



Location of destroyed antigenic sites of Gly m Bd 60 K after three processing technologies



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ABSTRACT

Gly m Bd 60 K, which is the α subunit of β -conglycinin, is a major soybean (*Glycine max*) allergen. We used high hydrostatic pressure (HHP), thermal techniques, and glycation to treat β -conglycinin, which can effectively reduce the antigenicity of β -conglycinin. β -conglycinin was used to immunize New Zealand rabbits, and the antiserum had a titer $> 1:1 \times 10^5$ and an IC_{50} of 2.254 μ g/mL. β -conglycinin was subjected to HHP, thermal techniques, and glycation and mixed with rabbit antiserum against β -conglycinin to obtain the site-specific antiserum. The overlapping gene fragments of Gly m Bd 60 K were amplified by polymerase chain reaction (PCR), then cloned into a T7 phage vector and packaged *in vitro*, the recombinant T7 phages were constructed. Indirect ELISA (iELISA) was used to locate the destroyed antigenic sites and, after three rounds of segment expression and identification, the C2-1 and C2-2 fragments were identified as destroyed antigenic sites of Gly m Bd 60 K. Allergenicity analysis showed that the C2-1 and C2-2 fragments reacted with allergic patients' serum, which indicated that the destroyed sites were allergic sites.

1. Introduction

A food allergy, which is a specific immune response caused by eating certain foods, is an allergic overreaction in the human immune system (Sicherer, 2010; Tiwari, Mitra, & Tiwari, 2018). It is also a problem for food safety worldwide, which seriously affects the health of allergic people (Lee, 2013). Approximately 8% of young children and 1–2% of adults suffer from food allergies, and this problem appears to have increased in prevalence (Li, Chen, Tong, & Wen, 2012; Tordesillas, Berin, & Sampson, 2017). Soybeans (*Glycine max*) are rich in unsaturated fatty acids, minerals, and vitamins. They are a major source of high-quality vegetable protein; therefore, soybeans are used widely in the food industry (De, Pilolli, Bavaro, & Monaci, 2017; Gagnon et al., 2010; Zheng, Qin, Chen, & Zhang, 2018). However, soybeans also contain many anti-nutritional factors, which include trypsin inhibitor, lectin, phytic acid, etc., and it is one of the major food allergens (Havenith et al., 2016; Ma, He, Sun, & Han, 2010; Wang, Qin, Sun, & Zhao, 2014). 11S and 7S globulins are the major soybean allergenic proteins (Shriver & Yang, 2011; Wang et al., 2014). β -conglycinin is a major component of 7S globulin, which is composed of different combinations of α' , α , and β subunits, all of which have been reported to be allergenic in humans (Talierto, Loveless, Turano, & Kim, 2014; Yoshino, Tsutsumi, & Kanazawa, 2015). The α subunit of β -conglycinin,

Gly m Bd 60 K, was the earliest recognized major allergenic protein, and almost 25% of soybean-sensitive patients were allergic to this protein (Ogawa, Bando, Tsuji, Nishikawa, & Kitamura, 1995). High hydrostatic pressure (HHP), thermal techniques, and glycation can decrease the antigenicity of β -conglycinin effectively (Bu et al., 2015; Li, Zhu, Zhou, & Peng, 2012; Liu, Xue, Mu, & Yan, 2005). However, there is still no relevant literature about the destroyed antigenic sites of Gly m Bd 60 K after HHP, thermal processing, and glycation processing.

Recently, many investigators have used different epitope mapping methods, such as peptide array, protein fragments, and phage display, to predict food allergens (Hansen et al., 2016; Longo et al., 2015; Mishra, Jain, & Arora, 2016). Also, phage display technology (PDT) has been used to screen for a variety of food allergens and epitope peptides (Chen & Dreskin, 2017; Li et al., 2012; Li, Yuan, He, Gao, & Chen, 2015; Schiller, Vieths, & Holzhauser, 2011). However, there is no information on the application of PDT to locate the allergenic sites of Gly m Bd 60 K in soybeans, especially for allergenic sites that have been destroyed after processing. Therefore, we explored a method to locate destroyed antigenic sites of Gly m Bd 60 K after HHP, thermal techniques, and glycation processing. Our results can be used as a theoretical basis to provide new processing methods for the food industry, and it should help to develop further the application products for rapid detection of the desensitization effect of processed foods.

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2. Materials and methods

2.1. Chemicals

The sequence of Gly m Bd 60 K was obtained from NCBI Genbank (accession number: LOC547464), synthesized by Sangon Biotech Co., Ltd (Shanghai, China), and stored in a plasmid vector. Most of the chemicals (Ex-Taq DNA premix, pMD18-T vector, *E. coli* JM109, *Eco*RI, and *Hind* III) were procured from Takara Bio Co., Ltd. (Dalian, China). The T7Select10-3b Cloning Kit and *E. coli* BLT5615 were purchased from Novagen Company (Madison, WI, USA).

2.2. Preparation of rabbit antiserum against β -conglycinin

Five New Zealand rabbits were purchased from the medical college of Zhengzhou University, Zhengzhou, China and housed in Henan Provincial Key Laboratory of Animal Immunology, Henan Academy of Agricultural Sciences. Rabbits were immunized with the β -conglycinin by subcutaneous injections at multiple locations. The first dose consisted of 300 μ g of immunogen as an emulsion of PBS (8.0 g NaCl, 0.2 g KH_2PO_4 , 2.13 g Na_2HPO_4 , and 0.2 g KCl per liter, pH 7.4) and Freund's complete adjuvant. After 14 d, 200 μ g of immunogen that was emulsified in Freund's incomplete adjuvant was injected. Then, two subsequent injections were given at 10 d intervals with the same dosage of immunogen emulsified in Freund's incomplete adjuvant. The last immunization was 400 μ g of the adjuvant-free antigen injected into the ear vein. Four days later, the serum was collected from the heart, mixed after centrifuged, and stored at -20°C .

2.3. Processing conditions of raw β -conglycinin

Before this study, we revealed that HHP treatment affected the antigenicity and structure of β -conglycinin (Xi & He, 2018). After this, we used response surface methodology to optimize the HHP treatment conditions and found that when HHP treatment pressure was 455 MPa, HHP treatment time was 18 min, and when the β -conglycinin concentration was 15 mg/mL (in 0.01 M pH 7.2 phosphate-buffered saline (i.e., PBS)), the antigenicity inhibition rate was lower, 49.59% (data not shown). The heat treatment conditions were a protein concentration of 10 mg/mL (in PBS buffer), heating temperature of 100°C , and heating time of 60 min, which resulted in an antigenicity inhibition rate of 38.4% (Fig. S1). The conditions for the glycosylation treatment were obtained from the published literature (Bu et al., 2015) and, on this basis, we obtained the conditions that minimized the antigenicity of β -conglycinin. Soy protein isolate (SPI)-glucose (3:1, w/w) conjugates, which was held at 60°C for 2.5 d, resulted in an antigenicity inhibition rate of 42.8% (Fig. S2).

2.4. The preparation of site-specific antiserum

The preparation procedure was the same as our previous work (Xi, He, & Pi, 2019). An excess of processed (HHP, thermal, and glycation treated) β -conglycinin antigen was added to rabbit anti- β -conglycinin antiserum, incubated at 37°C for 1 h, and the precipitate was removed by centrifugation (10000 g, 30 min, 4°C). The supernatant was the specific antiserum that recognized destroyed sites.

2.5. Expressing Gly m Bd 60 K and its overlapping fragments

2.5.1. Construction of the cloning vector of Gly m Bd 60 K and its overlapping fragments

The SWISS-MODEL webserver database (<https://swissmodel.expasy.org/>) was used to search for the homologous proteins of Gly m Bd 60 K. The highest homology alpha prime subunit of β -conglycinin (the similarity with Gly m Bd 60 K was 89.71%) was selected as the tertiary structure prediction template, and then the DiscoTope 2.0

server (<http://www.cbs.dtu.dk/services/DiscoTope/>) was used to predict the conformational epitopes. The results indicated that six potential epitopes were ²³⁹NQRSPQLQN²⁴⁷, ³¹⁷DNNE³²⁰, ³⁷⁹EEGQQQGEQ³⁸⁷, ⁴¹³SRK⁴¹⁵, ⁴¹⁸SSED⁴², and ⁴⁹⁷EQQQEEQQEEQ⁵⁰⁷. Based on these predictions, Gly m Bd 60 K was divided into four overlapping fragments, and a series of primers was designed by Primer Premier 5.0 and synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The overlapping Gly m Bd 60 K genes were amplified by PCR, ligated into a pMD18-T vector, and transformed into *E. coli* JM109. The recombinant plasmids were evaluated by PCR and *Eco*RI and *Hind* III enzyme digestion, and the DNA sequences were analyzed.

2.5.2. Phage packaging of Gly m Bd 60 K and its overlapping fragments

Double enzyme digestion and recovery of Gly m Bd 60 K and its overlapping fragments: Gly m Bd 60 K and its overlapping fragments were digested from the recombinant plasmids with *Eco*RI and *Hind* III. The digested products were separated using a 1% agarose gel, and the target fragments were recovered by a gel recovery kit after the DNA gels were cut out.

Phage packaging: The purified Gly m Bd 60 K gene and its overlapping fragments were recovered, and the purified genes were ligated to the T7 vector arms at a molar ratio of 1:1 to 3:1 (insert: vector). The ligation system was performed according to the kit manual.

2.5.3. Identification of recombinant phage

PCR identification of recombinant phage: The packaged products were cultured on agar plates, and multiple plaques were chosen randomly, dissolved in TE buffer (Tris-EDTA buffer solution), heated (65°C , 10 min), and centrifuged (12000 r/min). We used PCR amplification to detect the rate of phage recombination.

Sequence analysis of phage recombinants: PCR products were cloned into the vector to construct a recombinant plasmid for PCR and the restriction enzyme digestion. The method was the same as described above, and then the sequence was analyzed.

2.5.4. Amplification and preservation of phage lysate

We used the same method as in our previous work (Xi et al., 2019). The phage lysate was centrifuged, DNase I and RNase A (1 mg/mL) were added to the supernatant; after digestion, 10% (m/v) PEG 8000 and a final concentration of 1 M NaCl were added to an ice bath for > 1 h. The phage pellets were collected by centrifugation (4°C , 11,000 g, 10 min), and bacteriophages were resuspended in SM solution (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 mM MgSO_4). Finally, the phage was extracted with chloroform and separated by centrifugation (4°C , 3000 g, 15 min).

2.5.5. Identification of purified phage

After purified by liquid lysis, the phage particles were heated (65°C , 10 min), and the DNA bands were detected using 1% agarose electrophoresis. The immunological activity of phage protein was detected by the Western-blot method. The protein was separated by electrophoresis using a 12% gel and a 5% gel (Penas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011), transferred to a polyvinylidene difluoride (PVDF) membrane in a transfer buffer, and blocked with 5% skim milk overnight. After washing, the membrane was incubated with rabbit anti-serum against β -conglycinin for 1 h and rinsed several times in TBST (0.05% Tween 20 in 1 M pH 7.5 Tris-HCl buffered saline (i.e., TBS)) buffer. The membrane was then incubated with the goat anti-rabbit IgG-HRP for 1 h and, after washing, chemiluminescent HRP substrate (Millipore) was added.

2.6. The initial location of the destroyed antigen sites

All recombinant phages that expressed the overlapping fragments of Gly m Bd 60 K were coated in a microtiter plate. The indirect ELISA procedure was described in our previous work (Xi et al., 2019). The line

graph represented the reaction between phage-expressed protein and β -conglycinin antiserum. The greater the OD value, the stronger the antigenicity of the fragment. After processing, some of the antigen sites were destroyed when they reacted with the β -conglycinin antiserum; these destroyed antigenic sites did not bind to the antibody and existed in the form of free antibodies in the site-specific antiserum. However, the untreated antigen bound to the antibody and existed in the form of an antigen-antibody conjugate. Therefore, the reaction between the site-specific antiserum and the phage-expressed protein was stronger than that of the untreated antigen-absorbing antiserum. The destructive strength was equal to the subtraction value of OD 450 nm between process-treated antigen-absorbing antiserum and untreated antigen-absorbing antiserum; the larger the OD value, the more significant the destruction was.

2.7. The precise location of the destroyed antigen sites

The segment that was most destroyed was further divided into three fragments with a residue of 20 amino acids that overlapped; after it was expressed, its binding characteristics were measured with processing-destroyed site-specific antiserum. After three rounds, we located the precise site of the Gly m Bd 60 K antigen that was destroyed by the processing treatment.

2.8. Allergenicity analysis

At the location of destroyed sites, we used rabbit antiserum and finally located the antigenic sites. However, to determine whether these sites were sensitized, further allergic serum was needed to verify whether the located sites were allergic sites. The measurement procedure was the same as before, but the antiserum was from 12 Chinese adults with acute allergic symptoms after ingestion of soybeans, which were provided by Henan Provincial People's Hospital (Zhengzhou, China), and goat anti-human IgE conjugated with horseradish peroxidase was used as the enzyme-labeled anti-antibody.

2.9. Analysis of secondary structure

Secondary structure of the destroyed sites was analyzed by the GOR IV online server to determine the composition of the secondary structure.

2.10. Statistical analysis

The location of the destroyed antigen sites and allergenicity analysis were performed three times. An analysis of variance (ANOVA) was performed on the data using SPSS 16.0 (IBM Corporation, NY, USA), and differences were considered statistically significant if $p \leq 0.05$. Duncan's multiple range tests were used to detect differences among means.

3. Results and discussion

3.1. Analysis of rabbit antiserum against β -conglycinin

The antiserum has a titer $> 1:1 \times 10^5$ and an IC_{50} of 2.254 μ g/mL (Fig. 1), which could be used for later analysis.

3.2. Identification of site-specific antiserum

The Dot-Elisa method was used to detect the reaction of site-specific antiserum with untreated and process-treated β -conglycinin. We found that the site-specific antiserum reacted with β -conglycinin, but it did not react with process-treated β -conglycinin (Fig. 2). This indicated that by adding an excess of process-treated β -conglycinin to rabbit antiserum against β -conglycinin, the destroyed antigen sites did not bind to

the antibody, and the undestroyed antigenic sites bound to the antibody. Untreated β -conglycinin contained all the antigenic sites and, thus, it could be combined with the site-specific antiserum.

3.3. Expression and identification of Gly m Bd 60 K and its overlapping segments

3.3.1. Construction of the cloning vector of Gly m Bd 60 K and its overlapping fragments

The segmentation of Gly m Bd 60 K was as follows: A: A15-G230 (105–752 bp), B: E200-E370 (660–1172 bp), C: N321-E510 (1023–1592 bp), and D: I460-Y623 (1440–1931 bp). Primers were designed using DNA star and Primer Premier 5.0 software (Table 1).

Gly m Bd 60 K and its overlapping gene fragments obtained by PCR and agarose gel electrophoresis were consistent with the expected, and no nonspecific bands were observed (Fig. 3a). PCR and double-enzyme digestion of Gly m Bd 60 K and its overlapping fragments A, B, C, and D showed the size bands at 1800, 640, 510, 570, and 490 bp, respectively (Fig. 3b), which indicated that Gly m Bd 60 K and its overlapping fragments were inserted successfully into the pMD18-T vector, and the sequences of the inserted fragments were consistent with their gene sequences.

3.3.2. Phage display and identification of Gly m Bd 60 K and its overlapping fragments

Agarose gel electrophoresis showed that the recombination rate was $> 90\%$ (Fig. 3c). DNA sequencing showed that the exogenous DNA fragment was correct. The phage particles were heated at 65 °C after purification by liquid lysis, and only one brighter DNA band was observed in 1% agarose gel electrophoresis. However, there were multiple DNA bands in the unpurified phage lysate (Fig. 3d).

The capsid protein of T7 bacteriophage consisted of forms 10A (344 aa) and 10B (397 aa) (McCormick, Novy, & Mierendorf, 1996), and their molecular weights were 37.8 kDa and 43.7 kDa, respectively. The purified phage proteins were electrophoresed on SDS-PAGE and transferred to a PVDF membrane for an antigen-antibody reaction. The molecular weights were approximately 68 kDa, 63 kDa, 64 kDa, and 61 kDa for fragments A, B, C, and D, respectively, which were consistent with the expected fragment sizes (Fig. 3e). The purified overlapping segments of Gly m Bd 60 K reacted well with antiserum against β -conglycinin and exhibited immunological activity, although the negative phage control did not react with antiserum against β -conglycinin.

3.4. The initial location of the potential destroyed antigen sites

The reaction results (Fig. 4) showed that the most significant destroyed fragments due to HHP, heat treatment, and glycosylation were all in the C fragment, with an amino acid sequence of ³²¹NLRILTLAI-PVKNKPGRFESFFLSSTEAQQSYLQGFSSRNILEAS

YDTKFEINKVLFSREEGQQQGEQRLQESVIVEISKEQIRALSKRAKSS-SRKTISSEDKPFNLRSDPIYSNKLKGFETPEKNPQLRDLDFLSIVDMN-EGALLPHFNASKAIVIVINEGDANIELVGLKEQQEQEEQEEQPLE⁵¹⁰.

There were five potential N-glycosylation sites in β -conglycinin, which included the 199 th and 455 th Asn residues of the α subunit (Picariello, Amigo-Benavent, Del Castillo, & Ferranti, 2013) that was located in the C fragment. The C fragment was the most antigenic, and it was 30.13% higher than the A fragment, 63.74% higher than the B fragment, and 118.32% higher than the D fragment. Therefore, the C fragment was selected for further analysis.

3.5. Expression and identification of the C fragment gene and its overlapping fragments

The C fragment was divided into three segments, and each sequence was overlapped by approximately 20 amino acid residues. The

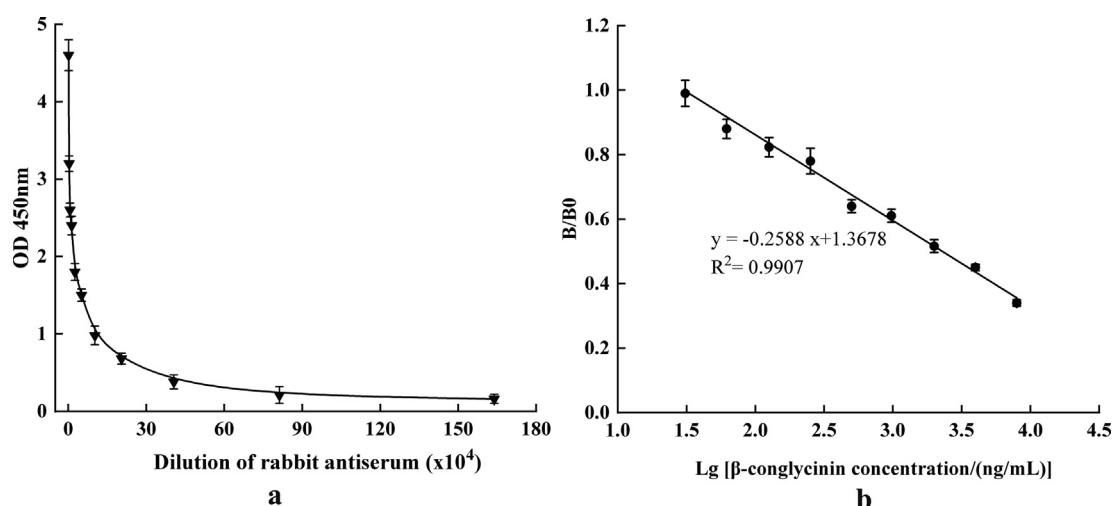


Fig. 1. Analysis of titer and sensitivity of rabbit antiserum against β -conglycinin. (a: the titer of antiserum against β -conglycinin; b: sensitivity of rabbit antiserum against β -conglycinin).



Fig. 2. Identification of site-specific antiserum in the study of destroyed sites. (1: β -conglycinin + a mixture of three processing-treated β -conglycinin-absorbing antiserum; 2: HHP-treated β -conglycinin + HHP treated β -conglycinin-absorbing antiserum; 3: heat-processed β -conglycinin + heat-processed β -conglycinin-absorbing antiserum; 4: glycosylated β -conglycinin + glycosylated- β -conglycinin-absorbing antiserum; 5: BSA + a mixture of three processing-treated β -conglycinin-absorbing antiserum).

Table 1
Primers for Gly m Bd 60 K and its overlapping fragments.

Primer	Sequence	Enzyme introduced
α A up	5'-AGTGAATTCCTGGCATCAGTTTCTGTC-3'	EcoRI
α A down	5'-CTGAAGCTTACCATATTGGTTTTTGAAG-3'	Hind III
α B up	5'-GGAGAATTCGAGTCTCAAAGAGAATTAC-3'	EcoRI
α B down	5'-CTAAAGCTTCTCCTCGAATTTGGTATC-3'	Hind III
α C up	5'-GCGGAATTCATCTCAGATTAATAACA-3'	EcoRI
α C down	5'-CTTAAGCTTTTCCAAAGGTGCTCTTC-3'	Hind III
α D up	5'-TTGGAATTCATTGTGGATATGAACGAG-3'	EcoRI
α D down	5'-CCTAAGCTTGTAAAGGCCCTCAAAT-3'	Hind III

segmentation of the C fragment was as follows: C1: N321-K400 (1023–1259 bp), C2: E380-L470 (1200–1472 bp), and C3: Q450-E510 (1410–1592 bp). Primers were shown in Table 2.

As seen in Fig. 5a, strips consistent with the expected fragment size were observed, and no nonspecific bands were observed. PCR and double enzyme digestion of the recombinant plasmids showed that the C1, C2, and C3 fragments exhibited the bands at 230 bp, 270 bp, and 180 bp, respectively (Fig. 5b), which indicated that the C fragment and its overlapping segments were inserted successfully into the pMD18-T vector. The recombination rate was about 100% (Fig. 5c). Sequencing results showed that the exogenous DNA fragment was correct, and we selected the overlapping segments of the C fragment. After purification, only one brighter DNA band was detected by 1% agarose electrophoresis (Fig. 5d).

3.6. More precise location of the destroyed antigen sites

The most significant destroyed fragments due to HHP, heat treatment, and glycosylation were all in the C2 fragment (Fig. 6). The amino acid sequence of the C2 fragment was ³⁸⁰EGQQQGEQRLQESVIVEISK-EQIRALSKRAKSSSRKTISSEDKPFLNLSRDPIYSNKLKGFEEIPEKNPQLR-DLDIFLSVDMNEGALL⁴⁷⁰. The C2 fragment was the most antigenic, and it was 17.25% higher than the C1 fragment and 30.45% higher than the C3 fragment, so the C2 fragment was selected for further analysis.

3.7. Expression and identification of the C2 fragment gene and its overlapping fragments

The C2 fragment was divided into three segments, and each sequence was overlapped by approximately five amino acid residues. The segmentation of the C fragment was as follows: C2-1: E380-R408 (1200–1286 bp), C2-2: A404-N448 (1272–1406 bp), and C2-3: T444-L470 (1392–1472 bp). Primers were shown in Table 3.

PCR results showed that strips were consistent with the expected fragment size, and no nonspecific bands were observed (Fig. 7a). Because the length of the C2 fragment was small, the C2 fragment and its overlapping fragments were recovered and purified directly by digestion without being linked to the vector. After digestion, the gel was recovered and purified. The PCR products and digested products were subjected to agarose electrophoresis to find that the length of the digested fragment was shorter than those that were amplified by PCR (Fig. 7b), which demonstrated that the digestion was successful. Agarose gel electrophoresis showed that the recombination rate was about 100% (Fig. 7c). After purification, there was only one brighter DNA band in the purified phage lysate (Fig. 7d).

3.8. The precise location of the potentially destroyed antigen sites

After further segmentation and expression of the C2 fragment, the reaction (Fig. 8) showed that the most destroyed fragments of HHP were in the C2-2 fragment; the most destroyed fragments from heat treatment and glycosylation were observed in the C2-1 fragment. The C2-1 and C2-2 fragments were the most antigenic, and they were 13.38% and 45.54% higher than the C3 fragment, respectively. Combining these three processing treatments, C2-1 and C2-2 fragments were the most significantly destroyed antigens, and these fragments also had the strongest antigenicity. The amino acid sequences of the C2-1 and C2-2 fragments were ³⁸⁰EGQQQGEQRLQESVIVEISK-EQIRALSKR⁴⁰⁸ and ⁴⁰⁴ALSKRAKSSSRKTISSEDKPFLNLSRDPIYSNKLKGFEEITPEKN⁴⁴⁸,

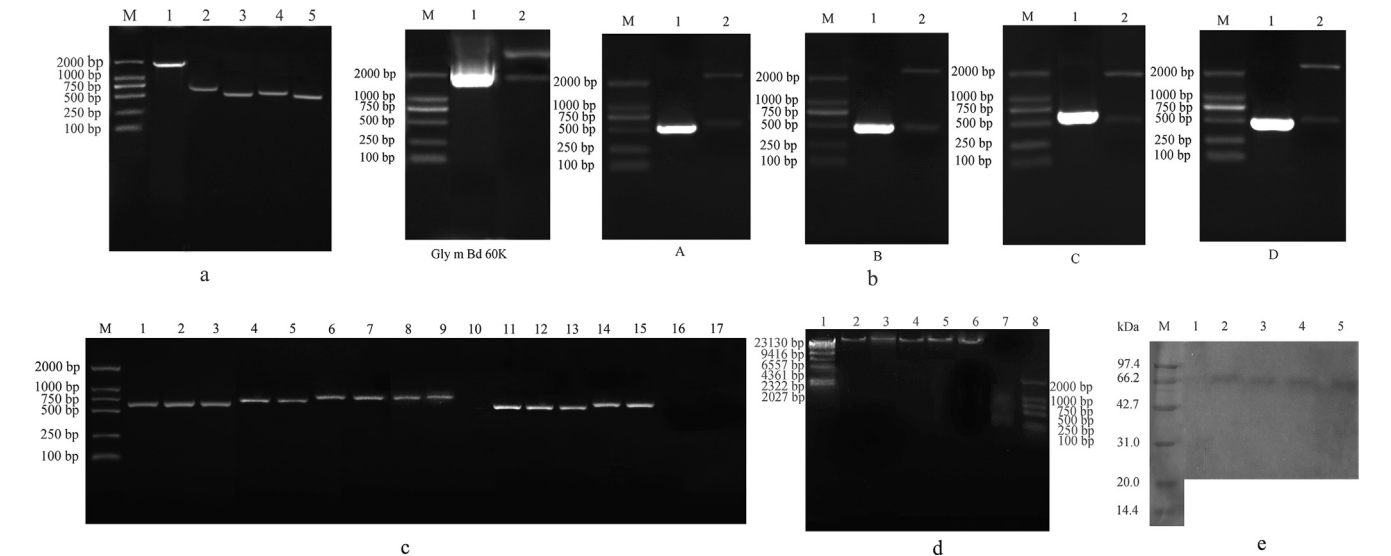


Fig. 3. Phage display and identification of Gly m Bd 60 K and its overlapping fragments. (a): PCR amplification of Gly m Bd 60 K and its overlapping fragments; b: identification of recombinant plasmids; c: identification of recombinant phages by PCR amplification; d: results of phage purification; e: Western-blot of purified phage) (a: M: DNA Marker; 1: Gly m Bd 60 K; 2: A; 3: B; 4: C; 5: D; b: M: DNA Marker; 1: PCR; 2: *EcoRI* + *Hind* III; c: M: DNA Marker; 1–15: recombinant phages; 16–17: negative phages; d: 1: DNA marker *Hind* III digest; 2: Gly m Bd 60 K; 3: A; 4: B; 5: C; 6: D; 7: unpurified phage; 8: DNA marker DL2000; e: M: Marker; 1: negative control; 2: A; 3: B; 4: C; 5: D).

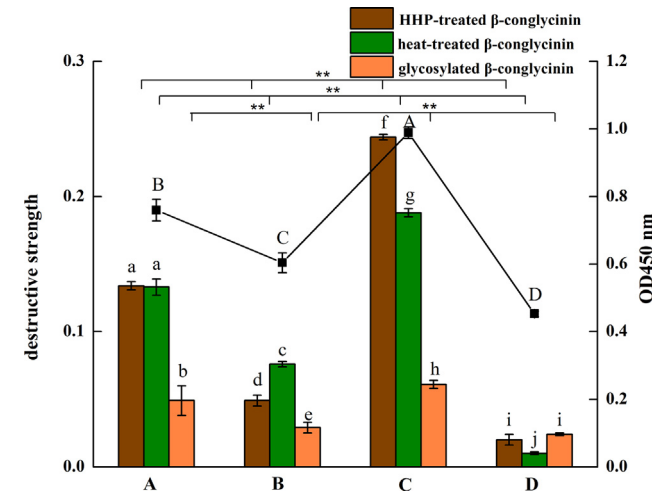


Fig. 4. The reaction of the recombinant phages and epitope-specific antiserum. (The reaction with antiserum against β-conglycinin indicated the antigenicity of recombinant phages, and the subtraction value of OD 450 nm between processing treated antigen-absorbing antiserum and untreated antigen-absorbing antiserum indicated the degree of destruction, and the larger the OD value, the more significant the destruction was.) (All values were mean ± standard deviation of three values, different superscripts in the same fragment indicate the significantly different at the p < 0.05 level; ** indicates p < 0.01, comparison of the same processing but different fragments (in the same color)).

Table 2
Primers for C segment and its overlapping fragments.

Primer	Sequence	Enzyme introduced
αC1 up	5'-GCGGAATTCATCTCAGATTAATAACA-3'	<i>EcoRI</i>
αC1 down	5'-CCTAAGCTTCTCGAGATTCACAAAT-3'	<i>Hind</i> III
αC2 up	5'-ATTGAATTCGAAGGCAGCAGCAAGG-3'	<i>EcoRI</i>
αC2 down	5'-CTGAAGCTTTAGAAGAAGAGCTCCCTC-3'	<i>Hind</i> III
αC3 up	5'-ATGGAATTCAGCTTCGGGACTTGGAT-3'	<i>EcoRI</i>
αC3 down	5'-GTCAAGCTTTTCCAAAGGTTGCTCTTC-3'	<i>Hind</i> III

respectively.

Ogawa et al. (1995) found that the site of the Gly m Bd 60 K protein was located on peptide 232–383, which was located in the B and C fragments in our research. Ye et al. (2006) reported that in the site region of Gly m Bd 60 K in pigs that the amino acid residues 185–231 were strongly sensitized, and they were located on the protein surface of the three-dimensional structure. The initial antigen in pigs was the S185-R231 region (Fu, Jez, Kerley, Allee, & Krishnan, 2007). Subsequently, Taliercio and Kim (2014) screened sera for IgG against β-conglycinins from pigs; they validated the major site using overlapping peptide sequences, and they found another major IgG binding site (Q589-Y621) in Gly m Bd 60 K. Sun, Shan, Yan, Zhang, and Guan (2013) predicted there were 15 peptides of Gly m Bd 60 K, using immunoinformatics tools and assembled by solid-phase synthesis. The dot-blot inhibition assay identified 11 sites, which reacted with more than half the samples tested of human sera. Among them, peptide 3 had a relatively higher capacity for binding to IgE compared with other peptides, and its amino acid sequence was ⁵⁵HEQREEQEWPRKEEKRGEGKSEEEDE⁸⁰. Compared to their research, we found that the C2-2 fragment was located on peptides 11 and 12, and these two peptides reacted more strongly to antiserum. However, the C2-1 fragment was not included in the predicted 15 peptides, probably because we used phage display technology to express Gly m Bd 60 K. In the future, we will use synthetic peptide technology to study the C2-1 and C2-2 allergen regions to determine whether the selected site is conformational or linear and to identify the key amino acid residues.

3.9. Allergenicity analysis

The reaction between recombinant phage-expressed proteins and serum of soybean allergy patients showed that β-conglycinin was highly reactive with serum from allergic patients. C2-1 and C2-2 fragments of recombinant phage-expressed proteins also reacted with allergic patients' serum and, thus, have allergenicity (Table 4).

3.10. Secondary structural

The α-helix content of the C2-1 fragment was 62.07%, the β-sheet content was 10.34%, the random coil content was 27.59%, and the

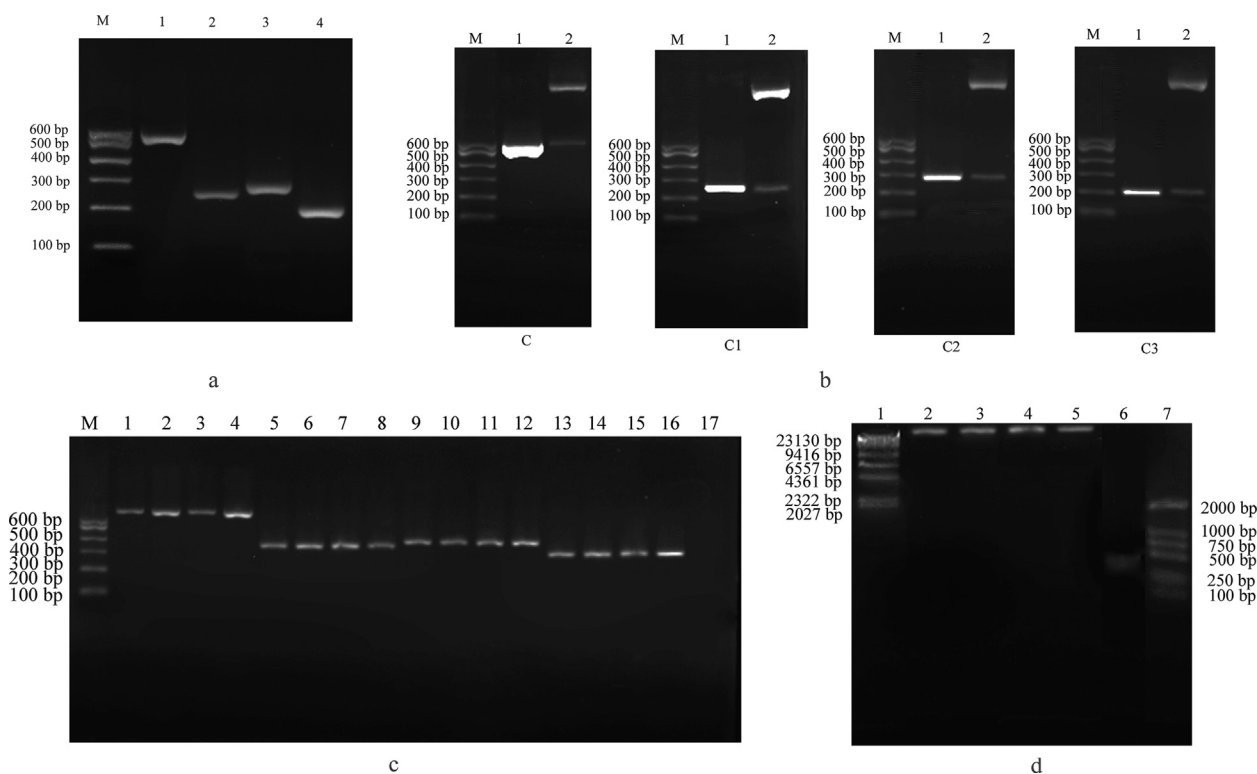


Fig. 5. Phage display and identification of the C fragment and its overlapping fragments. (a: PCR amplification of the C fragment and its segments; b: identification of recombinant plasmids; c: identification of recombinant phages by PCR amplification; d: results of phage purification) (a: M: DNA Marker; 1: C; 2: C1; 3: C2; 4: C3; b: M: DNA Marker; 1: PCR; 2: *EcoRI* + *Hind* III; c: M: DNA Marker; 1–16: recombinant phages; 17: negative phages; d: 1: DNA marker *Hind* III digest; 2: C; 3: C1; 4: C2; 5: C3; 6: unpurified phage; 7: DNA marker DL2000).

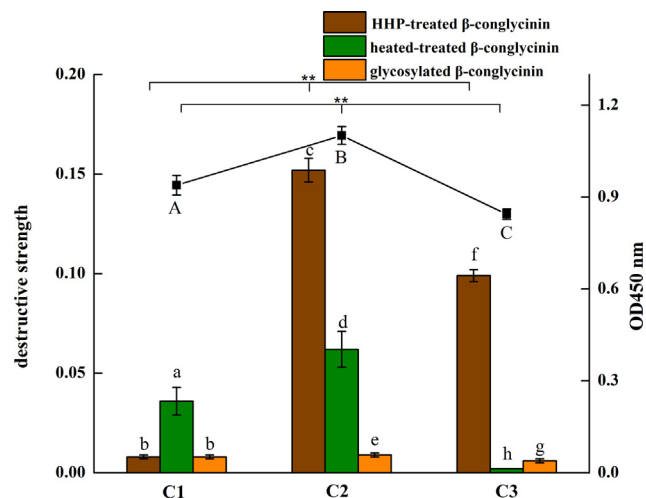


Fig. 6. The reaction of the recombinant phages and destroyed epitope-specific antiserum. (All values were mean $\pm$ standard deviation of three values, different superscripts in the same fragment indicate the significantly different at the $p < 0.05$ level; ** indicates $p < 0.01$, comparison of the same processing but different fragments (in the same color)).

main structure of the former was random coil. The β -sheet content of C2-2 fragment was 17.78%, and the random coil was 82.22% (Fig. 9).

4. Conclusions

The phage display technique has been used widely to locate antigenic sites. In our study, overlapping fragments of Gly m Bd 60 K were displayed by the T7 phage display technique. Indirect ELISA was used to locate the destroyed antigenic sites and, after three rounds of

Table 3

Primers for C2 segment and its overlapping fragments.

Primer	Sequence	Enzyme introduced
α C2-1 up	5'-GCGGAATTCAATCTCAGATTAATAACA-3'	<i>EcoRI</i>
α C2-1 down	5'-CCTAAGCTTCTTCGAGATTTCCACAAT-3'	<i>Hind</i> III
α C2-2 up	5'-ATTGAATTCGAAGGGCAGCAGCAAGGG-3'	<i>EcoRI</i>
α C2-2 down	5'-CTGAAGCTTTAGAAGAAGAGCTCCCTC-3'	<i>Hind</i> III
α C2-3 up	5'-ATGGAATTCAGCTTCGGGACTTGGAT-3'	<i>EcoRI</i>
α C2-3 down	5'-GTCAAGCTTTTCCAAAGGTTGCTCTTC-3'	<i>Hind</i> III

expression and location, the C2-1 and C2-2 fragments were identified as destroyed antigenic sites of Gly m Bd 60 K by HHP, thermal, and glycosylation technologies. These two fragments were also allergenicity sites. Therefore, the phage display technique not only located antigen sites of soybean, but it also located destroyed antigen sites, which can be used to provide a new method for allergen research. Research on the identification of process-destroyed antigenic sites of Gly m Bd 60 K is relevant for the identification of the molecular mechanism that causes food allergies and for the development of application products for rapid detection of the desensitization effect of processed foods.

CRedit authorship contribution statement

Jun Xi: Conceptualization, Methodology, Funding acquisition, Writing - review & editing. **Mengxue He:** Data curation, Formal analysis, Software, Investigation, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

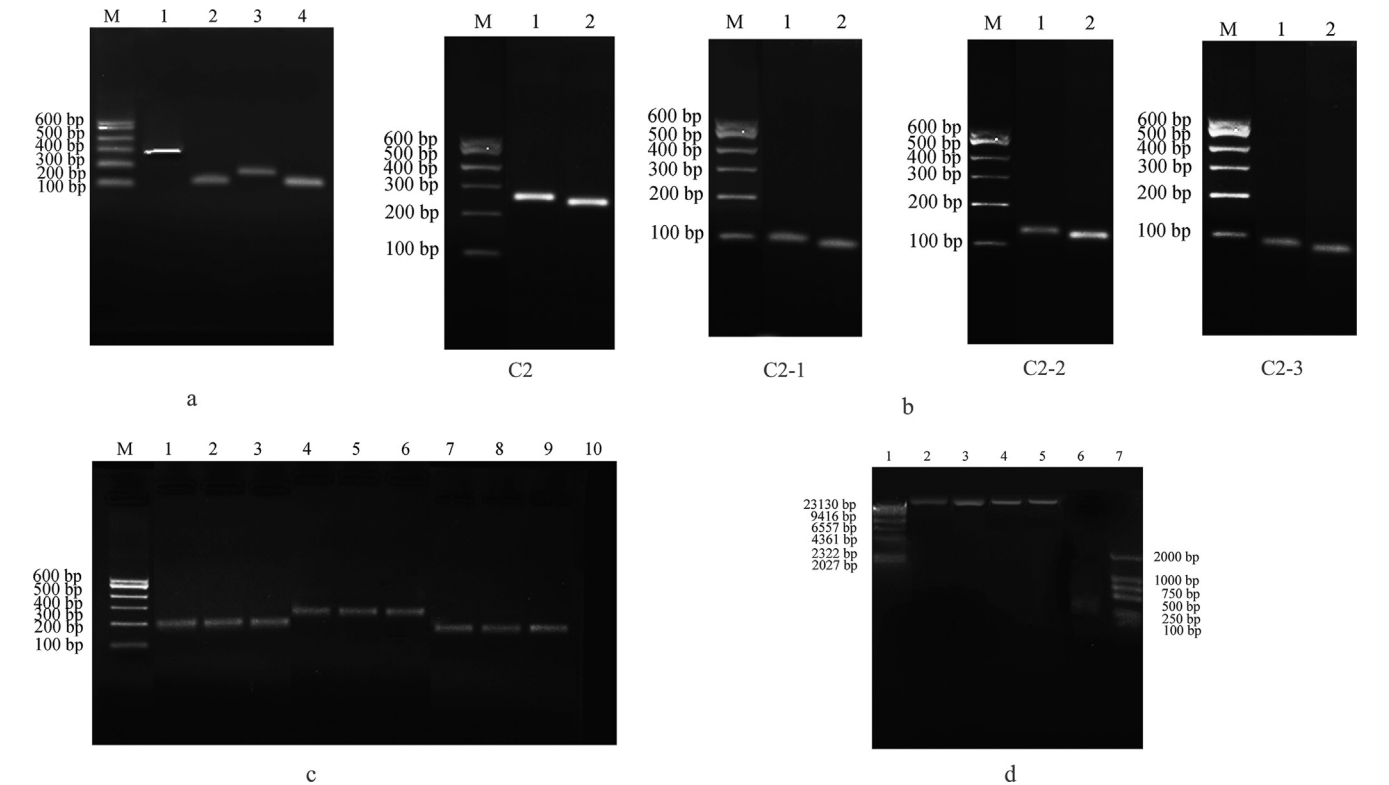


Fig. 7. Phage display and identification of the C2 fragment and its overlapping fragments. (a: PCR amplification of the C2 fragment and its segments; b: identification of recombinant plasmids; c: identification of recombinant phages by PCR amplification; d: results of phage purification;) (a: M: DNA Marker; 1: C2; 2: C2-1; 3: C2-2; 4: C2-3; b: M: DNA Marker; 1: PCR; 2: *EcoRI* + *Hind* III; c: M: DNA Marker; 1–9: recombinant phages; 10: negative phages; d: 1: DNA marker *Hind* III digest; 2: C; 3: C2-1; 4: C2-2; 5: C2-3; 6: unpurified phage; 7: DNA marker DL2000).

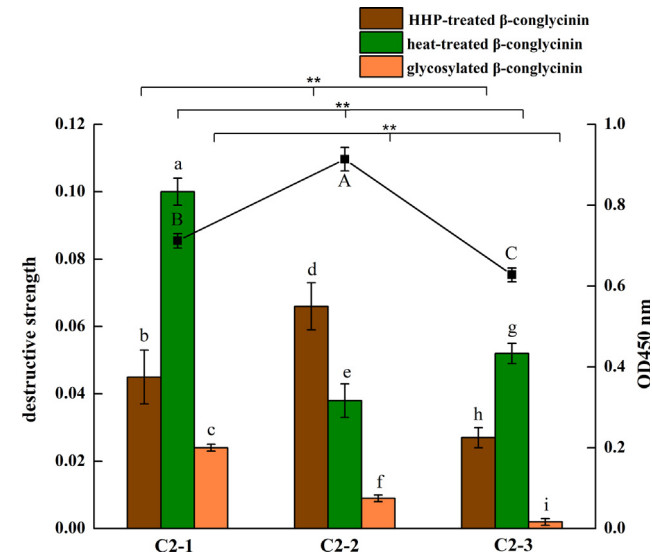


Fig. 8. The reaction of the recombinant phages and destroyed site-specific antiserum. (All values were mean $\pm$ standard deviation of three values, different superscripts in the same fragment indicate the significantly different at the $p < 0.05$ level; ** indicates $p < 0.01$, comparison of the same processing but different fragments (in the same color)).

influence the work reported in this paper.

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Table 4
Reaction of allergic patients' serum with phage-expressed proteins.

Coating proteins	C2-1	C2-2	β-conglycinin	BSA	Negative phage
Reaction intensity	+	+	++	–	–

The number of “+” represents intensity of the reaction of IgE-binding, and “–” represents negative binding.

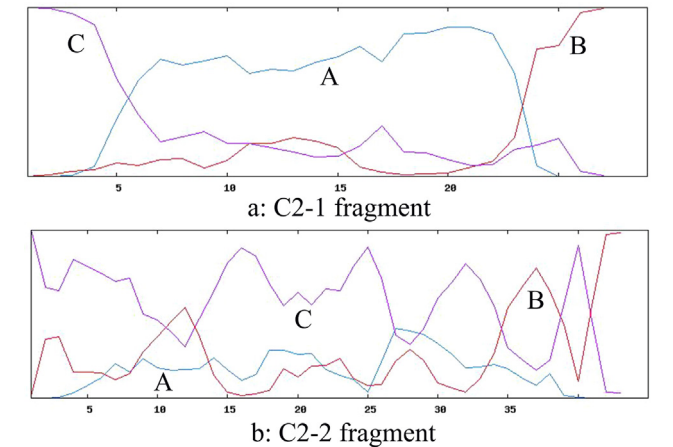


Fig. 9. Secondary structure analysis of destroyed allergen sites (a: C2-1 fragment; b: C2-2 fragment) (A: α-helix; B: the β-sheet content; C: random coil).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2020.109199>.

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