and structure of the antigenic peptides in soy protein hydrolysates. Therefore, it is meaningful to find a hydrolysis mode that attenuates the antigenicity of soy protein effectively and to analyze the structure of residual antigenic peptides in hydrolysates.

Ultrafiltration is a membrane separation technology (Loginov, Boussetta, Lebovka, & Vorobiev, 2013). The macromolecular substances in the solution are blocked by ultrafiltration membranes, but the small molecules are able to pass through. The proteins and peptides with specific ranges of molecular weights are separated and purified. Ion-exchange chromatography is another purification technique (Costa, Fernandes, Romão, & Ventura, 2014; Jungbauer & Hahn, 2009). Ion exchangers have different abilities to combine with various ions, which separates the various ions from the mixture. In this study, composite enzymatic hydrolysis of using Neutrase and Flavourzyme was applied to hydrolyze SPI to obtain the peptides with reduced allergenicity. Ultrafiltration and ion-exchange chromatography were applied to separate and to filtrate proteins with high antigenicity from hydrolysates. The primary structures of separated fractions with high antigenicity were determined by LC-MS, so we could explore the reason why residual soybean protein allergens can resist enzymatic hydrolysis. This study could contribute to the development of low-allergenic soybean peptide powder.

2 | MATERIALS AND METHODS

2.1 | Materials

SPI (protein content 84.45%) was obtained from Henan Kunhua Biological Technology Group Co., Ltd (Anyang, China). Neutrase was purchased from Novozymes Biotechnology Co., Ltd (Tianjin, China). Flavourzyme, TMB chromogenic single-component liquid, and DEAE Sepharose Fast Flow were obtained from Beijing Solarbio Science and Technology Co. Ltd (Beijing, China). β -conglycinin (C5868), glycinin (G3171), and HRP-labeled goat anti-rabbit IgG (A6154) were

obtained from Sigma Chemical Co. Ltd (St. Louis, MO, USA). The anti- β -conglycinin and anti-glycinin serum from rabbits were generated by our own laboratory. The gel preparation kit was obtained from Boster Biological Engineering Co., Ltd (Wuhan, China). Ultrafiltration membranes were purchased from Yuling Filter Equipment Co., Ltd (Shanghai, China).

2.2 | Hydrolysis of soybean protein

SPI was hydrolyzed by the combination of Neutrase and Flavourzyme based on the method in our previous research (Huang, Bu, & Chen, 2017). The suspension of SPI with a concentration of 100 g/L was preheated for 10 min and then proteases were added for SPI hydrolysis. The pH of the suspension was kept stable by adding 1 mol/L of NaOH continuously during the hydrolysis process. Flavourzyme (1,500 U/g) was added to the suspension that was kept at 60°C and pH 6.5 for 15 min. After inactivating Flavourzyme in boiling water for 5 min, Neutrase (2,000 U/g) was added and maintained at 55°C and pH 7.0 for 15 min. After hydrolysis, proteases were inactivated in boiling water for 10 min. The hydrolysates obtained were freeze-dried and saved at -20°C.

2.3 | Ultrafiltration

Ultrafiltration was used to separate the hydrolysates into different molecular weight fractions (Loginov et al., 2013). Based on our previous research techniques (Huang et al., 2017), we centrifuged hydrolysates at a concentration of 60 g/L at 5,000 r/min for 15 min, and the supernatant was separated by ultrafiltration at 0.15 MPa N_2 pressure. The ultrafiltration membranes with molecular weight cut-offs of 3, 5, 10, 30, and 50 kDa, which entrap macromolecules and allow small molecules to pass through, were used as the separation media. The ultrafiltration products were freeze-dried for further separation.

2.4 | Purification of ultrafiltration product

Ion-exchange chromatography (PuriFlash 450, Interchim, France) was applied to separate and purify ultrafiltration products with high antigenicity (Jungbauer & Hahn, 2009; Liu, Zhao, Chen, & Fang, 2015). The freeze-dried ultrafiltration products were dissolved in deionized water and then loaded onto a diethylaminoethyl cellulose (DEAE) Sepharose Fast Flow (FF) column (1.5 cm $\times$ 46 cm) equilibrated by 20 mmol/L of Tris-HCl buffer (pH 8.0). The column was eluted at a flow rate of 2.0 ml/min, with a linear NaCl gradient (0–1.2 mol/L) in a 20 mmol/L of Tris-HCl buffer (pH 8.0). An 8 ml of aliquot of the effluent was collected from each tube. Absorbance was measured at 280 nm using an online spectrophotometer to determine the elution profile of the sample. The fractions were concentrated and freeze-dried for the determination of antigenicity.

2.5 | Antigenicity assay

The antigenicity of samples was analyzed by indirect competitive ELISA (Bu, Zhang, & Chen, 2015; Shi et al., 2014). The 96-well microtiter plate (Costar 3590 High Binding, Corning, New York, USA) was coated with 100 μ l of antigen proteins β -conglycinin (0.3 μ g/ml) or glycinin (0.025 μ g/ml) in 50 mmol/L of carbonate buffer and incubated at 4°C overnight. The antigen concentration in the buffer solution was determined earlier by indirect ELISA. All hydrolyzed samples were diluted to 2 mg/ml (protein concentration) with phosphate-buffered saline solution (PBS, pH 7.4). Then, samples were incubated separately with rabbit anti- β -conglycinin serum (1:3,200) or anti-glycinin serum (1:1,600) diluted in 0.01 mol/L of phosphate-buffered saline (PBS, pH 7.4) that contained 10 g/L of BSA and 1 ml/L of Tween-20, at a ratio of 1:1 (v/v) at 4°C overnight. The following process followed the approach we reported earlier (Huang et al., 2017). The uninhibited serum sample (no sample) was used as a control. The absorbance was determined at dual wavelengths of 450 nm and 620 nm by a microplate reader (Thermo Fisher Scientific Instrument Co. Ltd, New York, USA). The antigenicity was represented by the inhibition percentages (%), which were calculated as follows:

$$\text{Inhibition rate (\%)} = (B_0 - B) / B_0 \times 100$$

where B is the absorbance measured in the presence of different hydrolyzed samples from the experiments and B_0 is the absorbance obtained with the uninhibited serum sample control. A low percentage of inhibition reflected a low antigenicity of samples for β -conglycinin or glycinin.

2.6 | SDS-PAGE analysis

SDS-PAGE was performed in a discontinuous buffered system according to the method of (Laemmli, 1970). SDS-PAGE analysis was performed using 5% stacking gels and 12% separating gels. The 10 μ l of samples (protein concentration 2 mg/ml) were added to 10 μ l of sample buffer, which contained 0.5 mol/L of Tris-HCl buffer, 10 g/L of SDS, 20 ml/L of β -mercaptoethanol, 200 ml/L of glycerol and 0.25 g/L of bromophenol blue, and heated for 5 min in boiling water before electrophoresis. The 10 μ l of mixed sample solution was loaded onto the gels. Electrophoresis was carried out at 20 mA for 40 min in the stacking gels and at 40 mA for 1.5 hr in the separating gels. After electrophoresis, gels were fixed for protein with a fixative solution that contained 500 ml/L of ethanol and 100 ml/L of glacial acetic acid for 1 hr and then stained with Coomassie brilliant blue R-250 for 1.5 hr. Finally, the gels were put into a destaining solution to decolor until the electrophoresis banding pattern became clear.

2.7 | Sequential analysis

LC-MS was applied to determine the amino acid sequence of fractions with high antigenicity (Lee & Kerns, 2010; Tomaszewski et al., 2014).

Fractions were first hydrolyzed into smaller peptides by trypsin and then purified by liquid chromatography. A C_{18} column was eluted at a flow rate of 300 nl/min for 65 min. Mobile phase A was 1 ml/L of formic acid, mobile phase B was 1 ml/L of formic acid and 800 ml/L of acetonitrile, and the B phase was increased from 50 ml/L to 900 ml/L as the effective gradient. The separated peptides were determined by mass spectrometer (Q Exactive mass spectrometer, Thermo Fisher) and the parameters of primary mass spectrometry were as follows: Resolution: 70,000; AGC target: 3×10^6 ; Maximum IT: 40 ms; Scan range: 350–1,800 m/z. The original MS file was converted by MM File Conversion and the MGF format file we obtained was searched in the UniProt database (<https://www.uniprot.org/taxonomy/3847>).

2.8 | Statistical analysis

All experiments were replicated three times and values given in the tables and figures are the means of these triplicates. Figures were drawn using OriginPro 8.5. Statistical significance of differences among means was performed by SPSS 16.0. The significance level was established at $p < .05$ for Duncan's test and univariate analysis of variance (ANOVA).

3 | RESULTS AND DISCUSSION

3.1 | Antigenicity of hydrolysates and ultrafiltration products

SPI was hydrolyzed by the combination of Neutrase and Flavourzyme. Enzymatic hydrolysis significantly reduced the antigenicity of soybean protein and the antigenicity of β -conglycinin was reduced by 64.46%, which was reported in our previous study (Huang et al., 2017). In this study, the hydrolysate was separated into different molecular weight fractions such as <3, 3–5, 5–10, 10–30, 30–50, and >50 kDa by ultrafiltration (Figure 1). As the molecular weight decreased, the antigen inhibition rate of glycinin was reduced. The 10–30 kDa peptides showed the lowest antigenicity compared with that of SPI. However, the ultrafiltration fraction of >50 kDa showed the highest inhibition rate of glycinin antigen. In fact, the ultrafiltration fraction of >50 kDa also showed the highest antigenicity of β -conglycinin in our previous study (Huang et al., 2017). Therefore, the ultrafiltration fraction of >50 kDa was separated further and purified by ion-exchange chromatography to explore the reasons for its high antigenicity.

3.2 | Separation and SDS-PAGE

Ultrafiltration fractions of >50 kDa were separated well by ion-exchange chromatography and five major fractions (marked as F_1 , F_2 , F_3 , F_4 , and F_5) were obtained (Figure 2a). The molecular weight distribution of fractions was determined by SDS-PAGE (Figure 2b), which illustrated that ultrafiltration fractions of >50 kDa (lane 0)

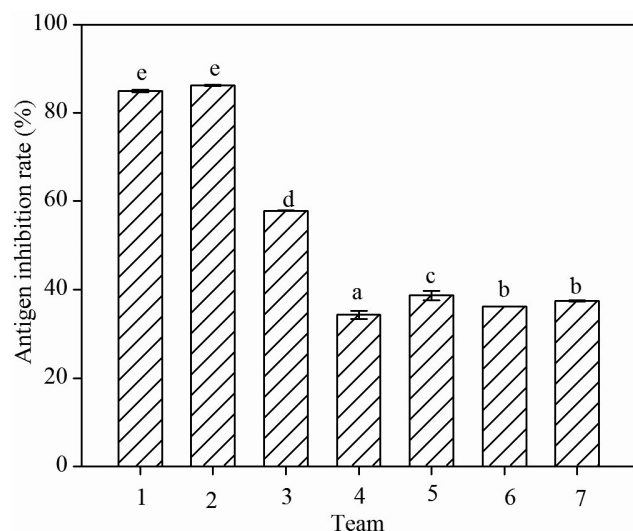


FIGURE 1 Antigen inhibition rate of glycine in ultrafiltration fractions. 1-7: Hydrolysate, >50 kDa, 30-50, 10-30, 5-10, 3-5, 3 kDa or <3 kDa ultrafiltration fractions, respectively. The same letters on the bars are not significantly different ($p > .05$)

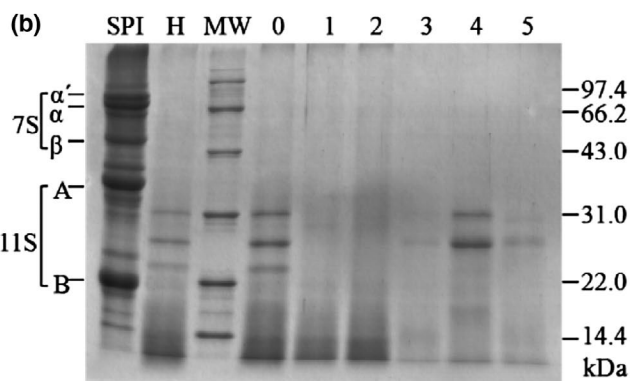
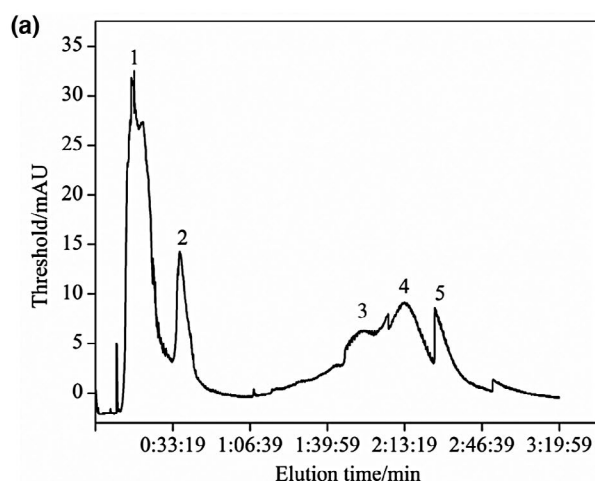


FIGURE 2 Chromatography separation of ultrafiltration fractions and SDS-PAGE. (a): Ion-exchange chromatography profile of ultrafiltration fractions of 50 kDa above. (b): Electrophoresis profile of chromatography fractions. MW: Molecular weight marker, H: Hydrolysate by Neutrase and Flavourzyme, Lane 0: Ultrafiltration fractions of 50 kDa above, Lanes 1-5: Fractions F_1 - F_5

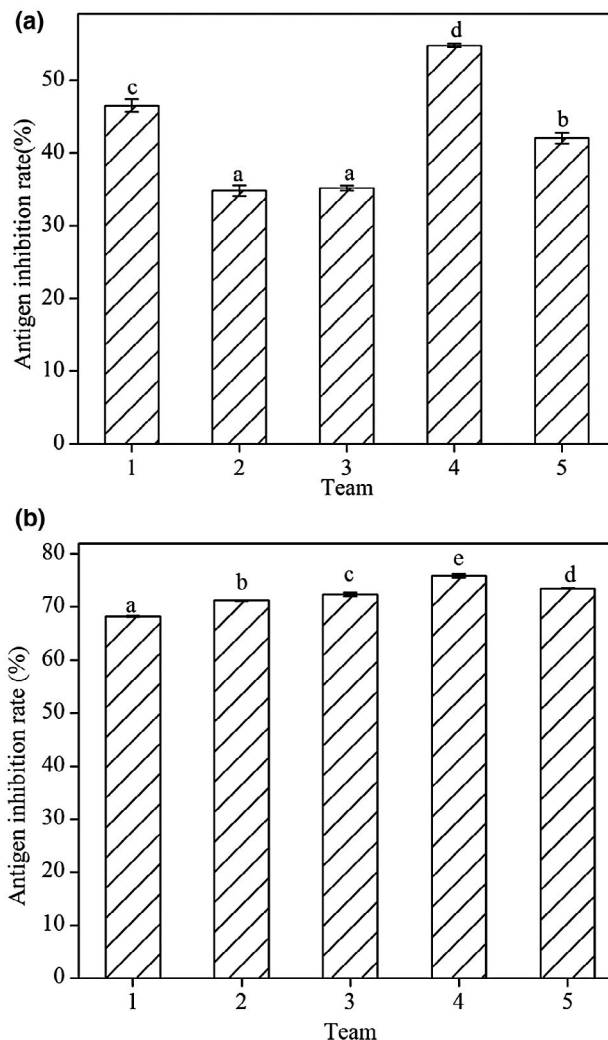


FIGURE 3 The antigenicity of β -conglycinin (a) and glycine (b) in chromatography fractions. 1-5: Chromatography fractions F_1 - F_5 . The same letters on the bars are not significantly different ($p > .05$)

showed peptide bands of 31, 26, 22, and <15 kDa. Fractions F_1 (lane 1) and F_2 (lane 2) contained peptide bands of <15 kDa, but F_4 (Lane 4) showed peptide bands of 31 and 26 kDa. Light peptide bands of 31 and 26 kDa were observed in lanes 3 and 5 that corresponded to fractions F_3 and F_5 .

3.3 | Antigenicity analysis

The antigen inhibition rate of β -conglycinin in fraction F_2 was the lowest, which was 34.81%, and fraction F_4 had the highest β -conglycinin antigenicity, which was 54.78% (Figure 3a). Fraction F_1 had the lowest inhibition rate of glycine of 68.25%, and the antigenicity of fraction F_4 was the highest, which was 75.92% (Figure 3b). Fraction F_1 had lower glycine antigenicity and Fraction F_2 had lower β -conglycinin antigenicity (Figure 3), and both of them contained peptide bands of <15 kDa (Figure 2b). Fraction F_4 showed the highest antigenicity (Figure 3) and it contained peptide bands of 31 and

TABLE 1 Antigen epitopes and allergen epitopes of soybean in the literature

Author	Literature	Years	Epitopes
Saeed et al.	Using patient serum to epitope map soybean glycinins reveals common epitopes shared with many legumes and tree nuts	2016	KQGQHQQQE GSVLSGFSKHFL EEEDQPRPDHPP QRPQDRHQK VHRFREGDLIAV GDLIAVPTGVAVWWMYNNE GSNILSGFAPEF KGGLRV QCVETDKGC
Xi and Yan	Epitope mapping and identification of amino acids critical for mouse IgG-binding to linear epitopes on Gly m Bd 28K	2016	EGGDKKSP RGE DPST GGAN KDD
Taliercio et al.	Identification of epitopes of the β subunit of soybean β -conglycinin that are antigenic in pigs, dogs, rabbits, and fish	2014	QNGRIRLLQRFNKRSP
Sun et al.	Prediction and characterization of the linear IgE epitopes for the major soybean allergen β -conglycinin using immunoinformatics tools	2013	ECEEGEIPRPRPQHPEREPQQPGEKEE RPIPFPRPQPRQEEHEQREEQEWPR QFPFPRPPHQKEERKQEEDEDEEQQR HEQREEQEWPRKEEKRGEGSSEEEDE DEEQQRESESESELSRRHKNKNPFL NKNPFLFGSNRFETLFKNQYGRIRVLQRF LQNLRDYRILEFNSKPNTLLLPNHAD TAILSLVNDDRRDSYRLQSGDALRVPSGT YYVVNPDDNNENLRLITLAIPVKNKGRFES FFLSSTEAQQSYLQGFSRNILE EQIRALSKRAKSSSRKTISSDKPFN SEDKPFNLRSRDPISYNSKLGKFFEITPEKN DPIYSNKLKGFFEITPEKNPQLRDLD NFFAIGINAENNQRNFLAGSQ QRESYFVDAQPKKKEEGNKGRKGPLSSILR
Liu et al.	Development of a competitive ELISA for the detection of soybean α subunit of β -conglycinin	2012	GEKEEDEDEQPR EQDERQFPFPR NDDRDSYRLQSGD KEEGNKGRKGP
Liu et al.	Prokaryotic expression and B-cell epitopes prediction of A3 acidic polypeptide of glycinin	2012	SRRGSRSQQLQDSHQK LDQNP PDIEHPET HGGRKQGQ YPPRRPSHGKHEDD RPDHPQRPSPRPEQQEPR
Shan et al.	Prediction of antigenic epitopes of soybean major allergen Gly m Bd 30K	2012	ANRKSP TDDD FSKK RVDHSP

(Continues)

TABLE 1 (Continued)

Author	Literature	Years	Epitopes
Xiang et al.	Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins	2002	GFAPEFLKEAFGVN
Helm et al.	Mutational analysis of the IgE-binding epitopes of P34/Gly m Bd 30K	2000	FLVLLLSLL PQEFSEKKTQY RCKANKIQDK INHFVLLVGY GYIWIQRNTG
Helm et al.	A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modeling	2000	KLVLSLCFLLFSGCF NGPQEIIYQQGNGIF QQGNGIFGMIFPGCP RFYLAGNQEQLFKY LKYQQQQGGSQSQK VKGGLRVTAPAMRKP LDFPALWLLKLSAQY LKLSAQYGSRLKKNAM MFVPHYTLNANSIY NANSIYALNGRALY QHTKNLKSQQARQVK
Beardslee et al.	Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3	2000	GGSLSGFTLEFLEHAFSV GAIVTVKGGLSVI
Hosoyama et al.	Epitope analysis of soybean major allergen Glym Bd 30K recognized by the mouse monoclonal antibody using overlapping peptides	1996	DKVTIDGYETLIMSDEST QGGCGRGWAFSATGAIEA

26 kDa (Figure 2b). To a certain extent, proteins with lower molecular weight indicated a lower antigen inhibition rate, which was the same as the antigenicity results of ultrafiltration fractions obtained by ELISA (Figure 1) and our previous results (Huang et al., 2017). Furthermore, fraction F_4 had the highest antigenicity, therefore, it was subjected to subsequent analysis by LC-MS.

3.4 | Sequential analysis

LC-MS was used to analyze the primary structure of chromatography fraction F_4 with the highest antigenicity. Uncharacteristic proteins or peptides were removed. Some peptides were picked up for further analysis if their scores were higher than peptide confidence thresholds. The characterized proteins or peptides mainly belonged to glycinin, β -conglycinin α subunit, 2S albumin, lipoxygenase, trypsin inhibitor, and so on (no data are shown).

Different studies have confirmed a number of soybean protein allergens and epitopes through research and analysis (Table 1) (Beardslee, Zeece, Sarath, & Markwell, 2000; Helm, Cockrell, Connaughton, Sampson, et al., 2000; Helm, Cockrell, Connaughton, West, et al., 2000; Hosoyama, Obata, Bando, Tsuji, & Ogawa, 1996; Liu, Teng, Yang, & Wang, 2012; Liu, Teng, Yang, Wang, & Wang, 2012; Saeed, Gagnon, Cober, & Gleddie, 2016; Shan et

al., 2012; Sun, Shan, Yan, Zhang, & Guan, 2013; Taliercio, Loveless, Turano, & Kim, 2014; Xi & Yan, 2016; Xiang, Beardslee, Zeece, Markwell, & Sarath, 2002). In this study, the peptide sequences determined by mass spectrometry were compared with these known allergen or antigenic epitopes, so that the peptides with epitopes in ion-exchange chromatography product F_4 were obtained (Table 2).

Seven peptides that contained antigenic epitopes were found in fraction F_4 (Table 2). There was no sensitized peptide that belonged to β -conglycinin, which indicated that enzymatic hydrolysis destroyed the epitopes of β -conglycinin (Table 2). This is consistent with the reduction in β -conglycinin antigenicity in this paper. Of the seven peptides that contained epitopes, four peptides belonged to glycinin subunits, one belonged to ribulose-1,5-diphosphate and two peptides were uncharacterized. Most peptides that contained epitopes that belonged to glycinin subunits, which included globulin subunit A3B4, which is a trimeric structure. Adachi et al. (2003) reported that glycinin was a hexamer structure, which was composed of A3B4 subunit, and the crystal structure of the internal surface of the disulfide bond is highly hidden. In addition, some allergenic proteins partially resisted the proteolytic activity of enzymes along the gastrointestinal tract (Moreno, 2007). Angelis, Pilolli, Bavaro, and Monaci (2017) found that glycinin subunits, especially the acid polypeptides, were more susceptible than β -conglycinin to simulated gastrointestinal enzymatic proteolysis. However, some studies

TABLE 2 Antigenic fragments in fraction F₄

Number	Protein description	Molecular weight	Peptide sequence
1	Glycinin A3B4 subunit	58,608	HEDDEDEDEEDQPRPDHPPQRPSRPEQQEPR
2	Uncharacterized protein	63,534	RGEKGSEEEDEDEEQDER
3	Uncharacterized protein	63,534	EPQQPGKEEDEDEQPRPIPFPRPQPR
4	Glycinin G2	54,927	QQUEENEGSNILSGFAPEFLK
5	Glycinin G2	54,927	NAMFVPHYTLNANSIYALNGR
6	Glycinin G2	54,927	GKQQUEENEGSNILSGFAPEFLK
7	Ribulose-1,5-bisphosphate	52,802	GGLDFTKDDENVNSQPFMR

above showed that after gastro-duodenal digestion, both β -conglycinin and glycinin protein fragments retained linear epitopes that were potentially able to trigger an immune reaction when exposed to intestinal mucosa.

Therefore, it is necessary to study glycinin and β -conglycinin peptides with epitopes further and to find effective ways to reduce their antigenicity. Compared with a single technology, the effects of multiple techniques were relatively stronger in modifying these proteins. Chen, Chen, Ren, and Zhao (2011) showed that ultrasonic pretreatment promoted the enzymatic hydrolysis of some subunits (α -7S and A-11S) in soy protein by papain. Jia et al. (2010) found that ultrasonic pretreatment promoted proteolysis of wheat germ protein. Therefore, the ultrasonic, co-enzymatic hydrolysis technique can be used as a method for the future treatment of peptides with epitopes.

4 | CONCLUSIONS

In this study, SPI was hydrolyzed with Neutrase and Flavourzyme. Enzymatic hydrolysis reduced the antigenicity of soybean protein. Hydrolysates were separated by ultrafiltration and the ultrafiltration fraction with the highest antigenicity (>50 kDa) was further separated by ion-exchange chromatography. Ion-exchange chromatography separated fraction F₄, which exhibited the highest antigenicity, was further analyzed by LC-MS. Mass spectrometry showed that most of the peptides that contained antigen epitopes in fraction F₄ belonged to glycinin subunits. These results revealed that glycinin in soybean protein showed resistance to enzymatic hydrolysis. So, further studies are needed to eliminate the antigenic fragments in glycinin.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31871748, 31201293); Foundation of Henan Educational Committee (19A550005); Science and Technology Project of Henan Province (182102110299); the Young-aged Backbone Teacher Funds of Henan Province of China (2019GGJS084); and the Project of Henan University of Technology Excellent Young Teachers. I would like to thank Thomas A. Gavin, Professor Emeritus, Cornell University, for help with editing this paper.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

ORCID

Guanhao Bu  <https://orcid.org/0000-0001-5867-986X>

REFERENCES

- Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., & Utsumi, S. (2003). Crystal structure of soybean 11S globulin: Glycinin A3B4 homo-hexamer. *Proceedings of the National Academy of Sciences of the United States of America*, 100(12), 7395–7400. <https://doi.org/10.1073/pnas.0832158100>
- Angelis, E. D., Pilolli, R., Bavaro, S. L., & Monaci, L. (2017). Insight into the gastro-duodenal digestion resistance of soybean proteins and potential implications for residual immunogenicity. *Food and Function*, 8(4), 1599–1610. <https://doi.org/10.1039/C6FO01788F>
- Beardslee, T. A., Zeece, M. G., Sarath, G., & Markwell, J. P. (2000). Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *International Archives of Allergy and Immunology*, 123(4), 299–307. <https://doi.org/10.1159/000053642>
- Bu, G. H., Zhang, N., & Chen, F. S. (2015). The influence of glycosylation on the antigenicity, allergenicity, and structural properties of 11S-lactose con-jugates. *Food Research International*, 76, 511–517. <https://doi.org/10.1016/j.foodres.2015.08.004>
- Businco, L., Bruno, G., & Giampietro, P. G. (1998). Soy protein for the prevention and treatment of children with cow-milk allergy. *American Journal of Clinical Nutrition*, 68(6), 1447S–1452S. <https://doi.org/10.1093/ajcn/68.6.1447S>
- Chen, L., Chen, J., Ren, J., & Zhao, M. (2011). Effects of ultrasound pretreatment on the enzymatic hydrolysis of soy protein isolates and on the emulsifying properties of hydrolysates. *Journal of Agricultural and Food Chemistry*, 59(6), 2600–2609. <https://doi.org/10.1021/jf103771x>
- Chiang, W. D., Tsou, M. J., Tsai, Z. Y., & Tsai, T. C. (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry*, 98(4), 725–732. <https://doi.org/10.1016/j.foodchem.2005.06.038>
- Costa, H. B., Fernandes, P. M. B., Romão, W., & Ventura, J. A. (2014). A new procedure based on column chromatography to purify bromelain by ion exchange plus gel filtration chromatographies. *Industrial Crops and Products*, 59, 163–168. <https://doi.org/10.1016/j.indcrop.2014.04.042>
- Fang, N., Yu, S., & Badger, T. M. (2004). Comprehensive phytochemical profile of soy protein isolate. *Journal of Agricultural and Food Chemistry*, 52(12), 4012–4020. <https://doi.org/10.1021/jf049842y>
- Fraczek, R. J., Kostyra, E., & Kostyra, H. (2008). Immunogenic potential of antigens isolated from trypsin pea protein hydrolysates. *Polish Journal of Food and Nutrition Sciences*, 58(4), 491–496.

- Friedman, M., & Brandon, D. L. (2001). Nutritional and health benefits of soy proteins. *Journal of Agricultural and Food Chemistry*, 49(3), 1069–1086. <https://doi.org/10.1021/jf0009246>
- Helm, R. M., Cockrell, G., Connaughton, C., Sampson, H. A., Bannon, G. A., Beilinson, V., ... Burks, A. W. (2000). A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modeling. *International Archives of Allergy and Immunology*, 123(3), 213–219. <https://doi.org/10.1159/000024446>
- Helm, R. M., Cockrell, G., Connaughton, C., West, C. M., Herman, E., Sampson, H. A., ... Burks, A. W. (2000). Mutational analysis of the IgE-binding epitopes of P34/Gly m Bd 30K. *Journal of Allergy and Clinical Immunology*, 105, 378–384. [https://doi.org/10.1016/S0091-6749\(00\)90091-5](https://doi.org/10.1016/S0091-6749(00)90091-5)
- Hosoyama, H., Obata, A., Bando, N., Tsuji, H., & Ogawa, T. (1996). Epitope analysis of soy bean major allergen Glym Bd 30K recognized by the mouse monoclonal antibody using overlapping peptides. *Bioscience Biotechnology and Biochemistry*, 60(7), 1181–1182. <https://doi.org/10.1271/bbb.60.1181>
- Huang, T., Bu, G. H., & Chen, F. S. (2017). The influence of composite enzymatic hydrolysis on the antigenicity of β -conglycinin in soy protein hydrolysates. *Journal of Food Biochemistry*, 42(1), e12544. <https://doi.org/10.1111/jfbc.12544>
- Jia, J., Ma, H., Zhao, W., Wang, Z., Tian, W., Luo, L., & He, R. (2010). The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. *Food Chemistry*, 119(1), 336–342. <https://doi.org/10.1016/j.foodchem.2009.06.036>
- Jungbauer, A., & Hahn, R. (2009). Ion-exchange chromatography. *Methods in Enzymology*, 463, 349–371. [https://doi.org/10.1016/s0076-6879\(09\)63022-6](https://doi.org/10.1016/s0076-6879(09)63022-6)
- Kasera, R., Singh, A. B., Lavasa, S., Prasad, K. N., & Arora, N. (2015). Enzymatic hydrolysis: A method in alleviating legume allergenicity. *Food and Chemical Toxicology*, 76, 54–60. <https://doi.org/10.1016/j.fct.2014.11.023>
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685. <https://doi.org/10.1038/227680a0>
- Lee, H. W., Keum, E. H., Lee, S. J., Sung, D. E., Chung, D. H., Lee, S. I., & Oh, S. (2007). Allergenicity of proteolytic hydrolysates of the soybean 11S globulin. *Journal of Food Science*, 72(3), C168–C172. <https://doi.org/10.1111/j.1750-3841.2007.00307.x>
- Lee, M. S., & Kerns, E. H. (2010). LC/MS applications in drug development. *Mass Spectrometry Reviews*, 18(3–4), 187–279. [https://doi.org/10.1002/\(SICI\)1098-2787\(1999\)18:3/4<187:AID-MAS2>3.0.CO;2-K](https://doi.org/10.1002/(SICI)1098-2787(1999)18:3/4<187:AID-MAS2>3.0.CO;2-K)
- Li, H., Zhu, K., Zhou, H., Peng, W., & Guo, X. (2013). Comparative study about some physical properties, in vitro digestibility and immunoreactivity of soybean protein isolate for infant formula. *Plant Foods for Human Nutrition*, 68(2), 124–130. <https://doi.org/10.1007/s11130-013-0358-9>
- Liu, B., Teng, D., Yang, Y. L., & Wang, J. H. (2012). Prokaryotic expression and B-cell epitopes prediction of A3 acidic polypeptide of glycinin. *Journal of Chinese Institute of Food Science and Technology*, 12, 13–20. <https://doi.org/10.16429/j.1009-7848.2012.03.015>
- Liu, B., Teng, D., Yang, Y. L., Wang, X. M., & Wang, J. H. (2012). Development of a competitive ELISA for the detection of soybean α subunit of β -conglycinin. *Process Biochemistry*, 47(2), 280–287. <https://doi.org/10.1016/j.procbio.2011.11.005>
- Liu, K., Zhao, Y., Chen, F., & Fang, Y. (2015). Purification and identification of Se-containing antioxidative peptides from enzymatic hydrolysates of Se-enriched brown rice protein. *Food Chemistry*, 187, 424–430. <https://doi.org/10.1016/j.foodchem.2015.04.086>
- Loginov, M., Boussetta, N., Lebovka, N., & Vorobiev, E. (2013). Separation of polyphenols and proteins from flaxseed hull extracts by coagulation and ultrafiltration. *Journal of Membrane Science*, 442(3), 177–186. <https://doi.org/10.1016/j.memsci.2013.04.036>
- Moreno, F. J. (2007). Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine & Pharmacotherapy*, 61(1), 50–60. <https://doi.org/10.1016/j.biopha.2006.10.005>
- Pinto, S. E. S. D. C., Neves, V. A., & Medeiros, B. M. M. D. (2009). Enzymatic hydrolysis of sweet lupin, chickpea, and lentil 11S globulins decreases their antigenic activity. *Journal of Agricultural and Food Chemistry*, 57(3), 1070–1075. <https://doi.org/10.1021/jf803108c>
- Saeed, H., Gagnon, C., Cober, E., & Gleddie, S. (2016). Using patient serum to epitope map soybean glycinins reveals common epitopes shared with many legumes and tree nuts. *Molecular Immunology*, 70, 125–133. <https://doi.org/10.1016/j.molimm.2015.12.008>
- Shan, X. H., Sun, X. L., Guan, L., Zhang, Y. Z., You, J. M., & Li, H. M. (2012). Prediction of antigenic epitopes of soybean major allergen Gly m Bd 30K. *Journal of Analytical Science*, 28(6), 771–774.
- Shi, J., Luo, Y., Xiao, Y., Li, Z., Xu, Q., & Yao, M. (2014). Effects of fermentation by *Lactobacillus casei* on the antigenicity and allergenicity of four bovine milk proteins. *International Dairy Journal*, 35(1), 75–80. <https://doi.org/10.1016/j.idairyj.2013.10.010>
- Sun, X. L., Shan, X. H., Yan, Z. H., Zhang, Y. Z., & Guan, L. (2013). Prediction and characterization of the linear IgE epitopes for the major soybean allergen β -conglycinin using immunoinformatics tools. *Food and Chemical Toxicology*, 56, 254–260. <https://doi.org/10.1016/j.fct.2013.02.014>
- Taliercio, E., Loveless, T. M., Turano, M. J., & Kim, S. W. (2014). Identification of epitopes of the β subunit of soybean β -conglycinin that are antigenic in pigs, dogs, rabbits and fish. *Journal of the Science of Food and Agriculture*, 94(11), 2289–2294. <https://doi.org/10.1002/jsfa.6556>
- Tomaszewski, M., White, C., Patel, P., Masca, N., Damani, R., Hepworth, J., ... Williams, B. (2014). High rates of non-adherence to antihypertensive treatment revealed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) urine analysis. *Heart*, 100(11), 855–861. <https://doi.org/10.1136/heartjnl-2013-305063>
- Verma, A. K., Kumar, S., Das, M., & Dwivedi, P. D. (2012). Impact of thermal processing on legume allergens. *Plant Foods for Human Nutrition*, 67(4), 430–441. <https://doi.org/10.1007/s11130-012-0328-7>
- Wang, J., & Sampson, H. A. (2007). Food anaphylaxis. *Clinical and Experimental Allergy*, 37(5), 651–660. <https://doi.org/10.1111/j.1365-2222.2007.02682.x>
- Xi, J., & Yan, H. (2016). Epitope mapping and identification of amino acids critical for mouse IgG-binding to linear epitopes on Gly m Bd 28K. *Bioscience Biotechnology and Biochemistry*, 80(10), 1–7. <https://doi.org/10.1080/09168451.2016.1165604>
- Xiang, P., Beardslee, T. A., Zeece, M. G., Markwell, J., & Sarath, G. (2002). Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins. *Archives of Biochemistry and Biophysics*, 408(1), 51–57. [https://doi.org/10.1016/s0003-9861\(02\)00534-9](https://doi.org/10.1016/s0003-9861(02)00534-9)
- You, J., Li, D., Qiao, S., Wang, Z., He, P., Ou, D., & Dong, B. (2008). Development of a monoclonal antibody-based competitive ELISA for detection of β -conglycinin, an allergen from soybean. *Food Chemistry*, 106, 352–360. <https://doi.org/10.1016/j.foodchem.2007.05.040>

How to cite this article: Bu G, Huang T, Li T. The separation and identification of the residual antigenic fragments in soy protein hydrolysates. *J Food Biochem*. 2020;00:e13144. <https://doi.org/10.1111/jfbc.13144>