



Prediction and characterization of the linear IgE epitopes for the major soybean allergen β -conglycinin using immunoinformatics tools

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ABSTRACT

The α subunit of β -conglycinin is a major allergen in soybean. The objective of this study was to predict and identify the linear immunoglobulin (Ig)E epitopes of the soybean α subunit of β -conglycinin. Three immunoinformatics tools were used to predict the potential epitopes and were confirmed by dot-blot inhibition using sera from soybean allergic subjects. As a result, 15 peptides were predicted and assembled by solid-phase synthesis. Eleven epitopes were identified by the dot-blot inhibition test. Moreover, peptide 3 had IgE binding capability with all sera (5/5) tested, while peptide 1, 4, 6, 8 and 12 could bind to 4/5 of the sera samples. Secondary structure prediction of peptide 3 and circular dichroism test validated that the structure of peptide 3 was a random coil.

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1. Introduction

Food allergy is caused by an immune response to antigens in food and is mediated in part by immunoglobulin (Ig)E. Food which can cause allergic reactions includes nuts, milk, eggs, fish, crustaceans, peanuts, soybeans and wheat (F.A.O., 1995; Wilson et al., 2005; Weiqi, 2003). Soybeans have long been recognized as a plant food that is relatively high in protein compared to other plants. Soy allergy is defined as the hypersensitivity to dietary substances from soy causing an overactivation of the immune system that leads to severe physical symptoms for millions of people worldwide. Of all food allergies, approximately 25% are caused by soybeans that can induce a number of pathologies including intestinal damage, stomach upset or allergic dermatitis, and may even cause anaphylactic shock and thus be life threatening, and among sensitive children, 6% are affected with soy allergy (Endres, 2001; Errahali et al., 2002; Meizhu et al., 2007).

Recently, the major soybean allergens glycinin and β -conglycinin (Gly m Bd 60 K) were identified (Wilson et al., 2005; Weiqi, 2003). The β -conglycinin of soybean is made up of 67 kDa α -, 71 kDa α' -, and 50 kDa β -subunits (Holzhauser et al., 2009). Using serum from 69 soy allergy patients Ogawa et al. identified the antigenicity of various components from soy proteins and noted that the allergens combined with IgE antibody. Three major allergens were described, GlymBd 28 K, GlymBd 30 K and α subunit from β -conglycinin (Ogawa et al., 1991; Verma et al., 2012; Wu et al., 2012). Many studies have focused on GlymBd 30 K and determined its glycosylation sites and IgE binding epitopes (Bnado et al., 1996;

Hosoyama et al., 1996). Studies on the α subunit of β -conglycinin have mainly focused on its role in digestion, the separation of its subunits and glycosylation sites, and IgG binding epitopes, but few studies have reported potential IgE binding epitopes (Bao et al., 2010; Astwood et al., 1996; Fu et al., 2007; Zheng et al., 2009). Therefore, the study of soybean α subunit of β -conglycinin epitopes is important.

Immunoinformatics, a newly emergent branch of bioinformatics, has already become a familiar and useful tool for selecting epitopes from immunologically relevant proteins, as well as for further development of information about different epitopes (Zheng et al., 2011). It combines molecular biology and related clinical data to collate, contrast and develop on the basis of homology modeling. It can effectively reduce the antigen epitope screening range and experimental workload, improve efficiency, allow the discovery of new epitopes between 10 and 20 times more efficiently, and thus save research funds whilst accelerating the process of new epitope discovery (Groot et al., 2002; Liu et al., 2011).

Here, we attempted to use three different immunoinformatics strategies for soybean α subunit conglycinin epitope prediction. After combining the predictions, the potential epitope peptides were synthesized and validated by inhibition test with sera from soybean allergic subjects. Secondary structure prediction and circular dichroism will allow a better understanding of the synthesized peptides.

2. Materials and methods

2.1. Human sera

Negative human serum, milk-allergic human serum and positive soy-allergic sera samples (No. 1–5) were obtained from WUXI Allergy Prevention and Diagnosis Centre (Wu Xi, Jiang Su, China). Negative serum was obtained from a non-allergic

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person. All five adult patients had a history of immediate hypersensitivity reactions after the ingestion of soybeans, and were diagnosed allergic to soybean by this centre. The primary inclusion criterion was a double-blind, placebo-controlled food challenge (DBPCFC) with soy or a convincing history of anaphylactic reactions to soy. The most relevant data necessary for interpretation of the *in vitro* results obtained in this study are summarized in Table 1. To set uniform and comparable criteria for the most severe symptoms, symptoms were recorded as experienced under challenge (Barbara et al., 2007). The severity of symptoms following soy ingestion was graded as follows: (1) mild: symptoms such as oral allergy syndrome, tightness of the throat, nausea or gastrointestinal pain; (2) moderate: symptoms such as rhinitis, flush, urticaria, angioedema, diarrhea, or emesis; and (3) severe: drop of blood pressure, or life-threatening laryngeal edema. The serum samples were stored at -80°C until use.

2.2. Amino acid sequence of α subunit from β -conglycinin

The amino acid sequence of α subunit of β -conglycinin in soybean was obtained from NCBI protein with the accession number: BAA23360. The primary structure of the α subunit contained 543 amino acids.

2.3. Epitope prediction

In order to enhance the veracity of epitope prediction in the α subunit of β -conglycinin, the complete amino acid sequence was analyzed using three immunoinformatics-based computational approaches including DNASTar Protean system, SOPMA Server and the BepiPred 1.0 Server.

In the DNASTar Protean system, four properties of the amino acid sequence were chosen as the parameters for epitope prediction including hydrophilicity, flexibility, accessibility and antigenicity. The hydrophilicity prediction and the properties of flexibility, surface accessibility and antigenicity were analyzed using strategies as previously described (Emini et al., 1985; Hopp and Woods, 1981; Jameson and Wolf, 1988; Karplus and Schulz, 1985; Kyte and Doolittle, 1982). Based on the results of the predicting methods, peptide regions with good hydrophilicity, high flexibility and surface accessibility, as well as high antigenic index were chosen as candidate epitopes of DNASTar prediction for further investigation. The secondary structure of α subunit of β -conglycinin was analyzed by Chou-Fasman (1978) and Garnier-Robson (Garnier et al., 1978) methods.

SOPMA Server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and the BepiPred 1.0 (<http://www.cbs.dtu.dk/services/BepiPred/>) server were also used to predict protein epitopes. SOPMA software (ID: 244de10e895f) is run by the Network Protein Sequence analysis (NPS) server and supported by Ministère de la Recherche (ACI IMPBio, ACC-SV13), CNRS (IMABIO, COMI, GENOME) and Région Rhône-Alpes (Programme EMERGENCE). These two servers only require the amino acid sequence of the allergen and provide more straightforward epitope

results, which are a combination of physicochemical properties of amino acids and secondary structure information (such as the hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity properties of the analyte). The ultimate consensus epitope results were developed by combining the results of the three immunoinformatics tools together using a previously published method (Yang and Yu, 2008). We chose the combined results from all three methods to determine the final potential epitope regions.

2.4. Peptide synthesis

Peptides corresponding to regions predicted as potential epitopic sites (Table 2) were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis by Sangon Ltd. (Shanghai, China). The purity of the peptides was greater than 95% as assessed by high performance liquid chromatography. The molecular mass of the synthesized peptides was confirmed by electrospray ionization-Mass Spectrometry and the peptides were stored at -20°C until use.

2.5. Purification of the allergen protein

Ground soybeans were weighed and then subjected to cold acetone three times to produce a nonfat soybean powder. Then, the acetone powder was extracted overnight with 10-fold 20 mM Tris-HCl (pH 7.5) containing 1 M potassium chloride and 5 mM 2-mercaptoethanol. The mixture was then centrifuged at 10000 rpm for 30 min and the resulting supernatant was dialyzed against double distilled water for 48 h with several changes of double distilled water. The extract was subjected to salting with ammonium sulfate (30–60% saturation), followed by isoelectric precipitation (pH 4.8). The purification of the allergen from soybean was performed as previously described and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. Dot blot inhibition experiment

A dot-blot inhibition experiment was performed to assay the binding of the potential epitope peptides with IgE. Nitrocellulose membranes (0.45 μm pore size) were immersed in double-distilled water for 10 min. When the membranes were semi-dry, the purified β -conglycinin samples which were dissolved with carbonate buffered saline (CBS, pH 9.6) were spotted on the membranes and left to dry at room temperature (5 μg for each dot). The spotted membranes were incubated for 2 h in PBST (0.1% Tween 20 in 0.01 M pH 7.4 phosphate-buffered saline i.e. PBS) containing 5% non-fat dry milk (NFDM) to block the non-specific binding sites at 37°C . The membranes were then incubated with human sera (diluted 1:40 with PBST/2.5% NFDM as the primary antibody), which was preincubated with the peptides at 37°C for 1 h. Following overnight incubation at room temperature, the membranes were washed for 10 min with PBST three times and further incubated

Table 1
Soy allergy patient data.

Serum number	Age (y)	Sex	Clinical evidence	Most severe symptom	Graded symptoms		Specific IgE (kU/l)	Total IgE (kU/l)
1	25	Female	Soybean DBPCFC	OAS, nausea	Mild	Objective	1.7	100–200
2	56	Female	Soybean DBPCFC	Drop of blood pressure	Severe	Objective	1.2	>200
3	27	Male	Soybean DBPCFC	GIT	Mild	Objective	2.1	>200
4	36	Male	Soybean DBPCFC	OAS	Mild	Objective	0.8	100–200
5	45	Male	Soybean DBPCFC	Flush, urticaria	Moderate	Objective	1.3	>200

OAS – oral allergy syndrome; GIT – gastrointestinal; DBPCFC – double-blind, placebo-controlled food challenge.

Table 2
Linear epitopes predicted by combined immunoinformatics methods.

Number	Source (position)	Sequence	Length of sequence
Peptide1	5–33	ECEEGEIPRPRPQHPEREPPQQGEKEE	29
Peptide2	40–65	RPFPFRPQPRQEEHEQREEQEWPR	26
Peptide4	90–115	QFPFRPPHQKEERKQEEDEDEEQQR	26
Peptide3	55–80	HEQREEQEWPRKEEKRGKEGSEEEDE	26
Peptide5	110–135	DEEQRESESESESLRRHKNKNPFL	26
Peptide6	130–158	NKNPFLFGSNRFETLFKNQYGRIRVLQRF	29
Peptide7	165–190	LQNLRDYRILEFNSKPNTLLPNHAD	26
Peptide8	200–229	TAILSLVNNDDRSYRLQSGDALRVPSGT	29
Peptide9	230–260	YYVNPDPNNENLRLITLAIPVKNKGRFES	29
Peptide10	260–280	FFLSSTEAQQSYLQGFNRNILE	22
Peptide11	320–345	EQIRALSKRAKSSSRKTISSEDKPFN	26
Peptide12	338–368	SEDKPFNLRSRDPYSNKLGKFFETPEKN	30
Peptide13	350–375	DPIYSNKLKGFETPEKNPQLRDL	26
Peptide14	460–480	NFFAIGINAENQNRNFLAGSQ	21
Peptide15	510–540	QRESYFVDAQPKKKEGNGKRGKPLSSILR	30

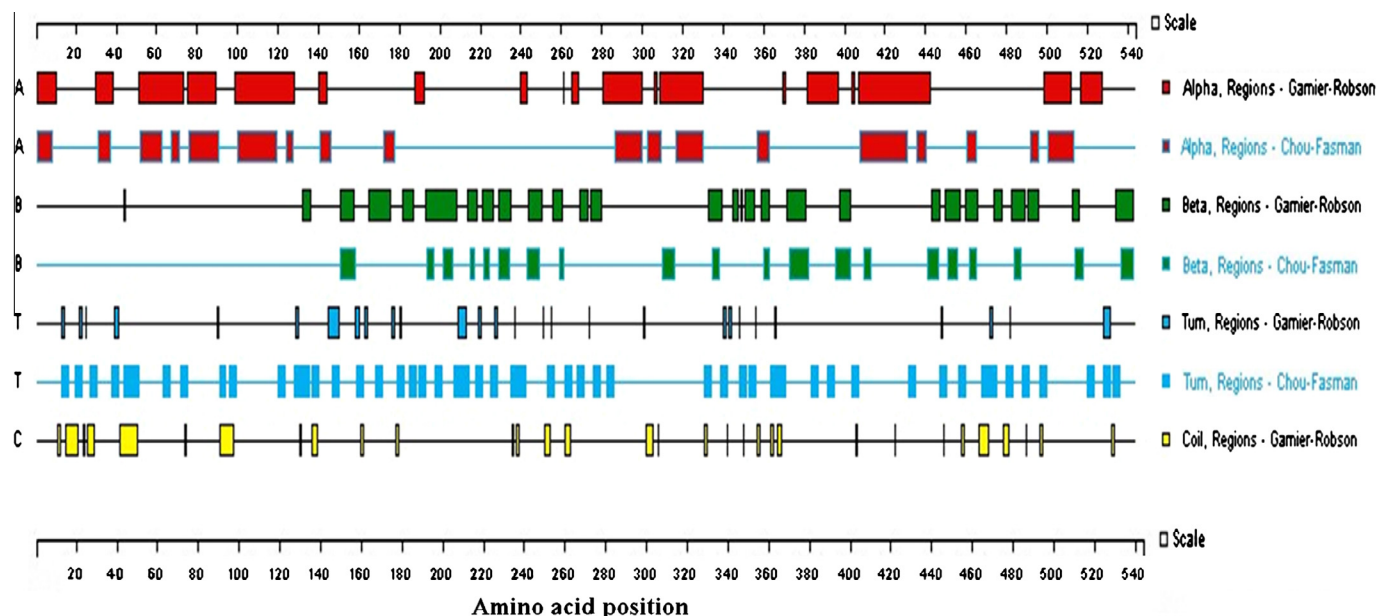


Fig. 1. The secondary structure of α subunit of β -conglycinin analyzed by Chou-Fasman and Gamier-Robson methods.

in PBST/1% NFD for 1 h containing goat anti-human IgE conjugated with horseradish peroxidase (Beijing Bios, Inc.) at 37 °C. The titer of anti-human IgE was obtained according to the supplier's suggested working dilution, which was 1:2000. Membranes were washed three times with PBST (10 min each) followed by two washes with PBS (5 min each) and incubated with 3,3'-diaminobenzidine (DAB) horseradish peroxidase chromogenic substrate for 3 min in darkness. The reaction was stopped by removal of the strips from the chromogenic solution and washed in double-distilled water.

2.7. Secondary structure prediction of the peptide

The peptide demonstrating the strongest IgE binding ability, using sera from the five allergy subjects, was used for secondary structure prediction. The GOR IV on-line server supplied by the institute of biology and chemistry proteins (IBCP) was used to predict the type and the ratio of the peptide secondary structure. The server only requires the amino acid sequence of the peptide and provided information including the ratio of the alpha helix, beta turn and random coil.

2.8. Verification of the peptide structure

Circular dichroism was used to evaluate the secondary structure of the peptide. The wavelength range of the CD spectra was 190–250 nm, which includes macromolecular main-chain conformation. The concentration of the peptide sample dissolved with phosphate-buffered saline (PBS, pH 7.4, 0.01 M) was 0.5 M. The peptide sample was added to a quartz groove where the optical path length is 1 mm. The result was analyzed by on-line server (<http://dichro-web.cryst.bbk.ac.uk/html/home.shtml>).

3. Results and discussion

3.1. Prediction and selection of the linear epitopes

The DNASTar provides prediction of the secondary structure of α -subunit of β -conglycinin. As shown in Fig. 1, the turn regions

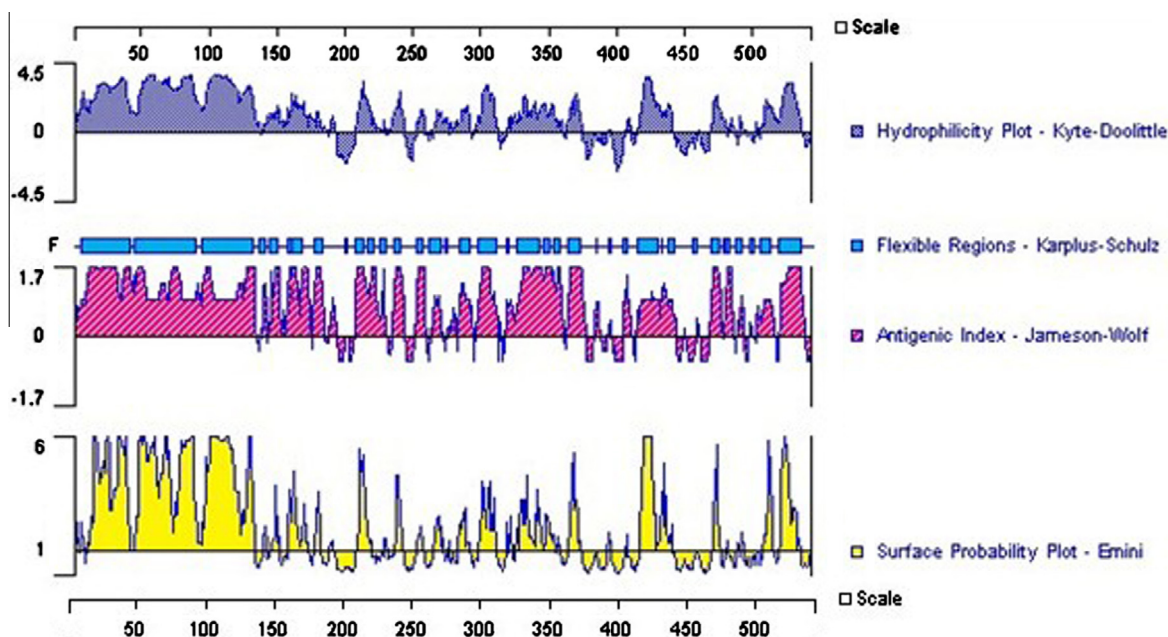


Fig. 2. DNASTar prediction results of the antigenic index, hydrophilicity, surface probability and flexible regions of α subunit of β -conglycinin.

Table 3

Predicted epitope site locations by three immunoinformatics tools.

Tools	Location of the prediction
DNAStar	9–31, 45–52, 62–90, 94–100, 119–132, 146–150, 158–169, 176–183, 209–218, 236–241, 252–256, 265–271, 274–276, 330–335, 338–345, 346–356,
Protean	364–372, 470–474, 519–536
SOPMA	9–31, 45–52, 62–77, 94–100, 125–132, 146–150, 176–183, 209–214, 236–241, 252–256, 265–269, 330–335, 338–345, 346–356, 364–369, 470–474, 519–536
BepiPred 1.0	5–14, 15–39, 40–44, 53–72, 74–86, 87–97, 98–105, 106–114, 117–123
Sever	

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1  vekeeecegeiprrprpqphepqpqqgekeedeedeqppipfprpqpqrqe eeheqreeq ewprkeekrgeksgseede-
81  dedeeqde rqpfprrpqpqhkeerkq eededeegqreseesedselr rhknknplfgsn rfe tlf knlqygrirv lqrf nq
161  rspqlnrdy rilefn skpn tllpnhadad ylivil ng la ilslv n ndbrdsyrdsgdalrps gtlvyvvpdrnt
241  nl rltla ipwnk pgrf esffl ssteaq qsylogfsmileasyd tk feeinkvlfs reegqqqgeqrqes vive is ke-
321  qiralskra ksssrkissedkpf nl rsrdpiys nkl gk ffe it pe knp qirldi flsiwd mn eg al lphfmsk aivi-
401  lwin eg dan ielv glkeqqqqeqq qeeqplewrkyrae ls eqd ifvi pagypvwnatsnlffaiginaennqrnflag-
421  q dnvisqpsqvqel af pgs aqaveklkn gr es yfv daqpkkke eg nkgrkgplssilrafy-

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Fig. 3. Secondary structures of α subunit of β -conglycinin protein as predicted by SOPMA. α -helix are shown by single underlined text (—); β -sheets are shown by double underlined text (====); β -turns are shown as boxed.

of α subunit from β -conglycinin had a frequent appearance of the whole sequence that indicated the high possibility of being an epitope. The turn regions and coil regions have an exposed outer edge and may easily bind antibody. The alpha regions and beta sheet regions were non-deforming and hard to integrate with antibody, suggesting these regions were not epitopes.

The DNAStar Protean system also provides other variables to obtain potential epitope regions. As shown in Fig. 2 the hydrophilic regions occupied a high proportion of the sequence indicating the high hydrophilicity of the protein. The frequent appearance of surface and flexibility regions indicated that the sequence was flexible, stretched, and easily exposed outside. The stretched structure was confirmed by flexibility and surface accessibility analysis. The antigenic index directly showed the epitope forming capacity of the protein sequence. The whole sequence had a high antigenic index that suggested a high tendency for the protein to form epitopes. Based on these sequence properties, the final predicting regions of DNAStar were obtained as shown in Table 3.

SOPMA Server provided secondary structure information and the BepiPred 1.0 Server provided more straightforward and integrative results were also employed. As shown in Fig. 3, the protein had the following composition: alpha helix (28.91%), beta sheet (20.07%), beta turn (6.81%) and random coil (44.2%). Table 3 showed the predicted results of the above two tools. The final potential epitopes of the soybean allergen were developed and selected on the basis of the results of these two tools and those from the DNAStar Protean system as previously described (Yang et al., 2008). The combined results of the three immunoinformatics tools predicted 15 peptides as potential linear epitopes of α -subunit of β -conglycinin. These peptides were named Peptide 1 to Peptide 15, and were synthesized according to the amino acid sequences shown in Table 2.

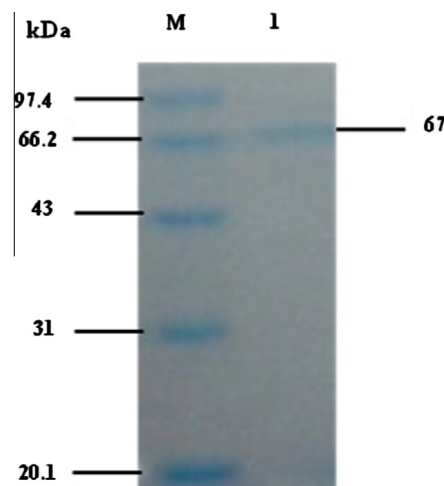
In this study, regions with high hydrophilicity, flexibility, composed of polar residues and with high antigenic propensity all appeared at the beginning of the whole protein sequence. However, the presence of these four properties was less frequent in the rest of the whole protein sequence. These results indicated the high possibility of epitopic regions at the beginning of the protein sequence. From the secondary structure prediction of DNAStar, we observed that β -turns and random coils occurred with a high

frequency in the beginning of the whole sequence and SOPMA Server results also shows that random coils occurred with a high frequency in the beginning of the whole sequence. And the presence of α -helix and β -sheets was more frequent in the rest of the whole sequence.

In addition, we used the empirical antigenicity method by Kolaskar et al. (Kolaskar and Tongaonkar, 1990) to confirm our prediction results. The antigenicity prediction results contained all the 15 peptides and the first five probability to be epitopes were peptide 1, 3, 6, 8 and 12 which corresponded with our prediction.

3.2. Validation of the peptides by dot-blot inhibition experiment

Dot-blot inhibition experiments were conducted with the synthesized peptides to demonstrate their IgE binding and to validate the potential epitopes predicted in the present study. Dot-blot immunoassay was performed on the basis of the reactivity

**Fig. 4.** SDS-PAGE of purified α subunit of β -conglycinin (stained with Coomassie brilliant blue R250). Lane M, prestained marker; lane 1, purified α subunit of β -conglycinin. The loading amount of the sample was 3 μ g.

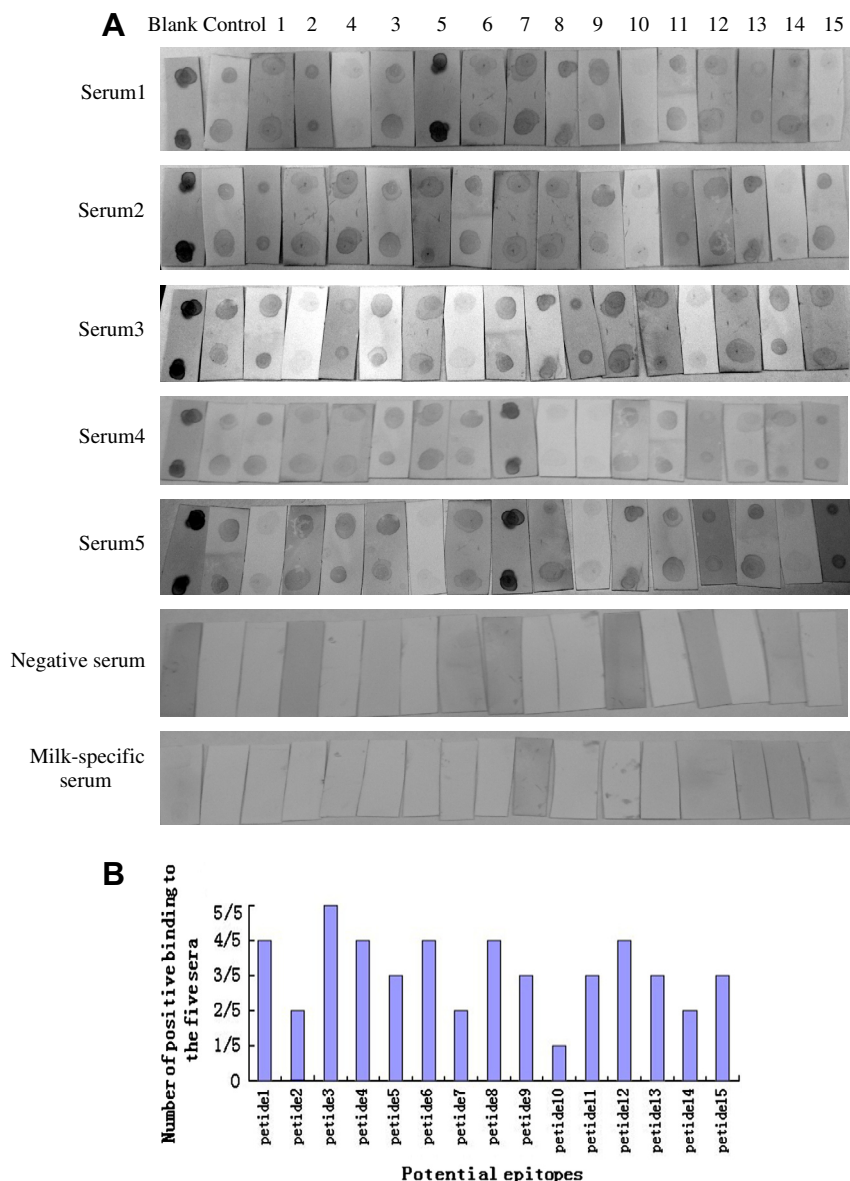


Fig. 5. (A) Results of dot-blot inhibition tests with sera from five soybean allergic subjects. The reactivity between β -conglycinin and sera of soybean allergic subjects was inhibited by preincubating the sera with potential epitope peptides (10 μ g) at 37 $^{\circ}$ C for 1 h. Blank: the antibody diluting buffer was used instead of potential epitope peptides; Control: β -conglycinin was used as the inhibitor; inhibitor 1 to inhibitor 15: peptide 1 to peptide 15, respectively. (B) IgE binding ability of the 15 peptides for sera from five soybean allergic patients.

between β -conglycinin (Fig. 4) and sera IgE of soybean allergic subjects that can be inhibited by preincubating the sera with potential epitope peptides (Fig. 5). As shown in Fig. 5A, strong inhibition was evident using the β -conglycinin allergen itself. Incubation with the peptides induced a different degree of inhibition among the five sera tested. Fig. 5B shows the binding times of IgE to the 5 test sera in the presence of 15 potential epitopes. Interestingly, 11 of the 15 potential epitopes reacted with more than half of the five sera, which mean 11 epitopes reacting with at least 3 test sera, and were considered major linear epitopes. Peptide 3 was critical for IgE binding as it bound to all five sera and consequently inhibited all reactions between β -conglycinin and serum IgE. In contrast, peptide 10 displayed less IgE binding activity, and its span regions had no intersection with the epitopes of α subunit of β -conglycinin.

Significant inhibition occurred when the dose of the competing peptide for each dot was 10 μ g. Interestingly, doses required for inhibition using the same synthesized peptide were different

among different sera. This implied that the IgE antibodies in different sera could recognize different epitopes. Eleven of the 15 predicted epitopic regions presented IgE binding ability, which validated the predicting accuracy of the tests used. We predicted 15 peptides among 543 amino acids and every peptide contain no more than 30 amino acids. And the accuracy was 11/15 which was similarity to the other study result 8/10 (Zheng et al., 2011). Peptide 8 was reported by previous researches that the sequence was validated to be the IgG binding peptide (Fu et al., 2007) and now it was also confirmed to be IgE binding peptide. In this study, another 10 IgE binding peptides were found. In addition, the strongest inhibiting peptide was located at the beginning of the protein sequence, thus validated the predicting accuracy further.

3.3. Secondary structure prediction of peptide

GOR IV (ID: 6db97b1fba60) was run on the NPS server. We used peptide 3 for secondary structure prediction as it had the strongest

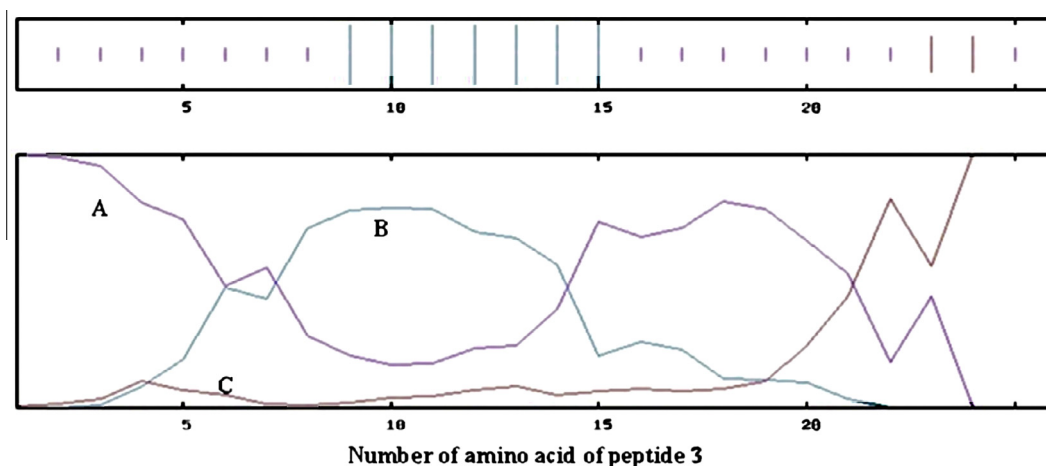


Fig. 6. Prediction map of the linear secondary structure of peptide 3. extended strand (A); α -helix (B); random coil (C). the peak of every line showed the region of the structure in the whole peptide. Peptide 3 sequence: HEQREEQEWPRKEEKRGEKGSEEEDE.

inhibitory effect. As is shown in Fig. 6, an α -helix occupied 26.92% of the middle part of the peptide and an extended strand occupied 7.69% of the end of the peptide. A random coil occupied 65.38% of the whole peptide demonstrating that the random coil dominated the whole peptide.

3.4. Verification of the peptide 3

The measurement of circular dichroism spectra can be used to evaluate the secondary structures of proteins and other biological macromolecules. CD spectra showed that a negative peak appeared at 198 nm and a small and wide positive peak appeared at 220 nm. Software (selcon3) calculation showed random coil occupied 72.15%, α -helix occupied 19.33%, and extended strand 9.33%. Experiment result coincided with GOR IV prediction results in Section 3.3, which validated the prediction accuracy. According to previous works, the random coil structure was mostly to be epitope region (Modrow et al., 1987; Saha and Raghava, 2006).

4. Conclusion

With the increasing availability of immunoinformatics tools and allergen sequences, the prediction of linear epitopes is becoming easier. These tools can also reduce the number of candidate epitopes that need to be synthesized and validated. Three-dimensional structures can also be considered to make the prediction more accurate. In the present study, we focused on an immunoinformatics-based approach as a means to predict the potential linear IgE epitopes of α subunit of β -conglycinin soybean, which could then be qualitatively validated by dot-blot inhibition experiments. Fifteen potential epitopic regions were predicted and peptides were synthesized. Eleven of the 15 potential epitopes were identified as major epitopes. Remarkably, peptide 3 presented a relatively higher IgE binding activity compared to the other synthesized peptides, and was circular dichroism confirmed its secondary structure was random coil. Therefore, the immunoinformatics approach used to predict linear epitopes in the present study has a high accuracy, which can be used to benefit allergen immunotherapy and reduce the frequency of allergic reactions.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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