

Identification of the linear immunodominant epitopes in the β subunit of β -conglycinin and preparation of epitope antibodies

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ABSTRACT

β -conglycinin is one of the major allergens in soybean protein. The purpose of this study was to predict and to identify the major linear epitopes of the β subunit of β -conglycinin. Potential linear epitopes were predicted and confirmed by three immunoinformatics tools combined with the Immune Epitope Database (IEDB). Ten potential epitope peptides were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis and were validated by the indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) using sera from soybean allergic patients. Polyclonal antibodies, which were prepared by immunizing rabbits with synthesized peptides, were used to confirm their binding ability with β -conglycinin through western blot and dot blot assays. The results showed that 10 peptides were screened as the main epitopes for the β subunit of β -conglycinin. All 10 peptides (P1-P10) presented IgG binding activity, and P2 and P6 were also validated as IgE binding peptides. Moreover, the results of dot blot showed that P5 and P8 might be located inside the protein molecule. Western blot indicated that most of polyclonal antibodies were bound effectively to the β subunit of β -conglycinin. In addition, few polyclonal antibodies exhibited an immune cross-reaction with the α and α' subunits.

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1. Introduction

Soybean seeds are important sources of protein and oil, and soybean protein is used widely as an ingredient in foods due to its rich functional properties [1,2]. However, soybean protein is one of eight major food allergens. Approximately 25% of food allergies are caused by soybeans, which generate serious physical health problems and limit the further use of soy protein in food [3,4]. In β -conglycinin, which is the main storage protein, all three subunits (α subunit of 70 kDa, α' subunit of 72 kDa, and β subunit of 52 kDa) could bind to the IgE antibody in patient serum [5–7]. The α subunit and β subunit of β -conglycinin are considered major allergens. The α subunit of β -conglycinin, which was recognized from serum of 25% of soy allergy patients, is firstly recognized as a major allergenic protein in soybean [8]. The β subunit has extensive amino acid sequence homology with α subunit, which consists of a core and a loop domain [9]. Recent studies have found that 19 IgE and 59 IgG binding sites were identified on the β subunit, which indicates that the sensitization of β subunit may be higher [10,11]. Therefore, it is very critical to find out the main sensitizing factors in β subunit of β -conglycinin.

B cell epitopes are determinant of food allergens, which consist of amino acid sequences. Linear epitopes are composed of sequential amino acid sequences, which can be recognized specifically by

antibodies [12]. Identification of linear epitopes is helpful to further understand the occurrence of food allergies and the changes in epitopes that occur during processing [13,14]. With the rapid development of bioinformatics in recent years, immunoinformatics has proved to be an effective method to study the information about antigen epitopes [4,14–17]. The Immune Epitope Database (IEDB) contains experimentally verified epitopes, and it also displays detailed information of all the collected epitopes, such as references, sources, and experimental results [18]. Immunoinformatics tools combined with epitope databases may be a valid way to predict dominant epitopes quickly and accurately.

In some studies, synthetic polypeptides have been used to validate the predicted epitopes by binding them to allergenic serum. Zheng et al. [14] predicted and synthesized 10 polypeptides; eight of these epitopes were identified with serum of patients from shrimp allergies. In addition, Fang et al. [19] used immunoinformatics tools to predict the antigenic epitopes of tropomyosin from oysters and identified the immunodominant epitopes with serum from allergic patients by an indirect enzyme-linked immunosorbent assay (i-ELISA); alanine was used to replace the amino acids at each position to seek out the key amino acids in epitopes in their research. Furthermore, 15 candidate epitopes in the α subunit of β -conglycinin were predicted using three immunoinformatics tools, and then they were assembled by solid-phase synthesis. Eleven epitopes were identified by dot blot using sera from soybean allergic patients [4]. At present, there are few reports on the prediction of the linear epitopes in the β subunit of β -conglycinin. Moreover, epitope antibodies have also been less reported, and the

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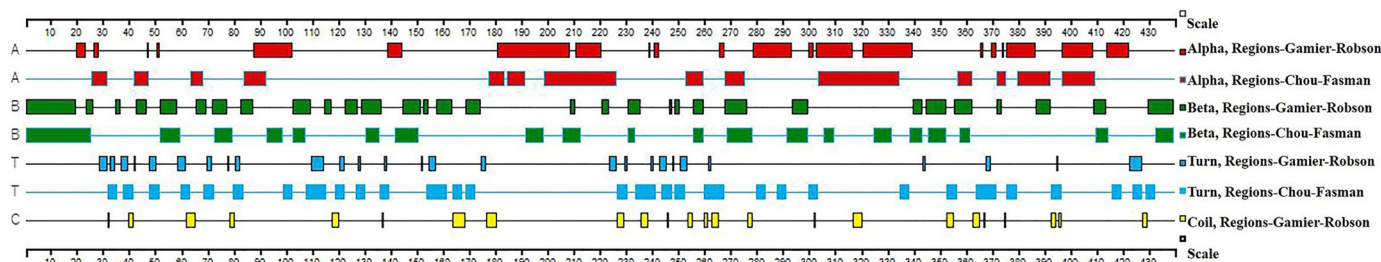


Fig. 1. The secondary structure of β subunit of β -conglycinin by DNASTar Protean system analysis.

preparation of epitope antibodies is contributed to the detection of allergenic proteins [20].

However, subunits in β -conglycinin showed high sequence homology, which could lead to cross-reactions in different soy proteins [21,22]. The presence of the same epitope in allergenic proteins from different sources is a key factor in causing cross-reactions [23,24]. So, the analysis of sequences of sensitized proteins helps to clarify the mechanism of immune cross-reaction between allergens [25].

In this study, our aim was to predict and screen potential epitopes of the β subunit of β -conglycinin by using three immunoinformatics tools combined the results of IEDB. The potential epitope peptides were synthesized and evaluated for their IgG and IgE binding abilities using ic-ELISA. In addition, synthesized peptides were coupled with keyhole limpet hemocyanin (KLH), and then they were used to immunize rabbits to produce polyclonal antibodies. The binding ability of polyclonal antibodies was confirmed by western blot and dot blot assays. Determination of dominant antigen epitopes and preparation of epitope antibodies will be important to explore the sensitization mechanism of β -conglycinin and to detect the changes in dominant epitopes during processing.

2. Materials and methods

2.1. Materials

Defatted soybean flour was obtained from Henan Kunhua Biological Technology Group Co. Ltd. (Anyang, China). HRP-labeled goat anti-human IgE (A9667), HRP-labeled goat anti-rabbit IgG (A6154), and β -conglycinin (C5868) were obtained from Sigma Chemical Co. Ltd. (St.

Louis, MO, USA). The 3,3',5,5'-tetramethylbenzidine (TMB) single-component substrate solution (PR1200) and 3-amino-9-ethylcarbazole (AEC) Peroxidase Substrate Kit (20 $\times$, A2010) were obtained from Beijing Solarbio Science and Technology Co. Ltd. (Beijing, China). The anti- β -conglycinin serum from rabbits was generated by our laboratory. All chemicals were analytical grade.

2.2. Human sera

Soy allergy sera and negative sera were obtained from Henan Children's Hospital (Zhengzhou, China). Negative sera were obtained from non-allergic subjects. Soy allergy sera were obtained from 38 child patients who had a history of immediate hypersensitivity reactions after the ingestion of soybeans. The binding ability of soy allergy sera to β -conglycinin was determined by i-ELISA (the corresponding results were shown in Fig. S1). Positive sera were collected and mixed into a positive serum pool and then stored at -80°C until used.

2.3. The extraction of β -conglycinin

β -conglycinin was prepared and purified from the defatted soy flour according to the method of Liu et al. [26], with some modifications. The low-temperature defatted soy flour was mixed with 30 mM Tris-HCl (pH 8.5) solution at the ratio 1:15 (m/v) and stirred at 45°C for 1 h. The protein solution was then centrifuged at 4°C (10,000 r/min, 20 min). NaHSO_3 (0.01 M) and CaCl_2 (5 mM) were added to the supernatant, adjusted to pH 6.4, and then centrifuged. The supernatant was adjusted to pH 5.5 with HCl (2 M) and centrifuged. Then, the supernatant was diluted with twice the volume of ice water and stirred for

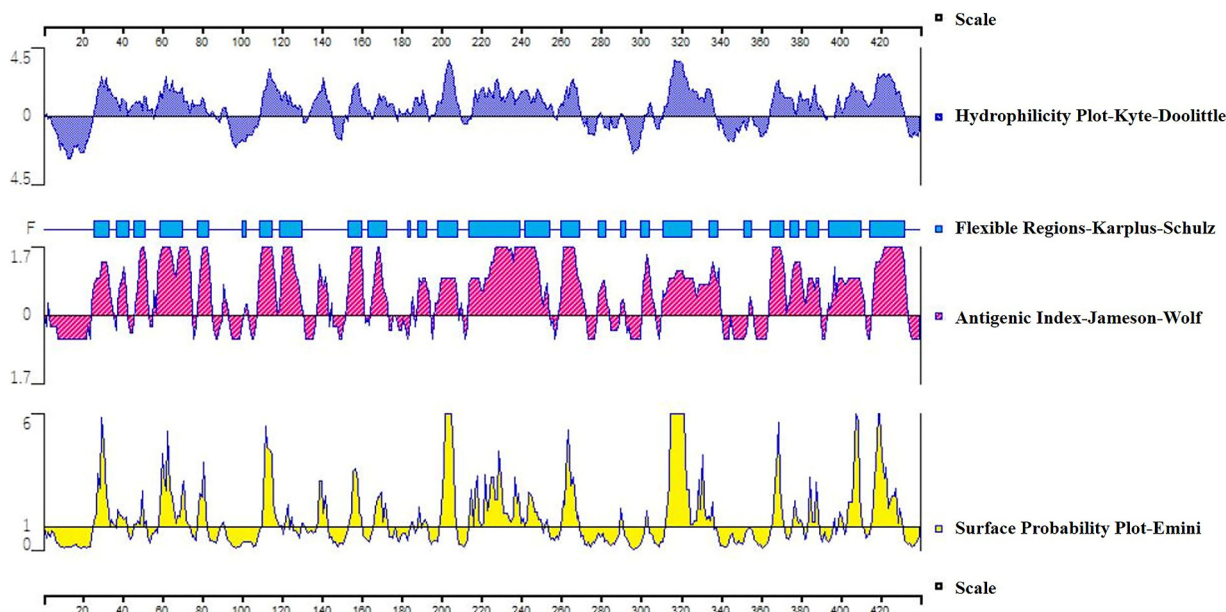


Fig. 2. Analysis results of the hydrophilicity, flexible regions, antigenic index and surface probability of β subunit of β -conglycinin by DNASTar.

Table 1

The epitope sites in β subunit of β -conglycinin predicted by three immunoinformatics tools.

Tools	Location of the predicted linear epitopes
DNASStar	38–41, 60–63, 110–114, 155–157, 227–230, 236–238, 244–246,
Protean	260–265, 367–369, 424–437
Bepi Pred 1.0	27–35, 61–67, 110–129, 138–140, 152–158, 167–171, 199–206,
	224–243, 247–250, 260–267, 313–327, 333–335, 365–368,
	377–379, 392–399, 412–432
NPS@	1–3, 8–11, 24–34, 37–51, 53–55, 60–64, 70–72, 79–82, 88–93,
	99–104, 109–122, 127–130, 136–142, 151–159, 162–169,
	174–188, 198–208, 225–254, 259–272, 278–292, 300–305,
	310–314, 317–324, 334–338, 343–347, 352–356, 364–370,
	374–378, 392–397, 407–410, 414–439

1 h, adjusted to pH 4.8, and centrifuged. The precipitate was collected and dissolved in 0.03 M Tris-HCl buffer and the pH was adjusted to 7.0. The protein solution was on dialysis for 2 d, and water was changed every 3 h. The extracted β -conglycinin was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [27]. The purity of proteins was analyzed by Gel Analyzer Software (Version 4.0). The extracted β -conglycinin was freeze-dried and stored at -20°C .

2.4. Amino acid sequence

The accession number of the β subunit of β -conglycinin was obtained from Allergen Online (<http://www.allergenonline.org/>), and it was P25974.1. The amino acid sequence of the β subunit of β -conglycinin was obtained from the National Center for Biotechnology Information (NCBI) with the accession number. The primary structure of the β subunit contained 439 amino acids.

2.5. Epitope prediction

To enhance the veracity of epitope prediction of the β subunit of β -conglycinin, the complete amino acid sequence was analyzed using three immunoinformatics-based computational approaches. These included the DNASStar Protean system, the Network Protein Sequence Server (NPS@), and the BepiPred 1.0 Server.

In the DNASStar Protean system, hydrophilicity, flexibility, accessibility, and antigenicity were chosen as the main parameters for epitope prediction of the β subunit of β -conglycinin [28–31]. Based on the results of the prediction, the peptide regions with good hydrophilicity (a value > 0), flexibility, high surface accessibility (a value > 1), and a high antigenic index (a value > 0) were chosen as candidate epitopes. In addition, the secondary structure of the β subunit of β -conglycinin was analyzed according to the methods of Chou and Fasman [32] and Garnier et al. [33]. The regions with the β -turn and random coil were chosen as candidate epitopes [4]. The overlap regions of these candidate epitopes were chosen as the prediction regions in DNASStar for further investigation.

The NPS@ Server with DSC, MLRC, PHD, and Predator methods in Consensus Secondary Structure Prediction (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html#Sopma) and the BepiPred 1.0 server (<http://www.cbs.dtu.dk/services/BepiPred-1.0/>) were also used to predict the secondary structure of the β subunit of β -conglycinin. Based on the results of these two servers, the regions with the β -turn and random coil were chosen as candidate epitopes.

The ultimate results of epitope prediction were determined by combining the results of the three immunoinformatics tools using a published method [34]. Furthermore, the verified linear epitopes of the β subunit of β -conglycinin were included in IEDB (http://www.iedb.org/home_v3.php), and these epitopes were searched and collected in our previous study [13]. The final potential epitope regions were determined by the combined results from immunoinformatics tools and the IEDB database.

2.6. Peptide synthesis

Peptides that corresponded to the regions that were predicted as potential epitopes were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis by Beijing Protein Innovation Co., Ltd. (Beijing, China). The purity of the peptides was $> 85\%$ as assessed by high performance liquid chromatography (HPLC). The molecular mass of the synthesized peptides was confirmed by Electrospray Ionization-Mass Spectrometry (ESI-MS).

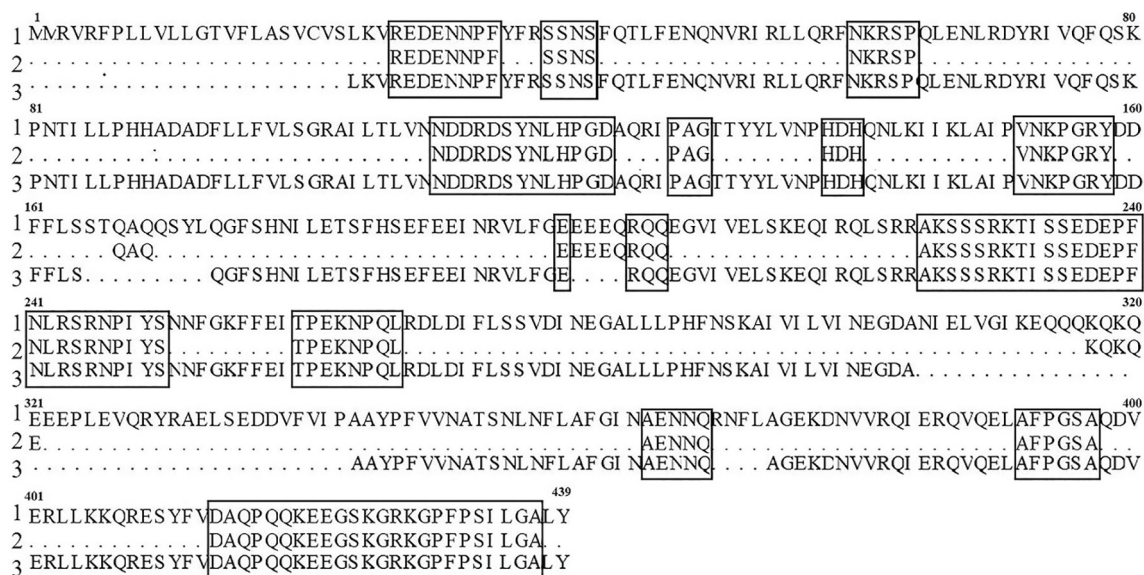


Fig. 3. The combined results of candidate epitopes and tested linear epitopes. Line 1: the amino acid sequence of β subunit of β -conglycinin; Line 2: the location regions of candidate epitopes; Line 3: the location regions of tested linear epitopes. The same amino acid sequence between three lines is shown in the box.

2.7. Preparation of epitope polyclonal antibodies

The peptides were first coupled with KLH, and then they were used as antigens for immunity to rabbits. These experiments were commissioned by Beijing Protein Innovation Co., Ltd. (Beijing, China). Peptides coupled with BSA were used to determine the titer of positive sera for all prepared polyclonal antibodies using the ELISA method. All epitope polyclonal antibodies were stored at -20°C until used.

2.8. The indirect competitive enzyme-linked immunosorbent assay

The IgG binding ability of peptides was analyzed by an ic-ELISA method [35]. Microtiter plates with 96 wells (Costar 3590 High Binding, Corning, NY, USA) were coated with $100\ \mu\text{L}$ /well of β -conglycinin antigen ($0.1\ \mu\text{g}/\text{mL}$) in $50\ \text{mM}$ carbonate buffer and incubated at 4°C overnight. Peptides coupled with BSA ($2\ \text{mg}/\text{mL}$, $10\ \mu\text{L}$) were mixed with an equivalent volume of rabbit anti- β -conglycinin serum ($1:3200$ dilution) diluted in PBST ($0.01\ \text{M}$ pH 7.4 phosphate-buffered saline with 0.1% Tween-20) at 4°C overnight. Meanwhile, blank control (serum only) and negative control ($10\ \mu\text{L}$ $2\ \text{mg}/\text{mL}$ BSA and serum) were performed. The following process followed the method we reported earlier [36]. An uninhabited serum sample (no peptides) was used as a control. The absorbance was determined at dual wavelengths of $450\ \text{nm}$ and $630\ \text{nm}$ by a microplate reader (Thermo Fisher Scientific Instrument Co. Ltd., New York, USA). The IgG binding ability of peptides was expressed by the inhibition percentages (%), which was calculated as follows: Inhibition rate (%) = $(B_0 - B)/B_0 \times 100$, where B is the OD of samples, and B_0 is the absorbance obtained with the uninhibited serum sample control. A low percentage of inhibition reflected a low IgG binding ability of peptides.

The IgE binding ability of peptides was analyzed by ic-ELISA [15,36]. Microtiter plates were coated with $100\ \mu\text{L}$ /well of β -conglycinin antigen ($10\ \mu\text{g}/\text{mL}$) in $50\ \text{mM}$ carbonate buffer and incubated at 4°C overnight. Peptides coupled with BSA ($2\ \text{mg}/\text{mL}$, $10\ \mu\text{L}$) were mixed with soy allergy human serum ($1:50$ dilution) diluted in PBST at 4°C overnight. Meanwhile, blank control (serum only) and negative control ($10\ \mu\text{L}$ $2\ \text{mg}/\text{mL}$ BSA and serum) were performed. The following procedure was the same as described above.

2.9. Dot blot

The binding ability of polyclonal antibodies with β -conglycinin was performed by dot blot [4]. The β -conglycinin samples, which were dissolved in carbonate buffer ($50\ \text{mM}$, pH 9.6), were spotted on the nitrocellulose membranes ($0.45\ \mu\text{m}$ pore size) and left to dry in a fume cupboard at room temperature. Each membrane had two dots, and each dot contained $5\ \mu\text{g}$ β -conglycinin. The spotted membranes were blocked for 2 h in PBST that contained 5% non-fat dry milk (NFD) at 37°C . After three washes with PBST (10 min each time), the membranes were incubated with polyclonal antibodies (diluted $1:1000$ with PBST) at 37°C for 1 h. The membranes were washed four times with PBST and incubated in PBST that contained 5% NFD with goat anti-rabbit IgG antibody ($1:5000$ dilution) at 37°C for 1 h. After five washes with PBST, the membranes were incubated with AEC horseradish peroxidase chromogenic substrate for 10 min. The reaction was stopped and washed in double-distilled water.

2.10. Western blot

The binding ability of polyclonal antibodies with different subunits of β -conglycinin was determined by western blot [37]. The subunits of β -conglycinin were first separated with SDS-PAGE [27] and transferred onto a pore-sized, polyvinylidene fluoride (PVDF) membrane. After washing three times (10 min each time) with PBST, the membrane was blocked with blocking buffer (PBST that contained 5% NFD) for 1 h at room temperature. Subsequently, the membrane was washed

Table 2

The ten dominant epitope sites by the combined immunoinformatics methods.

Number	Location	Sequence	Length
Peptide 1	27–41	REDENNPFYFRSSNS	15
Peptide 2	59–73	FNKRSPQLENLRDYL	15
Peptide 3	110–129	NDDRDSYNLHPGDAQIPAG	20
Peptide 4	150–164	IPVKNKPGRYDDFFLS	15
Peptide 5	197–208	FGEEEQRQKEG	12
Peptide 6	225–250	AKSSSRKTISSEDEPNLRSRNPIYS	26
Peptide 7	259–274	ITPEKNPQLRDLDFL	16
Peptide 8	355–369	NLNFLAFGINAENNQ	15
Peptide 9	389–404	QELAFPGSAQDVERLL	16
Peptide 10	414–437	DAQPQQKEEGSKGRKGFPSILGA	24

three times. Then, the membrane was incubated with a polyclonal rabbit antibody ($1:1000$ dilution) at 4°C overnight. After washing three times with PBST, the membrane was incubated in PBST that contained 5% NFD with HRP-labeled goat anti-rabbit IgG antibody ($1:20,000$ dilution) for 2 h at room temperature. After washing again as described above, the membrane was incubated with AEC horseradish peroxidase chromogenic substrate for 15 min. The reaction was stopped and washed in double-distilled water.

2.11. Sequence alignment

To analyze the sequence similarity between different peptides in α , α' , and β subunits of β -conglycinin, the sequence of peptides was searched against the NCBI database using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [38].

2.12. Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS Statistical Software, Inc., Chicago, IL, USA) and expressed as mean values $\pm$ standard deviation. Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Three determinations for

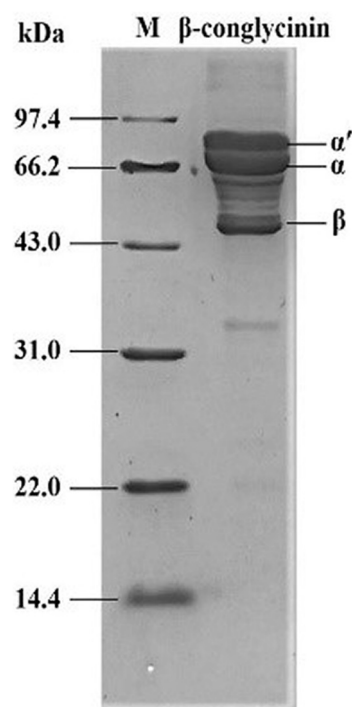


Fig. 4. SDS-PAGE of β -conglycinin. M: Molecular weight marker. The α , α' and β represent the α , α' and β subunit of β -conglycinin, respectively.

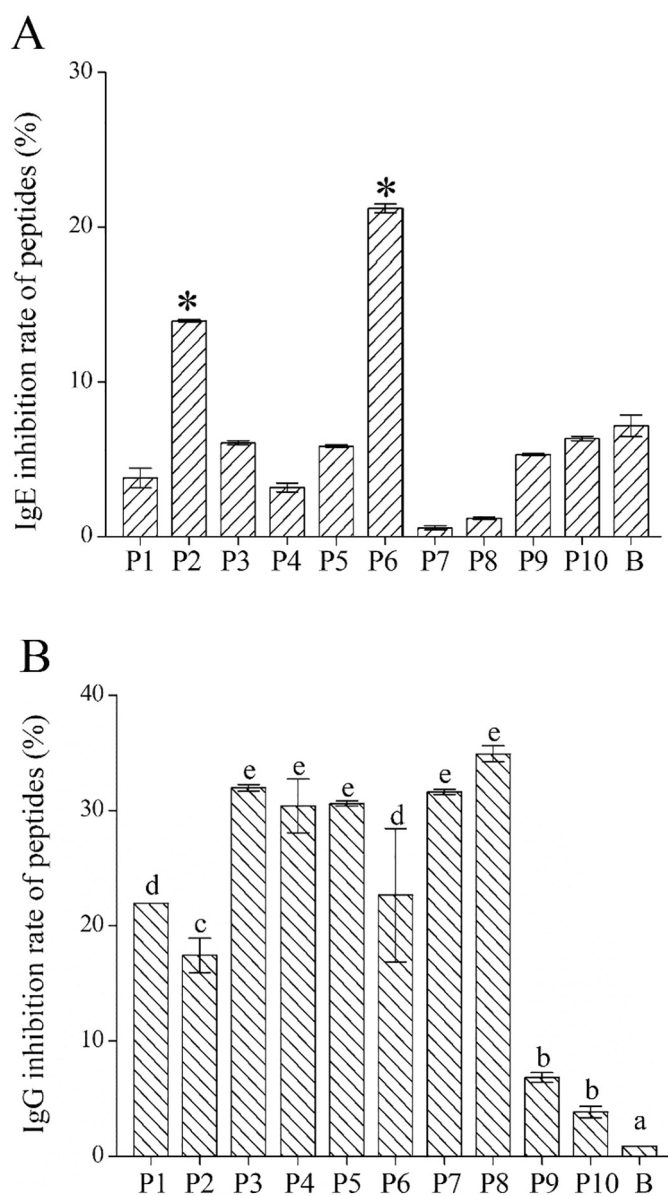


Fig. 5. (A) The IgE binding ability of the peptides. (B) The IgG binding ability of the peptides. P1 to P10: peptide 1 to peptide 10; B: BSA. * $P < 0.05$ shows that peptides have significant binding ability compared with BSA. The different lowercase letters indicate significant difference in values between the IgG binding ability of different peptides ($P < 0.05$).

each treatment were performed, and the significance level was set at $P < 0.05$.

3. Results and discussion

3.1. Prediction and selection of the linear epitopes

The DNASTar Protean system provided a prediction of the secondary structure, hydrophilicity, flexible regions, antigenic index, and surface probability for the β subunit of β -conglycinin. The predicted results of secondary structure were shown in Fig. 1. The turn regions and coil regions were exposed to the outside and bound easily with the antibody, so the sequences in these regions indicated the high possibility of being epitopes [4]. The analysis results of the hydrophilicity, flexible regions, antigenic index and surface probability of β subunit of β -conglycinin by DNASTar were shown in Fig. 2. The high hydrophilic regions (>0), high antigenic index (>0), and high surface probability (>1) were important features for predicting antigenic epitopes, and the flexible regions also indicated strong evidence for identifying epitopes [14]. Based on the sequence properties above, their overlapping regions were selected as candidate epitopes that were predicted by DNASTar.

The NPS@ Server was also used to predict the secondary structure. Furthermore, the BepiPred 1.0 server was applied to provide straightforward and integrative results of the candidate epitopes. The predicted results of the above three tools were shown in Table 1. Based on the method of Yang et al. [34], the final candidate epitopes of the β subunit of β -conglycinin were selected. The tested regions of linear epitopes for β -conglycinin were searched and collected with IEDB in our previous study [13]. The combined results of candidate epitopes and tested linear epitopes were shown in Fig. 3, and the final potential linear epitopes were selected based on the same amino acid sequences (Table 2). In addition, all the regions of linear candidate epitopes were contained in the regions of tested linear epitopes [13], which confirmed our previous predictions. An effective method for determining potential linear epitopes is to combine immunoinformatics tools with epitope databases. These selected peptides were named Peptide 1 (P1) to Peptide 10 (P10), and they will be synthesized further according to their amino acid sequences.

3.2. Validation of the linear epitopes by ic-ELISA

The protein content of extracted β -conglycinin was 85.2% using the kjeldahl method, and the purity of the protein was 93.3% (Fig. 4). To verify which were key linear epitopes among the selected 10 peptides for the β subunit of β -conglycinin, these peptides were synthesized and their IgE/IgG binding ability was evaluated by ic-ELISA. Compared with the BSA control in Fig. 5A, P2 and P6 inhibited the IgE binding ability between β -conglycinin and positive sera significantly, which

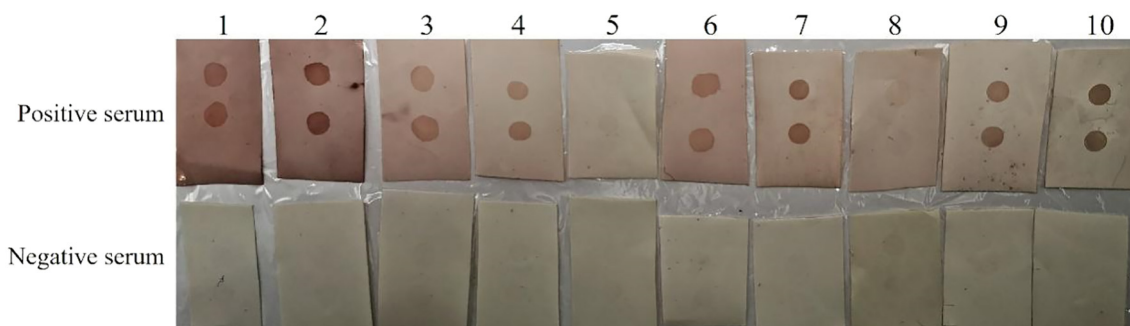


Fig. 6. The binding abilities of epitope polyclonal antibodies with β -conglycinin by dot blot analysis. 1–10: polyclonal antibodies PA-1 to PA-10; Positive serum: rabbit serum obtained by immunizing with 10 peptides; Negative serum: normal rabbit serum.

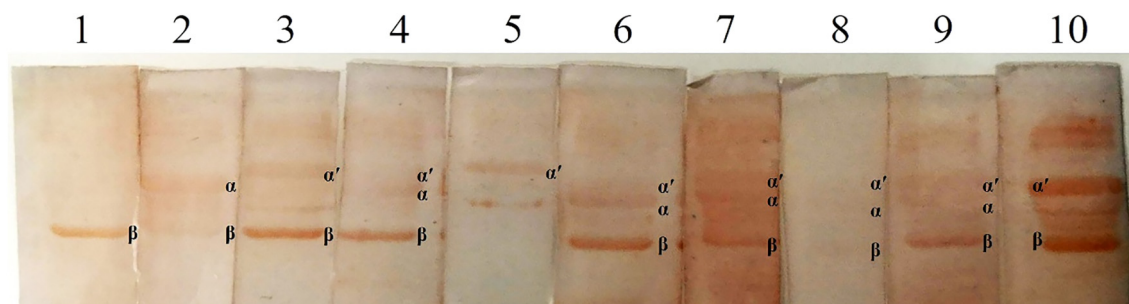


Fig. 7. The binding abilities of epitope polyclonal antibodies with β -conglycinin by western blot analysis. 1–10: PA-1 to PA-10.

Table 3

The amino acid sequences of peptides were aligned with α and α' subunits by BLAST programs.

Number	Blast result of peptides with α subunit	Blast Score	Blast result of peptides with α' subunit	Blast Score
Peptide 1	4 ENNPFYFRS 12 E+ PF RS 402 EDKPFNLRS 410	17.2 bits	4 ENNPFYFRS 12 E+ PF RS 418 EDKPFNLRS 426 11 RSSNS 15 RS NS 40 RSCNS 44	17.2 bits 13.8 bits
Peptide 2	1 FNKRSPQLENLRDYR 15 FN RSPQL+NLRDYR 220 FNQRSPQLNLRDYR 234 9 ENLR 12 ENLR 302 ENLR 305	46.0 bits 16.3 bits	1 FNKRSPQLENLRDYR 15 FNKR QL+NLRDYR 236 FNKRSPQLENLRDYR 250 9 ENLR 12 ENLR 318 ENLR 321	45.6 bits 16.3 bits
Peptide 3	1 NDDRDSYNLHPGDAQRIAG 20 NDDRDSY L+ GDA R+P G 271 NDDRDSYRLQSGDALRVPAG 290 1 NDDRDSY 7 N +RDSY 43 NSERDSY 49 17 IPAG 20 IPAG 508 IPAG 511	43.5 bits 19.3 bits 15.1 bits	1 NDDRDSYNLHPGDAQRIAG 20 NDDRDSYNL+ GDA R+PAG 287 NDDRDSYNLQSGDALRVPAG 306 17 IPAG 20 IPAG 524 IPAG 527	52.0 bits 15.1 bits
Peptide 4	1 IPVKNKPGRYDDFFLS 15 IPVKNKGR++ FFLS 311 IPVKNKPGRFESFFLS 325	41.8 bits	1 IPVKNKPGRYDDFFLS 15 IPVKNKGR++ FFLS 327 IPVKNKPGRFESFFLS 341	41.8 bits
Peptide 5	3 EEE - EQR - QQE 11 EEE EQR +QE 114 EEEHEQREEQE 124 4 EE - - - - EQRQQE 11 EE EQR QE 361 EEGQQQGEQRLQE 373	22.3 bits 18.9 bits	4 EEEQRQQE 11 E++QRQQ+ 495 EQQQRQQQ 502 3 EEEQRQQE 11 EEEE+ Q E 175 EEEEDQDE 183 2 GEEEEQ 7 G EEEQ 136 GSEEEQ 141 3 EEEEQ 7 EEEE+ 64 EEEEE 68	21.4 bits 18.9 bits 17.6 bits 17.2 bits
Peptide 6	1 AKSSSRKTISSEDEPNLRSRNPIYS 26 AKSSSRKTISSED PFNLRSR+PIYS 391 AKSSSRKTISSEDKPFNLRSDPIYS 416	76.6 bits	1 AKSSSRKTISSEDEPNLRSRNPIYS 26 AKSSSRKTISSED PFNLRSR+PIYS 391 AKSSSRKTISSEDKPFNLRSDPIYS 416	76.6 bits
Peptide 7	1 ITPEKNPQLRDLDFL 16 ITPEKNPQLRDLDFL 425 ITPEKNPQLRDLDFL 440	55.8 bits	1 ITPEKNPQLRDLDFL 16 ITPEKNPQLRDLDFL+FL 441 ITPEKNPQLRDLDFL 456	53.2 bits
Peptide 8	1 NLNFAFGINAENNQ 15 NLNF A GINAENNQ 521 NLNFFAIGINAENNQ 535 3 NFA 6 NFA 537 NFA 540	42.6 bits 15.9 bits	1 NLNFAFGINAENNQ 15 +LNF AFGINAENNQ 537 DLNFFAIGINAENNQ 551 3 NFA 6 NFA 553 NFA 556	44.8 bits 15.9 bits
Peptide 9	1 QELAFPGSAQDVERLL 16 QELAFPGSAQ VE LL 555 QELAFPGSAQAVEKLL 570	44.8 bits	1 QELAFPGSAQDVERLL 15 QELAFPGSA D+E L 571 QELAFPGSAKDIELN 585	37.1 bits
Peptide 10	1 DAQPQQKEEGSKGRKGPFPSSIL 22 DAQP KEEG KGRKGP SIL 580 DAQPKKKEEGKGRKGPLSSIL 601 4 PQQKEEGSKGRK 15 P+QKEE RK 159 PHQKEE - - - - RK 166	50.3 bits 21.0 bits	1 DAQPQQKEEGSKGRKGPFPSSIL 22 DAQPQQKEEG KGRKGP SIL 596 DAQPQQKEEGKGRKGPLSSIL 617 3 PQQKEE 9 QP+QKEE 151 QPHQKEE 157	59.6 bits 23.5 bits

indicated that P2 and P6 were IgE binding peptides. Meanwhile, all peptides inhibited the IgG binding ability of β -conglycinin and rabbit serum (Fig. 5B). Ten peptides showed variable inhibition in IgG binding ability of β -conglycinin, and the binding ability of IgG for P1–P8 was stronger than for P9 and P10.

In this study, all 10 predicted epitope regions exhibited IgG binding ability, and two of them also showed IgE binding activity. The verified results of linear epitopes indicated that it was an effective way to predict the linear epitopes of the β subunit of β -conglycinin using three immunoinformatics tools combined with IEDB. These 10 peptides were reported to have IgG binding ability in a previous study [11]. This meant that the method was accurate in predicting epitopes, and it required less time compared with traditional methods of locating epitopes. In addition, the IgE binding ability of P2 was validated in a previous research [9], but the IgE binding ability of P6 has not been reported. P6 is determined to be the IgE binding peptide in our study. P2 and P6 displayed both IgE and IgG binding activity, and thus, they were regarded as the dominant linear epitopes of the β subunit of β -conglycinin.

In the following experiment, all 10 peptides coupled with KLH were used to immunize rabbits to prepare polyclonal antibodies, which were used specifically to recognize the epitopes of β -conglycinin. Furthermore, the binding ability of all polyclonal antibodies was determined by western blot and dot blot assays.

3.3. The binding ability of epitope antibodies by blot assays

Dot blot was used to confirm the binding ability of the epitope polyclonal antibodies with β -conglycinin. Compared with the negative sera, PA-5 and PA-8 (polyclonal antibodies made of P5 and P8) barely recognized β -conglycinin (Fig. 6). This may be because the amino acid sequences of P5 and P8 in β -conglycinin existed inside the protein, and as a result, antibodies could not bind to the epitopes. To better evaluate the recognition sites of polyclonal antibodies, the polypeptide chain of β -conglycinin was unfolded by SDS-PAGE, and the binding ability of polyclonal antibodies was displayed by western blot. There were significant differences in the binding ability of polyclonal antibodies (Fig. 7). PA-1, PA-2, PA-3, PA-4, PA-6, PA-7, PA-9, and PA-10 could effectively bind to the β subunit of β -conglycinin and presented an obvious immune response band. Interestingly, the polyclonal antibody PA-5, which was derived from the amino acid sequence of the β subunit, showed no immunodominant results on the membrane. In addition, PA-1 recognized only the β subunit and PA-5 bound almost only to the α' subunit. PA-2 showed the binding with the β and α subunit, and PA-3 and PA-10 both exhibited the immunoreaction with the β and α' subunits. PA-8 showed a weak binding to three different subunits. Although the degree of binding ability of different epitope polyclonal antibodies to protein subunits varied, PA-4, PA-6, PA-7, and PA-9 could react with three subunits of β -conglycinin.

Western blot showed the recognition results of sequences of the β -conglycinin and polyclonal antibodies. Polyclonal antibodies were bound to not only the β subunit, but also to the other subunits (Fig. 7), which indicated that the verified linear antigenic epitopes were present in more than one subunit. The three subunits of β -conglycinin had high sequence homology, so there were cross reactions between them [21]. In addition, the IgG inhibitory ability of P9 and P10 was much lower than that of P5 and P8 (Fig. 5B), but the degree of binding with proteins for PA-9 and PA-10 was significant (Fig. 7). Differences in the degree of binding between polyclonal antibodies and subunits of β -conglycinin may be related to antibody titers (the titers of polyclonal antibodies were shown in Table S1), and the titers of PA-5 and PA-8 were lower. PA-5 was rarely bound to the β subunit, but could bind with the α' subunit easily (Fig. 7). This may be due to the presence of more recognition sites in the α' subunit.

3.4. Alignment of the linear epitopes in different subunits of β -conglycinin

To analyze further the binding ability of epitope polyclonal antibodies for β -conglycinin and to identify the sites that triggered the immune cross-reaction, the amino acid sequences of the 10 peptides were aligned by BLAST programs. The blast results of epitope peptides of β subunit aligned with α and α' subunits were shown in Table 3. P1 had fewer identical sequences to the α and α' subunits, which explained why the PA-1 was bound only to the β subunit. Appearance of an immune cross-reaction was associated with the same sequences between subunits, and these can be considered as epitopes when the length of sequences was >3 amino acids. Therefore, based on the experimental results from Table 3 and Fig. 7, the potential sites where an immune cross-reaction occurred were identified preliminarily. The four peptides RSPQL, SYNL, EEEEQRQQ, and DAQPQKEEG could be the main recognition sites for PA-2, PA-3, PA-5, and PA-10, respectively (Table 3). It was obvious that PA-4, PA-6, and PA-9 recognized three subunits, but the degree of their binding to the β subunit was significantly higher than that of the other two subunits (Fig. 7), which indicated that the main binding sites of the prepared polyclonal antibodies were located in different amino acid sequence regions between subunits. Moreover, there was little difference in the degree to which PA-7 and PA-8 were bound with the three subunits (Fig. 7), which meant that the same amino acid sequence regions between the β subunit and the other two subunits was the primary recognition site for antibodies.

4. Conclusions

With improvement in allergen databases and the increasing availability of immunoinformatics tools, the prediction and identification of major linear epitopes are becoming easier. In this study, three immunoinformatics tools were used to predict the linear epitopes in the β subunit of β -conglycinin. Then, the potential dominant epitope regions were determined by comparing the epitopes included in the IEDB. Ten potential epitope peptides were synthesized and validated by ic-ELISA. All peptides showed IgG binding activity, especially P2 and P6, which were validated as IgE binding peptides. Polyclonal antibodies were prepared by immunizing rabbits with 10 epitope peptides. Western blot and dot blot were used to confirm the binding ability of polyclonal antibodies with β -conglycinin. The results of dot blot indicated that the amino acid sequences of P5 and P8 might be inside the protein. Western blot showed that PA-1, PA-2, PA-3, PA-4, PA-6, PA-7, PA-9, and PA-10 were effectively bound to the β subunit. In addition, some polyclonal antibodies displayed immune cross-reactions with the α and α' subunits. This was related to the existence of the same amino acid sequence between subunits. Therefore, this is an effective way to predict and to identify linear epitopes using immunoinformatics tools and epitope databases. The preparation of epitope antibodies will contribute to further study the changes of epitopes in food allergens in processing.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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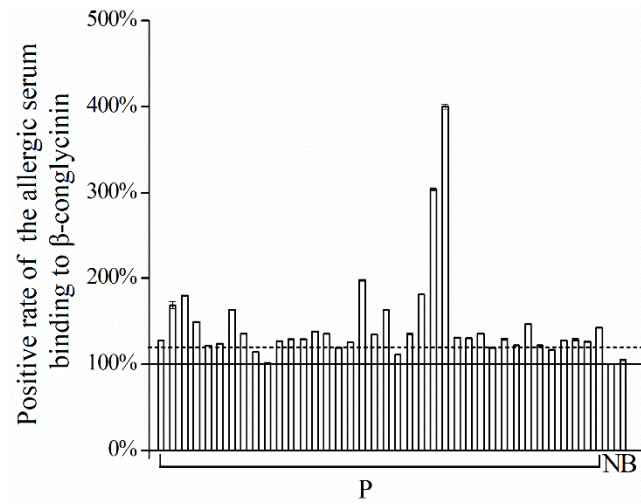


Fig. S1. The results of allergic serums binding to β -conglycinin by i-ELISA. P: The binding ability of 38 allergic patient serums; N: The binding ability of serums from non-allergic patient. B: No serum added. The positive ratio of the negative serum was 100%, serums with positive ratio greater than 120% were collected and mixed to prepare positive serum pool.

Table S1. The titers of epitope polyclonal antibodies by indirect ELISA.

Antibody	PA-1	PA-2	PA-3	PA-4	PA-5	PA-6	PA-7	PA-8	PA-9	PA-10
Titer	1:51200	1:51200	1:51200	1:51200	1:3200	1:102400	1:102400	1:3200	1:102400	1:102400