



Identification of an IgE epitope of soybean allergen Gly m Bd 60K

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ARTICLE INFO

Keywords:

Gly m Bd 60K
Overlapping peptide
IgE epitope
Critical amino acids

ABSTRACT

Soybeans (*Glycine max* L.) are one of the most important sources of food allergens, along with cow's milk and eggs; they are recognized as the "three major allergens" of children, which seriously affect children's health. β -conglycinin is the major soybean allergen, and the α subunit of β -conglycinin, Gly m Bd 60K, is an important allergen in susceptible populations; it was the earliest recognized major allergenic protein. Before this work, we confirmed that the processing treatment reduced the antigenicity of β -conglycinin, and we located the destroyed antigenic sites of Gly m Bd 60K after three processing technologies. In this paper, we used overlapping peptide technology to further study the destroyed antigenic sites of Gly m Bd 60 K. Finally, one epitope that contained both conformational and a linear IgE epitope was found with an amino acid sequence of ³⁸⁰EGQQQGEQRLQ³⁹⁰. Alanine scanning of this fragment documented that Q382, Q383, G385, and Q387 were the critical amino acids of this epitope.

1. Introduction

Soy protein has high nutritional value and important functional characteristics among plant proteins, and it has been used widely in the production of human food and animal feed (De Angelis, Pilolli, Bavaro, & Monaci, 2017; He et al., 2015). However, soybeans (*Glycine max* (L.) are also recognized as one of the eight major food allergens and is a common food allergen in humans (Taliencio, Loveless, Turano, & Kim, 2014; Wang, Qin, Sun, & Zhao, 2014). At present, there is no fundamental solution to soybean allergy, and we can only rely on avoiding ingestion to prevent allergic reactions. In addition, soybeans rely on valuable functional properties for their rich nutritional value; soy appears in many foods as a food additive, which undoubtedly poses a potential threat to people who are susceptible to soybean allergies (Boxtel, Broek, Koppelman, Gruppen, & Gruppen, 2008; You et al., 2009). β -conglycinin is a major storage protein in soybeans, which comprises about 30% of soybean protein, and it is the main food allergen in soybeans (Xi & He, 2018). β -conglycinin is a conjugated triglycan protein composed of the three subunits α' (76 kDa), α (72 kDa), and β (52 kDa) through hydrophobic interaction, and these three subunits of β -conglycinin are allergenic. The homology of the amino acid sequences of the α subunit and α' and β subunits are 90.4% and 76.2%, respectively (Krishnan, Kim, Jang, & Kerley, 2009). Among them, α subunit was the most sensitizing and affected 25% of soybean sensitive patients (Ogawa, Bando, Tsuji, Nishikawa, & Kitamura, 1995). In recent years,

researchers have conducted in-depth studies on soybean antigen proteins. As a result, glycinin, β -conglycinin, Gly m Bd 28K, and Gly m Bd 30K antigen epitopes have been discovered as important contributors to protein sensitization-antigen epitopes (Saeed, Gagnon, Cober, & Gledie, 2016; Sun, Shan, Yan, Zhang, & Guan, 2013; Taliencio & Kim, 2014).

The research methods for discovering antigen epitopes mainly include epitope mapping and epitope prediction. Epitope prediction is the analysis of the structure or physicochemical properties of proteins through bioinformatics methods to predict regions that may become epitopes. Epitope mapping technologies mainly include phage display technology (PDT), protein fragments, mass spectrometry (MS), peptide scanning, and site-directed mutations of amino acids. Some studies identified the linear epitopes of Gly m Bd 60K, IgG and IgE, using a peptide library and immune informatics tools (Sun et al., 2013; Taliencio et al., 2014; Wu, Fu, Wang, & Tsay, 2007). In our previous work, we confirmed that the processing treatment reduced the antigenicity of β -conglycinin, and we located the destroyed antigenic sites of Gly m Bd 60K using PDT; the antigenic site had amino acid sequences of ³⁸⁰EGQQQGEQRLQESVIVEI

SKEQIRALSKR⁴⁰⁸ and ⁴⁰⁴ALSKRAKSSSRKTISSDKPFLNLSRDPIYS NKLKFF

EITPEKN⁴⁴⁸ (Xi & He, 2020). In the present paper, we used overlapping peptides to further locate linear epitopes, and we synthesized six overlapping peptides to react with antiserum from soybean allergic

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Table 1
The synthesized overlapping peptides.

No.	Sequence
1	(C) ³⁸⁰ EGQQGEQRLQESVIVE ³⁹⁶
2	(C) ³⁹¹ ESVIVEISKEQIRALSKR ⁴⁰⁸
3	(C) ⁴⁰⁴ ALSKRAKSSSRKTTISSEDK ⁴²²
4	(C) ⁴¹⁷ ISSEDKPFNLRSD ⁴³⁰
5	(C) ⁴²⁵ NLRSDPIYSNKLK ⁴³⁸
6	(C) ⁴³³ YSNKLKGFFEITPEKN ⁴⁴⁸

subjects. Finally, we obtained one IgE epitope and, at the same time, we determined the critical amino acids in this epitope. It is expected that the key amino acids will serve as the target to carry out amino acid substitutions to obtain hypoallergenic foods directly, which would eliminate the impact of processing on the flavor and nutrition of the food.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), *N,N*-Dimethylformamide (DMF), and ethylenediamine tetraacetic acid (EDTA) were procured from Sangon Biotech Co., Ltd (Shanghai, China). Goat anti-human HRP-IgE, bovine serum albumin (BSA), and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Sulfo-SMCC and nitrocellulose membranes were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Trifluoroacetic acid (TFA) and Acetonitrile (ACN), which were high-performance liquid chromatography (HPLC) grade, were purchased from Thermo Fisher Scientific (Waltham, USA). The antiserum with soybean allergy patients was obtained from Henan Provincial People's Hospital (Zhengzhou, China) from 12 Chinese adults with acute allergic symptoms after ingestion of soybeans.

Table 2
Molecular weight and purity of synthetic peptides.

NO.	Molecular Weight (Da)	Purity (%)
1	2061.47	90.18
2	2190.45	96.60
3	1627.17	90.40
4	1767.28	86.42
5	1737.75	98.01
6	2019.64	88.68

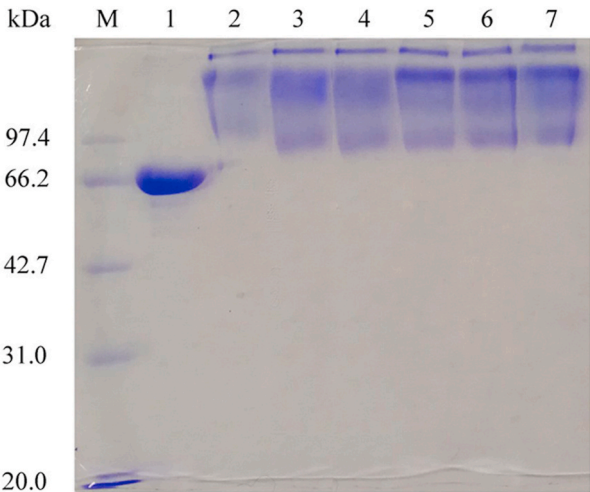


Fig. 2. Identification of conjugated peptides (M: Marker; 1: BSA; 2: peptide NO.1; 3: peptide NO.2; 4: peptide NO. 3; 5: peptide NO.4; 6: peptide NO.5; 7: peptide NO. 6).

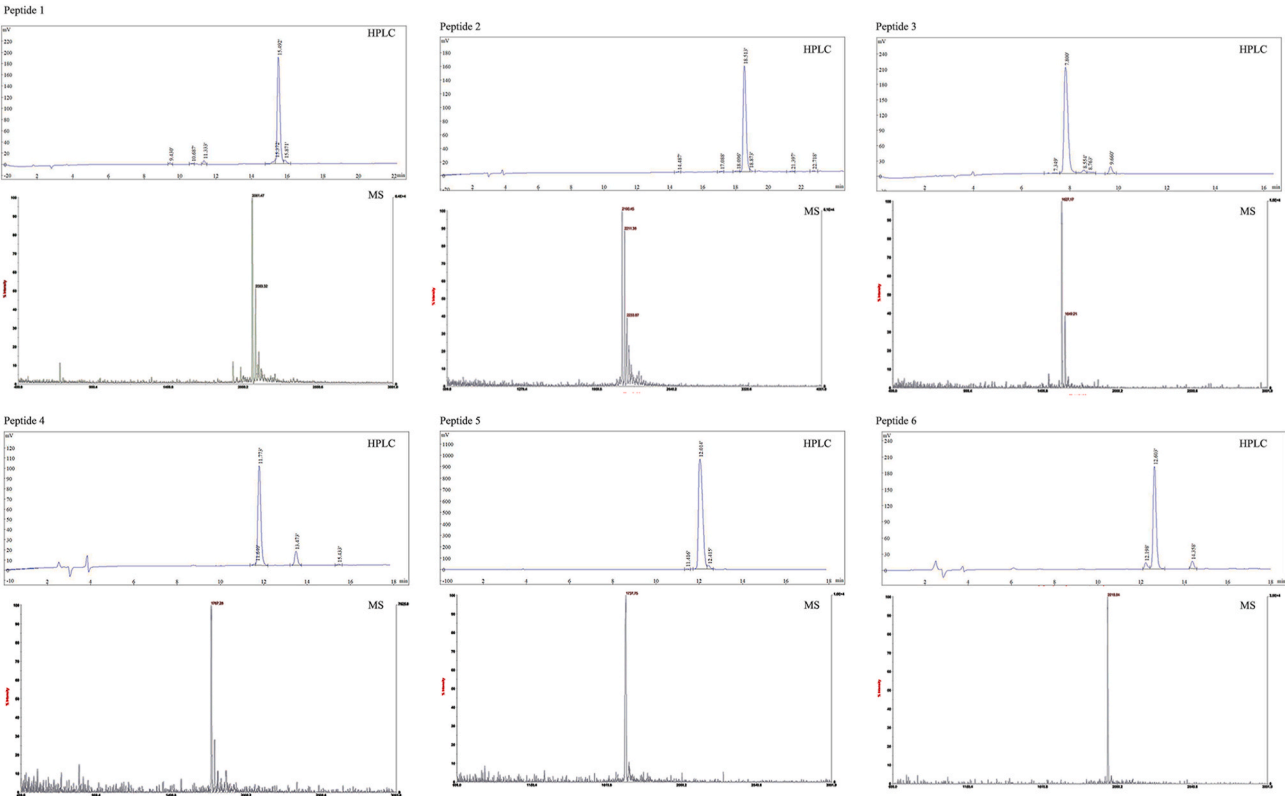


Fig. 1. Identification of synthesized peptides.



Fig. 3. The reaction between overlapping peptides and antiserum (1: peptide NO.1; 2: peptide NO.2; 3: peptide NO. 3; 4: peptide NO.4; 5: peptide NO.5; 6: peptide NO. 6; 7: BSA; 8: β-conglycinin; 9: PBS).

2.2. Peptide synthesis

We designed and synthesized six overlapping peptides (Table 1), which contained the sequences ³⁸⁰EGQQQGEQRLQESVIVEISKEQIR-ALSKR⁴⁰⁸ and ⁴⁰⁴ALSKRAKSSSRKTISSEDKPFNLRSRDIPIYSNKLKGF-FEITPEKN⁴⁴⁸ in the protein of Gly m Bd 60K, with six amino acid

overlaps. The peptides were synthesized by Zhongke Co., Ltd (Beijing, China) using the Fmoc method.

2.3. Peptide identification by HPLC and MALDI-TOF/MS

The synthesized peptides were identified by HPLC and mass spectrometry (MS). The chromatographic separation was performed on a C18 column (250 × 4.6 mm (i.d); particle size 5 μm) from Agilent Technologies (California, USA), and the operating temperature was 35 °C. The mobile phase was composed of two solvents: (A) 0.05%TFA + 2% ACN and (B) 0.05% TFA + 90% ACN, with a flow rate of 1 mL/min. The linear gradient started with 10% B was set to 32% B at 20 min and held for 2 min.

MALDI-TOF conditions were as follows: mode: voyager; instrument mode: linear positive; control mode: manual; voltages: accelerating 20000V; grid: 95%; guide wire: 0.05%; delay time: 900 nsec; spectrum acquisition: 50 (shots/spectrum); mass rang (Da) 1500 to 6000; low mass gate (Da) 300; and calibration: matrix α-Cyano-4-hydroxycinnamic

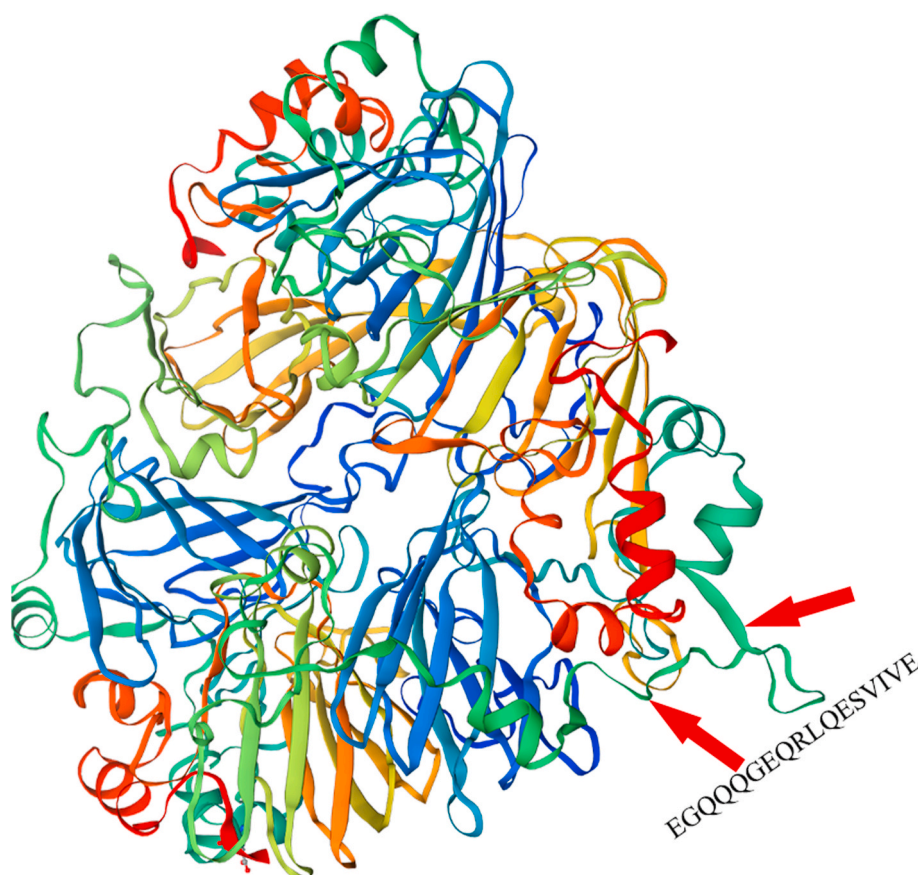


Fig. 4. Tertiary structure of peptide No. 1.

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MARFPLLLGVVFLASVSFSFGAYWEKQNPKNKCLQSCNSERDSYRNQACHARNLLKVEKEECEEGEIPRPRPQH 80
PEREPQPGKEEDEDQPRPIFPRPQRQEEHEQREEQEWPRKEEKRGEKGSEEQDGREHPRPHQPHDEEEQDE 160
RQFPFRPPHQKESEERKQEEDEEEQQRSESESESSQRELRRHKKNKPFHFGSNRFETLFKNQYGRIRVLQRFNQRS 240
PQLQNLRDYRILEFNSKPNLTLLPNHADADYLIAILNGTAILSLVNNDDRDSYRLQSGDALRVPSTTYVVNPNNENL 320
RLITLAIPVKNKPGRFESFSLSTEAAQQSYLQGFNRNILEASYDTKFEINKVLFSREEGQQQGEQRLQESVIVEISKEQI 400
RALSKRAKSSSRKTISSEDKPFNLRSRDIPIYSNKLKGFPEITPEKNPQLRDLDFLSIVDMNEGALLPHFNKAIVLV 480
INEGDANIELVGLKEQQEEQEEQPLEVRKYRAELSEQDIFVIPAGYPVVVNATSNLNFFAIGINAENNQRNFLAGSQD 560
NVISQIPSQVQELAFGLSAQAVEKLLKNQRESYFVDAQPKKKEEGNKGKRGKGLSSILRAFY

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Fig. 5. The amino acid sequence of Gly m Bd 60K and the reported B cell epitopes in humans (Epitopes were shown in red (Sun et al., 2013) and green (Kern et al., 2019), “___” indicated the epitope reported by both articles).



Fig. 6. Homology comparison of α' , α (Gly m Bd 60K) and β subunit (We identified epitope is shown in red).

Table 3
Molecular weight and purity of mutated peptides.

NO.	Molecular Weight (Da)	Purity (%)
1	1346.24	90.65
2	1418.78	92.53
3	1347.77	91.86
4	1347.94	85.61
5	1347.51	87.25
6	1418.51	91.58
7	1345.77	93.47
8	1347.22	90.51
9	1341.36	85.36
10	1362.95	93.28
11	1347.85	97.06

acid.

2.4. Peptide conjugation and identification

A heterobifunctional reagent (Sulfo-SMCC) was used to connect the –SH of the N-terminal of the cysteine-added polypeptide to the –NH₂ on the carrier protein of BSA (Chan & White, 2000; Xi & Yan, 2016). The steps were as follows (Xi, He, & Pi, 2019): First, 5 mg of IgG-free BSA protein was dissolved in 0.5 mL conjugation buffer (0.15 mol/L NaCl, 0.1 mol/L PB pH 7.2, and 1 mol/L EDTA), and 1 mg of Sulfo-SMCC was dissolved in 50 μ L DMSO solution, then mixed with BSA solution and reacted at room temperature for 30 min. The solution was dialyzed to remove excess coupling agent and then diluted with conjugation buffer to 5 mg/mL protein. Two milligrams of peptide was dissolved in 50 μ L DMF solution, and 150 μ L PB (pH 7.2) was added to prepare a peptide storage solution. Next, we diluted the peptide solution with an equal volume of PB (pH 7.2) buffer, then added two volumes of SMCC-BSA solution, and reacted at room temperature for 30 min. Finally, we

adjusted the peptide concentration to 1 mg/mL in PB (pH 7.2) buffer for further assay.

The conjugated peptide was identified by SDS-PAGE to see whether it conjugated to BSA. A 20 μ L aliquot of 1 mg/mL conjugated peptide and BSA solution were mixed with an equal volume of 2 $\times$ SDS-PAGE loading buffer, and the electrophoresis procedure was as described previously (Nakagawara et al., 2013). The molecular weight of the conjugated peptides was observed with BSA as a control.

2.5. Dot-blot assay for IgE-binding

Two micrograms of conjugated peptides was spotted on a nitrocellulose membrane with BSA protein as the control, dried at 37 $^{\circ}$ C for 30 min, and washed with PBST solution (0.05% Tween 20 in 0.01 mol/L pH 7.2 PBS). Then, 0.2% gelatin (in PBST buffer) was used to block the non-specific binding sites at 37 $^{\circ}$ C for 2 h. After washing with PBST, the membrane was incubated with the antiserum (1: 100 dilution (PBS) at 37 $^{\circ}$ C for 30 min and washed in PBST solution. Next, the membrane was incubated with goat anti-human HRP-IgE at 37 $^{\circ}$ C for 30 min. Finally, the membranes were washed and AEC was added; after color development was completed, the reaction was terminated with distilled water.

2.6. Identification of the critical amino acids

To determine the critical amino acids on the positive peptide, a series of related peptides with alanine substitutions was synthesized. After conjugation and identification of peptides, their IgE binding capacity was evaluated.

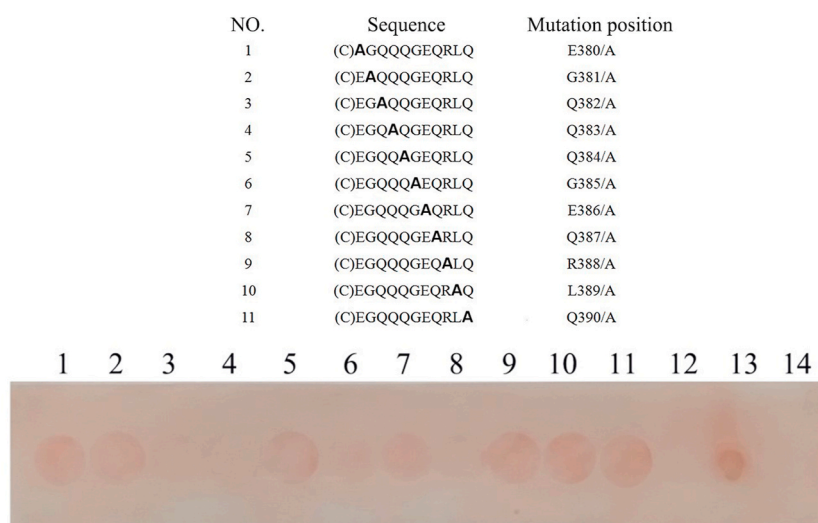


Fig. 7. The reaction between mutated peptides and antiserum (1: NO.1; 2: NO.2; 3: NO. 3; 4: NO.4; 5: NO.5; 6: NO. 6; 7: NO.7; 8: NO.8; 9: NO. 9; 10: NO.10; 11: NO.11; 12:BSA; 13: β -conglycinin; 14: PBS).

3. Results and discussion

3.1. Peptide synthesis

The synthesized peptides were identified by HPLC and MS (Fig. 1). The molecular masses of six peptides were well matched with their theoretical molecular masses, and the purity was >86% (Table 2), which meant that they could be used for further study.

3.2. Peptide conjugation and identification

Molecular weights of the conjugated peptides were larger than those of the BSA standard protein, which indicated that the peptides and carrier proteins were conjugated successfully, and the number of peptides that could be conjugated to a BSA protein was not fixed (Fig. 2). A BSA protein can connect to 30–35 peptides (Hermanson, 2008). Taking peptide No. 4 as an example, the MS results indicated that its molecular weight was 1.737 kDa (Fig. 1), the electrophoresis results showed that the molecular weight of BSA was 66.2 kDa, and the molecular weight of conjugated peptide No. 4 was 97.4–126 kDa (Fig. 2). We estimated that the number of conjugated peptides was 18–36.

3.3. Dot-blot assay for IgE-binding

The reaction of the overlapping peptides with antiserum showed that only peptide NO.1 reacted with the antiserum, which indicated that this peptide was a linear epitope, and its amino acid sequence was ³⁸⁰EGQQQGEQRLQESVIVE³⁹⁶ (Fig. 3). Peptide No.2 did not react with the antiserum, so we did not consider using this any further. Finally, we chose the sequence ³⁸⁰EGQQQGEQRLQ³⁹⁰ for further research. In our previous work, we predicted that ³⁷⁹EEGQQQGEQ³⁸⁷ was a conformational epitope (Xi et al., 2019), which indicated that this amino acid contained both conformational and linear epitopes.

The swiss-model webserver database (<https://swissmodel.expasy.org/>) was used to obtain the tertiary structure of ³⁸⁰EGQQQGEQRLQESVIVE³⁹⁶ on Gly m Bd 60K (Fig. 4). Most of the amino acids were located in the random coil and existed on the surface of the protein, which was easy to bind to the antibody.

Ogawa et al. (1995) pointed out that the epitope of α subunit in β -conglycinin was located in the N-terminal residues 232–383. Fu, Jez, Kerley, Allee, and Krishnan (2007) used planar cellulose support technology (SPOT) to show that S185-R231 was essential for sensitization. Subsequently, Taliercio et al. (2014) verified this major epitope using

overlapping peptide analysis; they obtained another major IgG binding epitope with an amino acid sequence of ⁵⁸⁹QRE-SYFVDAQPKKKEEGNKGRKGPLSSILRAF⁶²¹. However, there are few studies on human IgE epitopes. The B cell antigen epitope of Gly m Bd 60K was searched using the IEDB database (<http://www.iedb.org/>). All 82 IgE epitopes that were retrieved were linear (Fig. 5). Sun et al. (2013) predicted 15 linear epitopes using bioinformatics software, and they verified that 11 peptides were linear IgE epitopes; among them, peptide 3 was considered to be the main epitope with amino acids ⁵⁵HEQREEQEWPKEEKRGEKGSEEEDE⁸⁰.

Recently, Kern et al. (2019) identified more than 400 potential epitopes of soybean, of which > 60% had not been described as potential epitopes. Of these peptides, 56 were bound by antibodies, 55 were bound by serum IgE, 43 were bound by serum IgG, and 30 were bound by both IgE and IgG. So far, there are few reports on the conformational IgE epitope of Gly m Bd 60K. The fragments we found in this research were both linear and conformational epitopes. At present, the research on the allergenicity of β -conglycinin has reached the level of subunits, and there is a high homology similarity among α' , α , and β (Fig. 6). Studying the allergic epitope of α subunit can also provide a reference for other subunits. We searched the IEDB database, but there were no results for conformational epitopes of α' , α , and β subunits. In our previous work, we used PDT and found a conformational epitope; we further synthesized overlapping peptides to react with antiserum from soybean allergic subjects. Finally, we obtained one IgE epitope, which indicated that this fragment contained both linear and conformational epitopes.

3.4. Identification of critical amino acids

We used alanine to replace the amino acids on the peptide ³⁸⁰EGQQQGEQRLQ³⁹⁰ of Gly m Bd 60K, and 11 related peptides were synthesized. We identified the purity and molecular weight using HPLC and MS (Table 3), and we found that the molecular masses of 11 peptides were well matched with their theoretical molecular masses, and the purity was >86%. After conjugation with BSA, the reaction with antiserum was detected using the dot-blot method. The alanine substitutions were at the 3rd, 4th, 6th, and 8th positions, and its ability to react with the antiserum decreased; Q382, Q383, G385, and Q387 were the critical amino acids on the ³⁸⁰EGQQQGEQRLQ³⁹⁰ of the Gly m Bd 60K protein (Fig. 7). This may be because glutamine and glycine are hydrophilic amino acids, which were located on the surface of Gly m Bd 60K that easily became epitopes (Huang, Lu, Wei, Guo, & Liu, 2011).

4. Conclusion

In this paper, we further investigated the destroyed antigenic sites of Gly m Bd 60 K using peptide synthesis technology, which had amino acid sequences of ³⁸⁰EGQQQGEQRLQESVIVEISKEQIRALSKR⁴⁰⁸ and ⁴⁰⁴ALSKRAKSSSRKTISSSD

KPFNLRSDPIYSNKLKGKFFETPEKN⁴⁴⁸. Finally, we found an amino acid (³⁸⁰EGQQQGEQRLQ³⁹⁰) that contained both conformational epitopes and linear epitopes. Using bioinformatics software to simulate its tertiary structure, most of the amino acids on this peptide were located at random coils. Alanine substitutions were performed on the positive peptide fragments to determine the critical amino acids, which included Q382, Q383, G385, and Q387. Processing treatment can destroy the allergenicity of soybean protein. We have located destroyed allergenic sites of processing technologies, and we analyzed the structure of destroyed allergenic sites and the key amino acids at these site (or, these sites?). It is expected that key amino acids would be the targets to carry out amino acid substitutions to directly obtain hypoallergenic foods, which would eliminate the impact of processing on the flavor and nutrition of the food.

CRediT authorship contribution statement

MengXue He: Data curation, Formal analysis, Software, Investigation, Visualization, Writing - original draft, Writing whole paper, including commentary or revision. **Jun Xi:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest. This article does not contain any studies with human subjects.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC, 31671778), the National Key Research and Development Program of China (2017YFC1600402), the Youth Key Teachers from Henan University of Technology, the Henan Provincial Scientific and Technological Research Project (182102110002), the Opening Topics of Engineering Technology, Research center for Grain and Oil Food of State Administration (GA2018009), and the Integration of Science and Education (26400109). I would like to thank Thomas A. Gavin, Professor Emeritus, Cornell University, for help with editing this paper.

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