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Identification and mutational analysis of continuous, immunodominant epitopes of the major oyster allergen *Crag* 1

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ABSTRACT

Shellfish, including oysters, often cause allergic reactions in children and adults. Oysters are inevitably consumed because of its delicacy and nutritional benefit, leading to frequent occurrence of severe clinical symptoms observed in patients with oyster hypersensitivity. We aimed to identify the immunodominant epitopes of oyster tropomyosin and crucial amino acids for IgE binding, which will help us to further understand the immunochemical characteristics of *Cra g* 1. The potential epitopes were predicted by immunoinformatics tools and the resultant immunodominant epitopes were identified by inhibition ELISA with pooled sera and individual serum from oyster allergic patients. Surprisingly, homologous substitution of multiple amino acids led to obviously decrease affinity of IgE antibodies, but this manner did not abrogate binding completely. Five major linear epitopes were evenly distributed on the surface of homology-based *Cra g* 1 model and hydrophilic residues appeared to be the most important for IgE binding. These results not only offer a better understanding of the molecular mechanism of interaction between *Cra g* 1 and oyster-specific IgE but also have significance in clinical diagnosis and immunotherapy.

1. Introduction

Food allergy is allergic reaction after people intake of specific food composition, it can cause clinical symptoms in sensitized subjects [1]. With the development of society, the dietary structure has been changed. The incidence of food-induced allergic diseases is increasing, which poses a great threat to people's health [2]. Unlike most other food allergies, the reactions to shellfish (crustaceans and mollusks) mediated by IgE continue throughout life. It is well documented that the ingestion of oyster, skin and mucosal contact and the inhalation of aerosolized oyster proteins can cause a large variety of clinical symptoms in sensitized patients, such as urticaria, angioedema, atopic dermatitis, asthma, rhinitis, vomiting, diarrhea and anaphylaxis [3–5]. Until now, some IgE-binding epitopes from major allergens of peanut, soy, milk, and shrimp have been identified [6–11], but oyster, which constitutes the most common mollusc food item, its B cell epitopes of major allergen are few reported.

At present, six shellfish allergens have been well explored including tropomyosin, sarcoplasmic calcium-binding protein, arginine kinase, triose phosphate isomerase, myosin light chain and troponin C [10–19]. Among them, tropomyosin is the major allergen with a frequency of sensitization at 80% in shellfish-allergic patients [20]. Ishikawa et al. [21] purified an allergen from oyster (*Crassostrea gigas*), named *Cra g* 1, and was confirmed as tropomyosin on the basis of composition of amino acids and molecular weight. Tropomyosins, without complex secondary structure over almost the whole molecules, are simple coiled-coil dimers that are responsible for regulating muscle contraction [10]. Thus, it is a very flexible protein which can spontaneously unfold and expose certain epitopes to bind IgE of allergic subjects [10,20].

Based on the computer technology and biological databases, immunoinformatics has already been a useful method to predict epitopes from immunological proteins. Instead of overlapping synthetic peptides used to validate IgE reactivity in previous studies, epitope mapping by immunoinformatics tools may decrease the number of synthesized peptides and working time [22,23]. Now, with an increasing availability of these tools such as DNAStar, BepiPred, BEPIYOPE and BcePred, selecting protein epitopes with immunoinformatics tools is becoming more popular and easier, this approach is also gradually used in clinical diagnosis of food allergy [24].

Tropomyosin is highly conserved protein, the sequence homology is 93.8% in the crustaceans, 77.2% in the mollusks and over 49% between invertebrate and vertebrate [20], vertebrate tropomyosins have been

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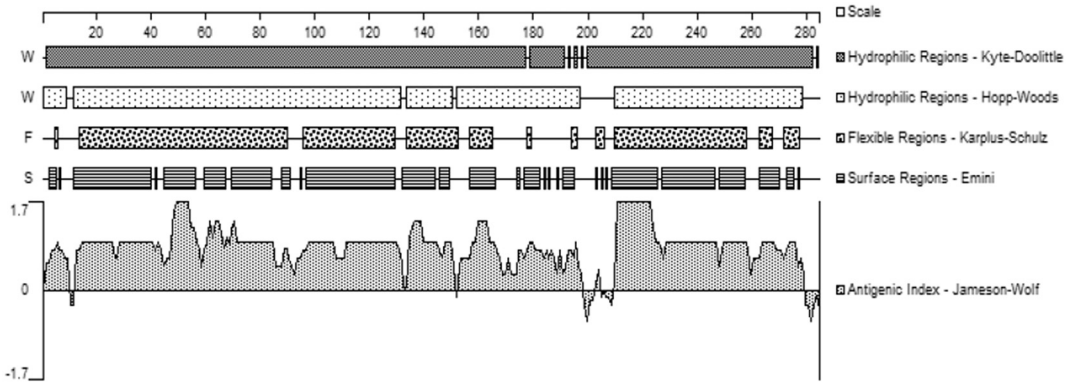

Fig. 1. Analysis results of the whole amino acid sequence of *Cra g* 1 by DNAstar Protean system.

Table 1
Results predicted by three immunoinformatics tools.

Tools	Amino acid regions
DNAstar protean	14–40, 45–56, 60–67, 70–84, 97–129, 134–144, 157–165, 210–225, 227–246, 248–257, 263–275
BPAP	42–49, 61–69, 83–95, 140–146, 148–155, 166–176, 181–202, 205–211, 243–255, 257–263, 266–272
BepiPred 1.0 server	15–25, 27–41, 51–66, 69–85, 99–108, 110–126, 134–143, 156–165, 209–224, 233–245

Table 2
IgE-binding epitopes finally predicted by combined immunoinformatics methods.

Number	Amino acid sequence	Position	Length of sequence
Peptide 1	KENAQDRAEQL	15–25	11
Peptide 2	QQLRDTEEQKAKIE	27–40	14
Peptide 3	TSLQKK	44–49	6
Peptide 4	SNLENE	51–56	6
Peptide 5	VNEKYQEC	60–67	8
Peptide 6	TKLEEAEKTASEAEQET	69–85	17
Peptide 7	MERSEERLQT	99–108	10
Peptide 8	TEKLEEASKAADESERN	110–126	17
Peptide 9	NNASEERTDVL	134–144	11
Peptide 10	EADKKYDEA	157–165	9
Peptide 11	VQNDQASQREDSYEET	209–224	16
Peptide 12	KDAENRATEAERTV	233–246	14
Peptide 13	KLQKECDR	248–255	8

regarded as nonallergenic molecules [25]. Therefore, this protein provides an excellent model to convert allergenic epitopes of tropomyosin into nonallergenic form while retaining as much of function and structure as possible. Such mutated proteins or peptides will have significance for food allergy treatment.

In this communication, we tried to use different immunoinformatics strategies to predict oyster (*Crassostrea gigas*) tropomyosin epitopes. After combining prediction, potential epitope peptides were synthesized and validated by inhibition ELISA with the pooled sera and randomly selected individual serum from oyster allergic patients. Moreover, the crucial amino acids within each of *Cra g* 1 epitope that played an important role for IgE binding were determined. We also constructed a homology-based three-dimensional structure model of *Cra g* 1 to elucidate the positional distribution of IgE-binding epitopes in the whole molecule. These results may enable us to improve immunotherapy and reduce the risk of oyster-induced allergic reactions by lowering the IgE binding capacity of this allergen.

2. Materials and methods

2.1. Human sera

Serum samples from 15 oyster-allergic subjects (mean age, 25 years) were used to determine wild-type and mutant *Cra g* 1 epitopes. All 15 subjects had a clinical history of oyster anaphylaxis (severe wheezing, urticaria, angioedema, vomiting, nausea or diarrhea), immediate positive skin prick test (wheal > 3 mm) to cooked oyster, oyster-specific IgE antibodies (from 25 to > 100 kU L^{-1}, median > 100 kU L^{-1}) measured by the CAP system FEIA (Pharmacia Diagnostics, Sweden) and strong IgE reactivity to purified oyster tropomyosin by immunoblot analysis. 10 ml of venous blood was collected from each subject and a serum pool was made by mixing equal aliquots of serum IgE. Sera from 5 normal volunteers without any previous history of shellfish allergies were used as negative controls. Sera were stored in aliquots at −80 °C until used. All experiments have been approved by medical ethics committee at the China National Research Institute of Food and Fermentation Industries.

2.2. Amino acid sequence of Cra g 1 and alignment

The amino acid sequence of tropomyosin in *Crassostrea gigas* was obtained from protein database of NCBI (https://www.ncbi.nlm.nih.gov/) with the accession no. AB444943. Multiple sequence alignment of the tropomyosins from invertebrate and vertebrate was carried out by the use of ClustalW, which their sequences were also obtained from NCBI.

2.3. Epitopes prediction

To improve the accuracy of prediction, the complete amino acid sequence of IgE linear epitopes in *Cra g* 1 was analyzed by three immunoinformatics-based tools, including DNAstar Protean system, BepiPred 1.0 Server (http://www.cbs.dtu.dk/services/BepiPred/) and Bioinformatics Predicted Antigenic Peptides website (http://immunax.dfci.harvard.edu/Tools/antigenic.html). In the DNAstar Protean system, predicting linear epitopes is mainly based on the hydrophilicity, surface accessibility, flexibility and antigenicity of the amino acid sequence. Furthermore, Bioinformatics Predicted Antigenic Peptides website and BepiPred 1.0 Server could provide more direct epitopes information according to physicochemical properties of sequence. Combining the results of the above mentioned three tools as well as the approach of Yang et al. [26], the final potential epitope regions that have good hydrophilicity and antigenic index, proper polarity and exposed surface together with high accessibility and flexibility were

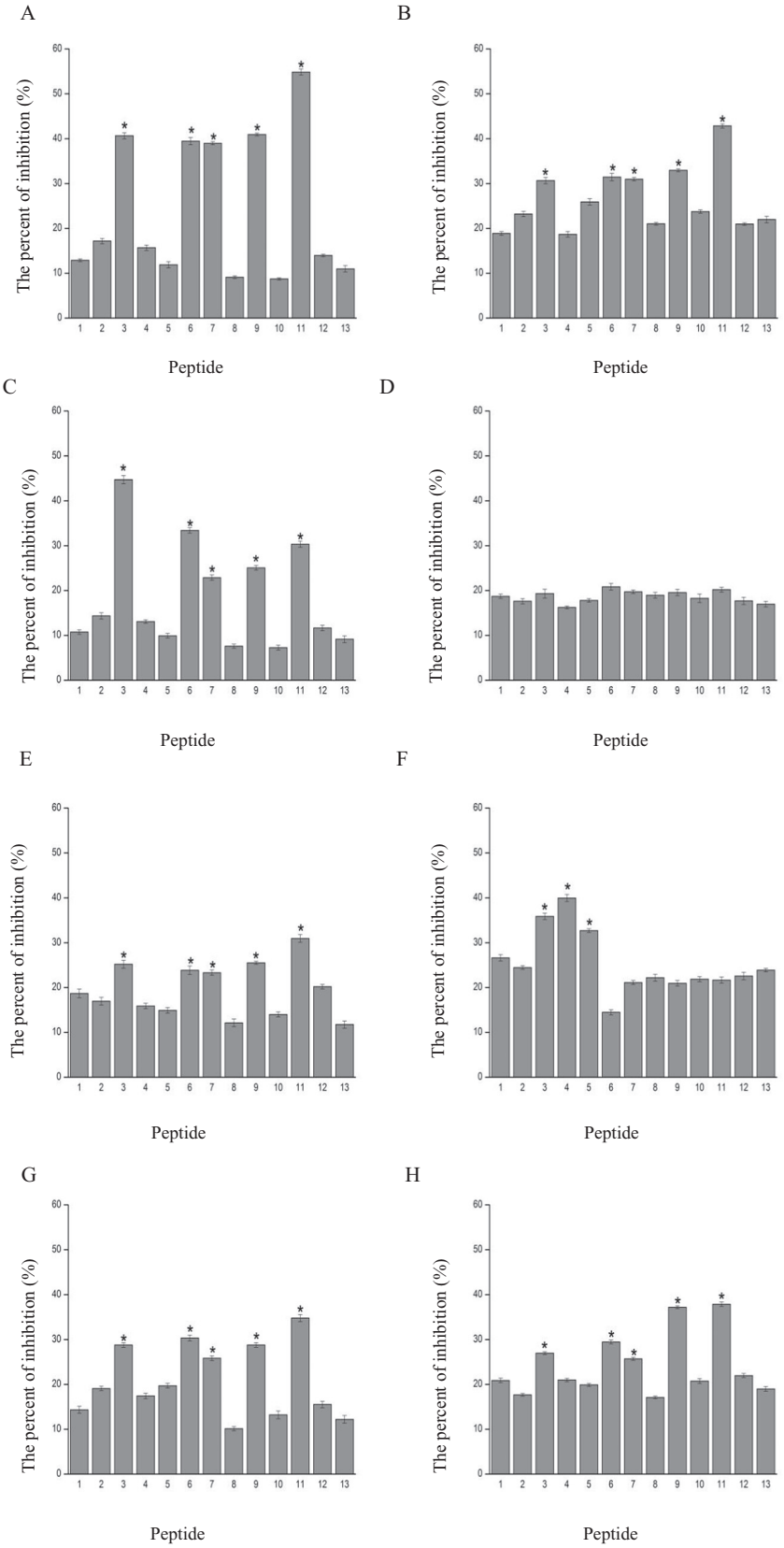

Fig. 2. Determination and amino acid composition of *Cra g* 1 major epitopes. (A) Histogram of the IgE binding reactivity against the *Cra g* 1 peptides as determined by inhibition ELISA using the pooled sera from 15 oyster-allergic subjects. (B–I) Histogram of the IgE binding reactivity against the *Cra* g 1 peptides as determined by inhibition ELISA using individual serum from 8 randomly selected subjects whose serum was included in the pool. All data are presented as the mean ± SD (n = 3). (J) Amino acid composition of *Cra g* 1 major epitopes.

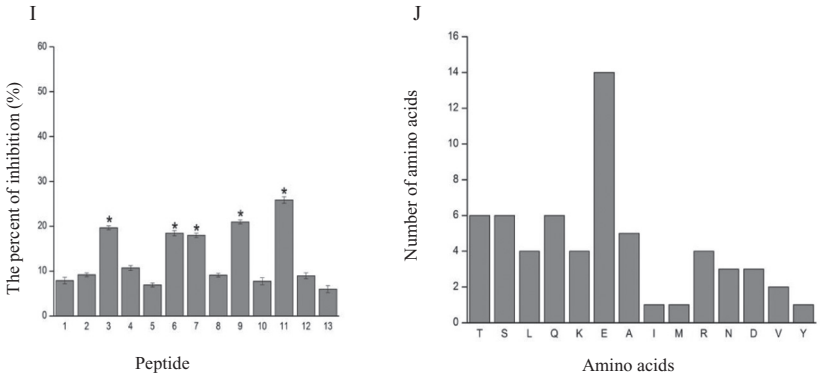

Fig. 2. (*continued*)

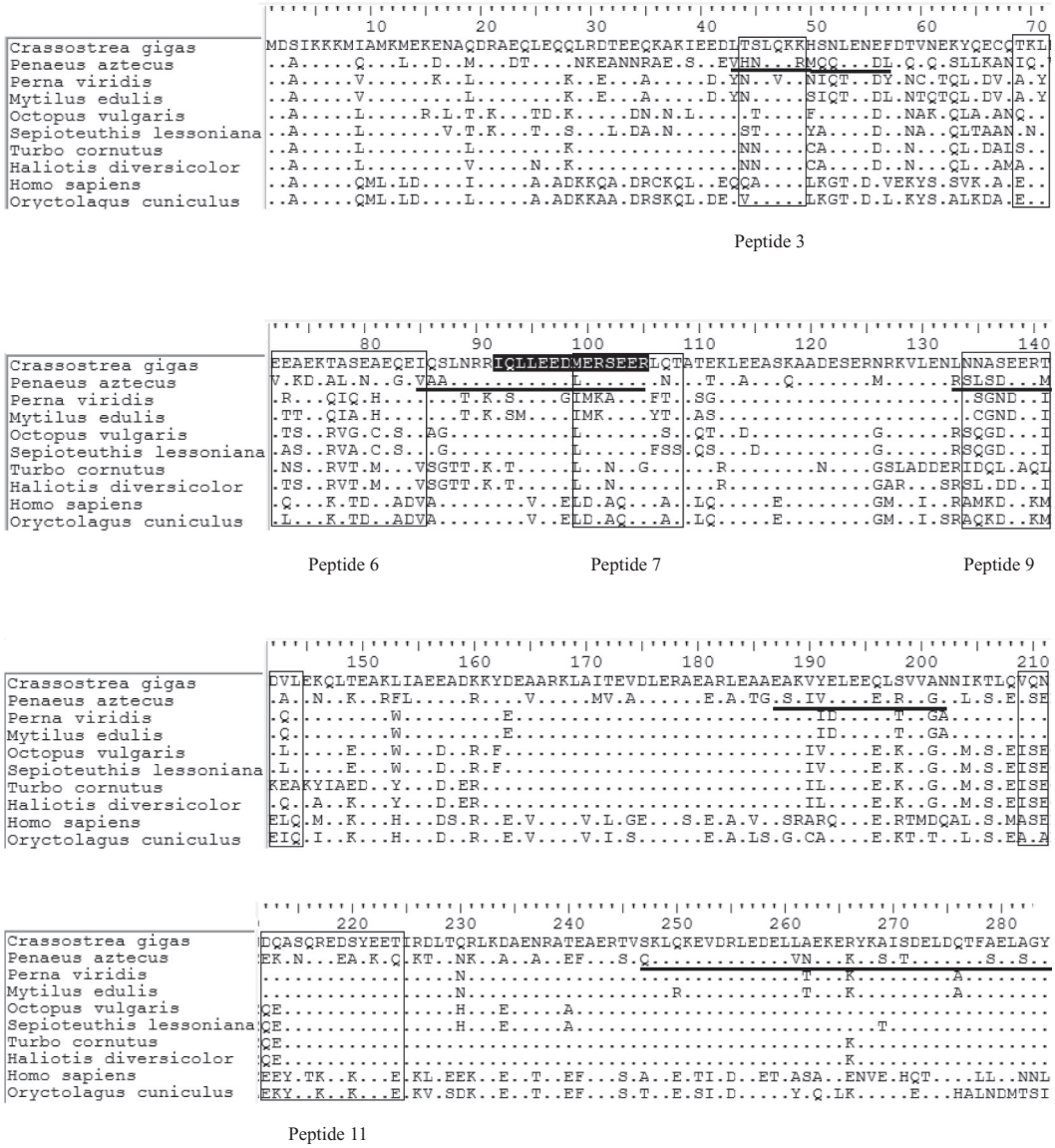

Fig. 3. Amino acid sequence alignment of different tropomyosins. Alignment of different tropomyosin sequences was from ten species (NCBI accession numbers): *Crassostrea gigas* (AB444943), *Penaeus aztecus* (AAZ76743), *Perna viridis* (AAG08988), *Mytilus edulis* (U40035), *Octopus vulgaris* (BAE54433), *Sepioteuthis lessoniana* (AB218914), *Turbo cornutus* (BAH10149), *Haliotis diversicolor* (AAG08987), *Homo sapiens* (AAB59509.1) and *Oryctolagus cuniculus* (gi|157,787,199). Identical residues are shown as ".". Sequences of the 10 tropomyosins that correspond to the five major IgE-binding epitope regions of *Crassostrea gigas* tropomyosin are shown in white boxes. Sequences that are underlined represent IgE-binding linear epitopes proposed for *Penaeus aztecus* and the previously identified epitope of *Cra g* 1 is displayed in black box.

Table 3
Mutagenesis of *Cra g* 1 major epitopes.

Mutant peptide	Amino acid sequence	Substitution position from to		
Peptide 3–1	**QA**LQKK	44,45	T/S	Q/A
Peptide 3–2	**VA**LQKK	44,45	T/S	V/A
Peptide 6–1	TKLE**L**AEKTAS**D**AEQ**D**I	73,80,84	E/E/E	L/D/D
Peptide 6–2	**E**KLEEAEK**K**ASEAEQEI	69,77	T/T	E/K
Peptide 6–3	TKLEEAEKTA**T**EAE**A**EI	79,83	S/Q	T/A
Peptide 6–4	**E**KLEEAEKTA**T**EAE**A**EI	69,79,83	T/S/Q	E/T/A
Peptide 7–1	MER**A**EERL**A**T	102,107	S/Q	A/A
Peptide 7–2	M**D**RS**Q**ERLQT	100,103	E/E	D/Q
Peptide 7–3	M**D**R**AQ**ERL**A**T	100,102,103,107	E/S/E/Q	D/A/Q/A
Peptide 9–1	NNA**D**EE**KM**DV**Q**	137,140,141,144	S/R/T/L	D/K/M/Q
Peptide 9–2	NNA**D**EE**K**TDVL	137,140	S/R	D/K
Peptide 9–3	NNASEER**M**DV**Q**	141,144	T/L	M/Q
Peptide 11–1	**AQ**A**D**K**K**ASQ**K**ED**K**YEE**E**	209,211,213,217, 220,224	V/N/Q/R/S/T	A/A/K/K/E
Peptide 11–2	**AQ**A**D**K**K**ASQREDSYEET	209,211,213	V/N/Q	A/A/K
Peptide 11–3	VQNDQAS**Q**K**ED**K**YEE**E	217,220,224	R/S/T	K/K/E
Peptide 11–4	VQNDQAS**TK**ED**K**YEET	216,217,220	Q/R/S	T/K/k

Note: The mutant amino acids are shown as the bold, underlined residues.

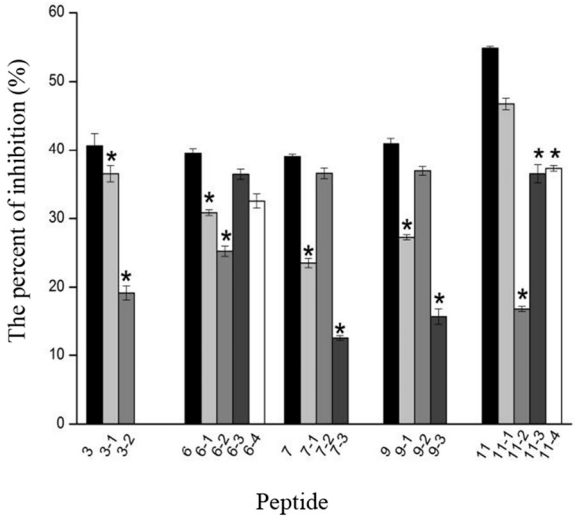

Fig. 4. Identification of the critical amino acids within the major *Cra g* 1 epitopes. Wild-type and mutated peptides were synthesized and probed with the pooled sera from 15 patients with oyster hypersensitivity. $^{*}P < .05$ *vs.* corresponding wild-type peptide. Data represent the mean ± SD (n = 3).

chosen as candidate epitopes (Table 2).

2.4. Peptide synthesis

The epitopes were synthesized by GL Biochem (Shanghai) Ltd. using Fmoc solid phase method. The purity and molecular weight of these peptides were determined by high performance liquid chromatography (HPLC) and Electrospray Ionization-Mass Spectrometry (ESI-MS), respectively. The purity of the peptides was > 98% and they were stored at −20 °C until used.

2.5. Design and synthesis of mutant peptides

Amino acid sequences of vertebrate tropomyosins (*Oryctolagus cuniculus,* α-tropomyosin, NCBI accession no. gi|157,787,199; *Homo sapiens,* NCBI accession no. AAB59509.1) were used as templates to design the mutations. Instead of an alanine scan, amino acids located at homologous positions of nonallergenic tropomyosins were introduced into allergenic epitopes, and the number and location of homologous substitution were determined by homologous sequences of human and rabbit. In previous reports, the results of amino acid substitutions analysis of major shrimp allergen and milk allergen illustrated that a single substitution was not sufficient to markedly reduce IgE binding [20,27]. Thus, at least two amino acid substitutions were introduced into each *Cra* g 1 epitope to inhibit IgE reactivity according to the homologous sequences of nonallergenic muscle tropomyosins from human and rabbit. Then, a set of modified peptides was synthesized for individual identified major epitope.

2.6. Purification of allergen protein

The muscle tissue of fresh oyster purchased from local market was homogenized in the buffer (PH 8.0) containing 1 mM KCl and 20 mM Tris-HCl at 4 °C, followed by centrifugation and made acetone powder with cold acetone. Then, the acetone powder was extracted overnight with 20 mM Tris-HCl (pH 7.5) containing 1 M KCl and 10 mM DTT. The mixture was centrifuged at 18,000 × *g* for 30 min and supernatant was dialyzed against distilled water for 24 h. Subsequently, the extract was subjected to salting-out with ammonium sulfate (40–60% saturation), followed by isoelectric precipitation (pH 4.6). Finally, the crude extract of tropomyosin was purified through anion-exchange chromatography using an ÄKTA fast protein liquid chromatography (FPLC) system. The purified protein was further dialyzed in PBS at 4 °C, concentrated by an Amicon spin column (Merck, USA) and stored at −80 °C until used.

2.7. Inhibition enzyme-linked immunosorbent assay (ELISA)

The inhibition ELISA was carried out according to the method of Wang et al. [28] with some modifications. Briefly, purified *Cra g* 1 (5 μg mL^{-1}) was used to coat 96-well EIA/RIA plate (Costar, USA) in 0.05 M CBS (pH 9.6) overnight at 4 °C and blocked with 5% skim milk for 3 h. Pooled sera from 15 oyster-allergic subjects or 8 randomly selected individual serum from the same pool (diluted 1:50 with skim milk) were incubated with an equal volume (50 uL) of wild-type or mutated peptides that were diluted with CBS overnight at 4 °C. The mixed solution was added to wells and incubated for 2 h at room temperature. After that, plates were washed and followed by the addition of peroxidase-labeled goat anti-human IgE antibody (diluted 1:2000; KPL Inc., USA). The plates were incubated at 37 °C for 1 h and washed in PBS. Subsequently, each well was developed with TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate for 5 min, the reaction was terminated by 2 M H_2SO_4. The absorbance was measured at 450 nm with spectrophotometer (Dynex Technologies Inc., USA). Percent inhibition was determined using the following equation [29,30]: Inhibition rate (%) = (O.D. $_{\text{no inhibitor}}$ − O.D. $_{\text{inhibitor}}$)/O.D. $_{\text{no inhibitor}}$ × 100%. The negative sera were screened to determine the extent of nonspecific binding, which was subtracted from test data. ELISA was performed in triplicate and data were given as mean values.

2.8. Isothermal titration microcalorimetry (ITC)

The crucial amino acid residues were determined by a MicroCal iTC200 microcalorimetry (GE Healthcare, USA). The titration was performed at 25 °C with pooled sera and mutated peptides dissolved in 10 mM Tris buffer. All the samples were degassed properly prior to the experiment. The measurements were carried out in a cell with a volume of 200 uL with 20 injections of 2 uL peptides at 180 s intervals. Concentration of peptides and sera was 400 uM and 40 uM, respectively. Control experiments were measured by titrating peptides into 10 mM Tris and negative sera under the same conditions, which was subsequently subtracted from test data. The results were analyzed using ORIGIN® software provided with the calorimeter.

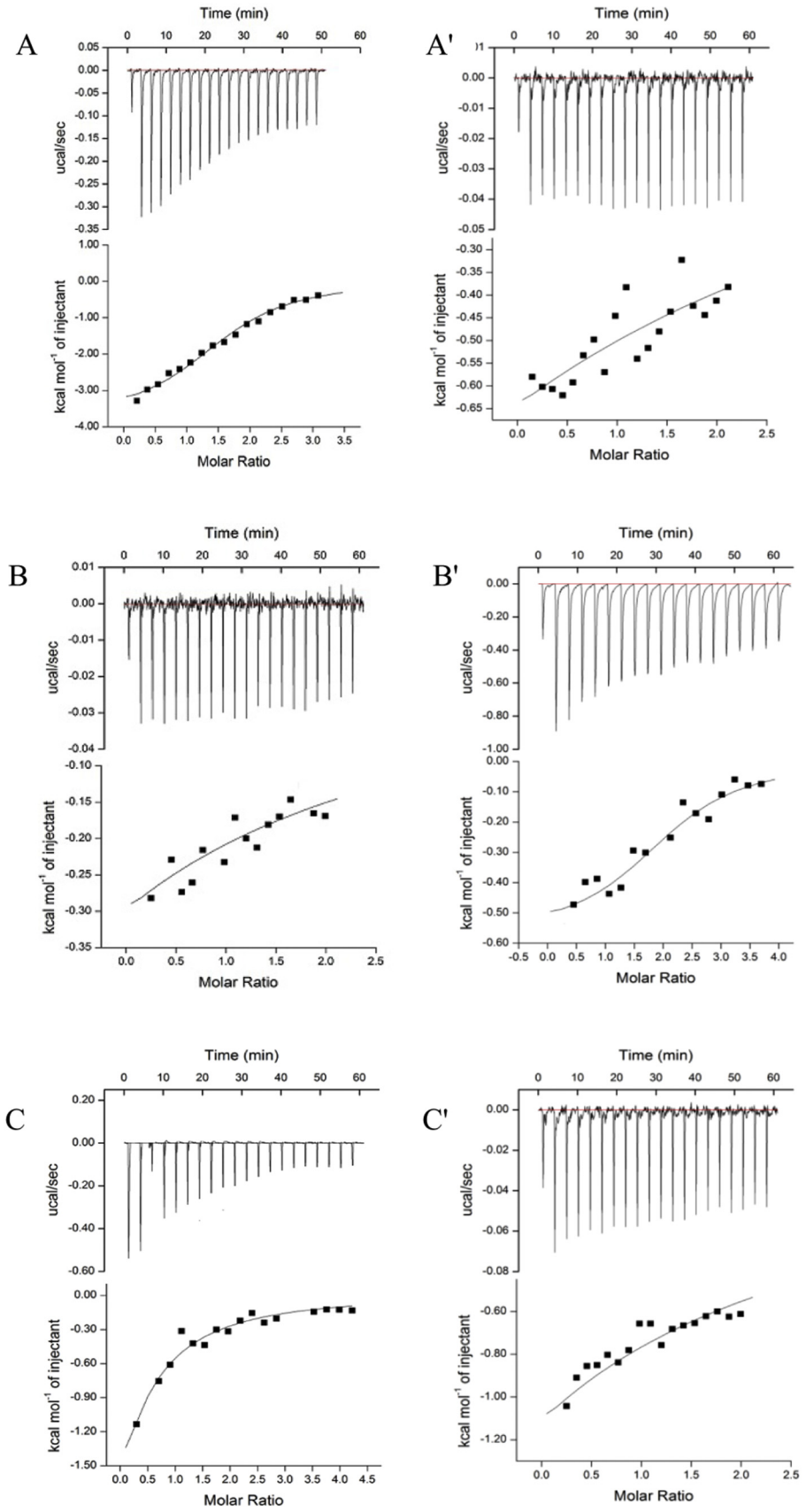

Fig. 5. Binding analysis of peptides to the pooled sera using ITC. The IgE-binding capacity to wild-type peptide 3 (A), 6 (B), 7 (C), 9 (D), 11 (E) and mutated peptide 3–2 (A'), 6–2 (B'), 7–3 (C'), 9–3 (D'), 11–2 (E') was deliberated by the use of ITC. The top panel shows the raw titration data measured in μcal/s. Each peak corresponds to a single injection of peptide into the pooled sera. The bottom graph shows integrated heat data after correction for background effects.

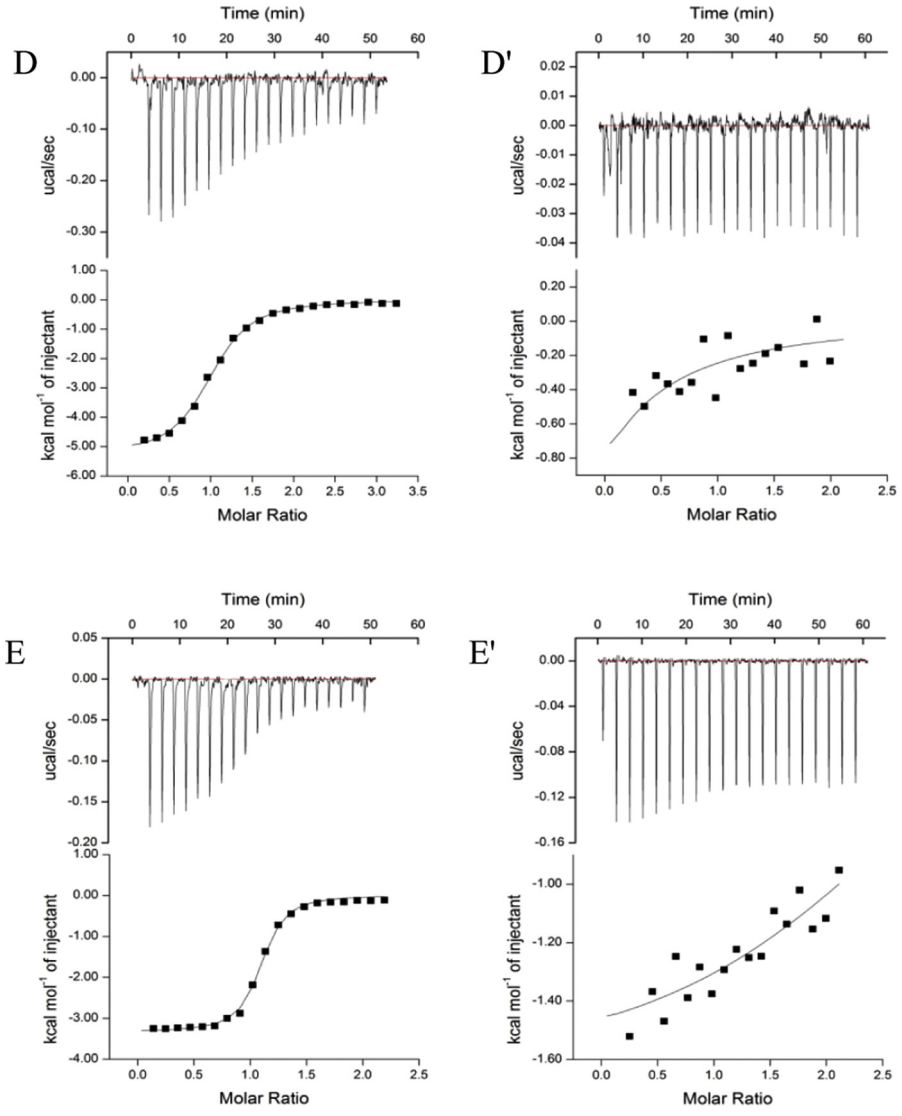

Fig. 5. (*continued*)

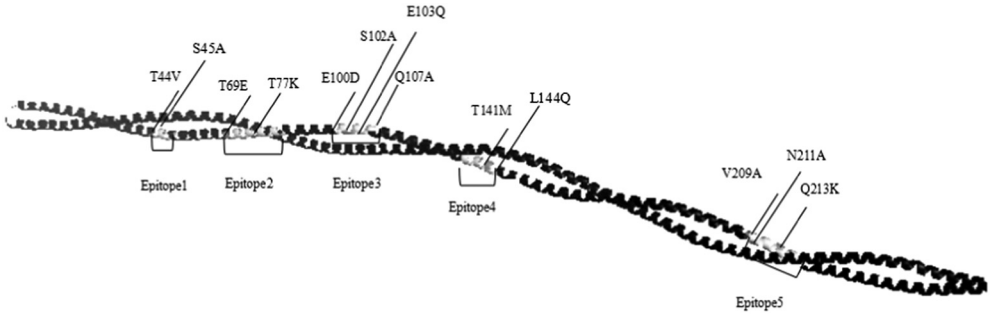

Fig. 6. The three-dimensional structure of *Cra g* 1 based on homologous simulations was drawn by cartoon image. The major IgE-binding epitope regions and critical amino acid positions that were mutated were labeled.

2.9. Homology-based model of Cra g 1

Because the three-dimensional structure of *Cra g* 1 has not been characterized, the crystal structure of tropomyosin from *Sus scrofa* (PDB code 1C1G) was used as the template for homology-based modeling. *Cra g* 1 was modeled as dimer using SWISS-MODEL online program (https://swissmodel.expasy.org/interactive). Images of *Cra g* 1 and positional distribution of each IgE-binding epitope were drawn by Pymol.

2.10. Statistical analysis

All data were expressed as means ± standard deviation. Statistical analysis was performed using one-way analysis of variance, followed by Student's t-test. Significant difference in means between the samples was determined at a 5% confidence level ($P < .05$).

3. Results and discussion

3.1. Prediction of the IgE-binding epitopes

Hydrophilicity, accessibility, flexibility and antigenicity of protein provide important information for epitope prediction. DNAstar Protean system could obtain different epitopes using the above variables. As shown in Fig. 1, the hydrophilic regions occupy > 95% of the sequence while hydrophobic regions are only a small part, indicating that *Cra g* 1 is a hydrophilic molecule. Furthermore, surface and flexible regions that almost distributed throughout the amino acid sequence and high antigenic index demonstrated that the tropomyosin was ape to form linear epitopes. Based on these characteristics, eleven epitopes, with 8 to 33 residues in length, were yielded by DNAstar Protean system (Table 1). Moreover, Using Bioinformatics Predicted Antigenic Peptides (BPAP) system, eleven antigenic regions, between 7 and 22 residues in length, were recognized as B cell epitopes. Ten epitopes from 10 to 17 residues in length were defined by the BepiPred 1.0 Server (Table 1). Altogether, the final predicted epitopes were developed with the approach of Yang et al. [26] in accordance with the results of three immunoinformatics tools. Thirteen peptides from 6 to 17 residues in length, named peptide 1 to peptide 13, will be the potential IgE-binding epitopes of *Crassostrea gigas* tropomyosin and be synthesized on the grounds of amino acid sequence (Table 2).

3.2. Identification of the immunodominant Cra g 1 epitopes

In an effort to find out which of the 13 predicted epitopes were immunodominant, these synthesized peptides were probed with a pool of sera from 15 oyster-allergic subjects and individual serum from 8 randomly selected subjects whose serum was included in the pool. Serum IgE from control subjects with no oyster hypersensitivity did not specifically bind any of the 13 peptides tested (data not shown). Using the pooled sera, five peptides showed greater inhibition of IgE binding than other peptides ($P < .05$) by inhibition ELISA (Fig. 2 A), implying that peptide 3, 6, 7, 9 and 11 contained the immunodominant *Cra g* 1 epitopes with position at *Cra g* $1^{44–49}$, *Cra g* $1^{69–85}$, *Cra g* $1^{99–108}$, *Cra g* $1^{134–144}$ and *Cra g* $1^{209–224}$, respectively. On the other side, the majority of individual serum showed similar results that were found with the pooled sera (Fig. 2 B, C, E, G, H and I), except 2 patient's serum (Fig. 2 D and F). The same pattern of inhibitive IgE binding was observed in 6 of 8 individual patients as well as the pooled sera, suggesting this approach should be adequate for determining the clinical dominance of the epitopes. Additionally, amino acid composition was analyzed in the five major epitopes. Fourteen amino acids were found existing in these epitopes with the absence of tryptophan (W), cysteine (C), histidine (H), glycine (G), phenylalanine (F) and proline (P), while glutamic acid (E), threonine (T), serine (S) and glutamine (Q) were relatively abundant (Fig. 2 J). Hence, it might be deduced that negatively charged amino acids (E) and polar, uncharged amino acids (T, S, Q) are easily to form IgE-binding regions in oyster tropomyosin.

Epitopes include linear and three-dimensional epitopes. Tropomyosin of invertebrate is apt to form linear epitopes, because it is a flexible and stretched molecule without complicated advanced structure. Mapping linear epitopes has been deployed extensively through various immunoinformatics strategies instead of overlapping peptides scan. In present study, five major *Cra g* 1 epitopes have been identified, which are almost distributed along the whole protein. Peptide 3, 7 and 9 spanning from six to eleven amino acid residues are similar to IgE-binding regions of other allergens in length [6,7,31], whereas peptide 6 and 11 contained 17 and 16 residues may be longer than their epitopes and the smallest sequences to bind IgE should be further identified. Previous literature have reported that latex allergen *Hev b* 5 and shrimp allergen *Pen a* 1 present amino acid sequences motif XEEX and LEXXL [10,31], respectively. Motif XEEX are also shared by the immunodominant *Cra g* 1 epitopes with the exception of peptide 3. However, amino acid sequence of type XEEX was also found in peptide 2 and 8 that showed weaker inhibition of IgE reactivity (Fig. 2), thus this motif was not critical amino acid sequence for recognition of sera IgE from oyster-allergic patients.

Cra g 1 is highly conserved protein and contains 284 amino acid residues. Amino acid sequence of *Cra g* 1 shared 61.6–78.9% identity and 75.7–88.4% similarity compared with those from selected shellfish, and exhibited over 49.6% identity and 70.8% similarity to the human and rabbit (Fig. 3). Currently, several IgE-binding regions of shellfish tropomyosins have been partially determined, such as *Pen a* 1 from shrimp [10], *Tur c* 1 from snail [32] and *Cra g* 1 from oyster [14]. In this study, peptide 7 partially overlaps with previously characterized epitope of *Cra g* $1^{92–105}$ (IQLLEEDMERSEER) [14], but other four major epitopes (peptide 3, 6, 9 and 11) have not been reported. Besides, three main epitope regions (peptide 3, 7 and 9) were found to partially overlap with those of *Pen a* 1, which would provide further molecular basis for observed clinically relevant cross-reactivity between crustaceans and mollusks. Remarkably, *Tur c* $1^{245–284}$ and *Pen a* $1^{247–284}$ seemed to be of particular importance, because the sera from snail-allergic and shrimp-allergic patients could significantly bind this region, respectively [10,32]. However, there was no immunodominant epitope in the same region of *Cra g* 1, although the identity and similarity of homologous sequences were high (Fig. 3).

3.3. Critical amino acids determined for five major epitopes

The most frequently used method to determine the amino acids critical for IgE binding is to substitute with alanine at each position [6,8,33]. Instead of conservative substitutions, amino acids located at homologous positions of vertebrate tropomyosins were introduced into allergenic epitopes, which would retain its natural structure. Sequence identity and similarity between *Cra g* 1 and other tropomyosins (*Homo sapiens* and *Oryctolagus cuniculus*) ranged from 49.6–53.5% and 70.8–73.9%, respectively (Fig. 3). As a result, a total of 16 mutant peptides were designed by replacing the amino acids in *Cra g* 1 major epitopes with homologous amino acids of human and rabbit tropomyosin, which was summarized in Table 3. These peptides were probed with pooled 15 patients' serum to elucidate whether the changes would affect oyster-specific IgE binding. Fig. 4 shows the results of inhibition ELISA containing the native and mutant peptides of five epitopes. In comparison with the native peptides, the combinatorial substitutions (position 44, 45 in peptide 3; 69, 77 or 73, 80, 84 in peptide 6; 100, 102, 103, 107 or 102, 107 in peptide 7; 137, 140, 141, 144 or 141, 144 in peptide 9 and 209, 211, 213 or 216, 217, 220 or217, 220, 224 in peptide 11) showed significant reduction of IgE-binding capacity ($P < .05$) especially modified peptide 3–2, 6–2, 7–3, 9–3 and 11–2, but they could not be totally mutagenized to non-IgE binding peptides with the substitutions. There was no obvious position in each peptide that would lead to diminish IgE binding.

Next, the IgE reactivity to mutant peptides that had the most significant effects was further deliberated by isothermal titration microcalorimetry (ITC). ITC is a calorimetric analysis technique. The changes in heat of the reaction system are measured, which could be used to indicate the affinity between receptors and ligands. The top diagram demonstrated that IgE and native peptide reacted rapidly, while the bottom diagram indicated the effective binding isotherms of IgE-peptide interactions after correction for background (Fig. 5 A, C, D and E). On the contrary, the modified peptides dramatically reduced binding with the pooled sera compared with native peptides (Fig. 5 A', C', D' and E'). The results agreed with those of inhibition ELISA. Surprisingly, an interesting finding was the peptide 6–2 seemed to increase IgE reactivity than peptide 6 in ITC assay (Fig. 5 B and B'), which was contrary to the experimental results of ELISA. This discrepancy might be due to the characteristics of peptide 6 and different conditions in the two assays.

In previous study, the results of amino acid substitutions analysis of

major shrimp allergen (*Pen a* 1) illustrated that a single substitution was not sufficient to markedly reduce IgE binding [20]. Therefore, at least two amino acid substitutions were introduced into each *Cra g* 1 epitope to inhibit IgE reactivity. Similar observation was obtained for the cow's milk allergen, α_{s1}-casein, for which combination of two or more substitutions was necessary to render epitopes non-IgE binding [27]. On the other hand, among a total of 60 amino acids presented in the five immunodominant epitopes that were studied, 25 were charged (42%), 11 were hydrophobic residues (18%) and 49 were polar (82%) (Fig. 2 J). At least 22 amino acids were identified as being critical, of which polar residues appeared most frequently (20/22) especially glutamic acid (5/22) and threonine (5/22). This phenomenon was consistent with results previously obtained in the analysis of critical amino acids of shrimp *Penaeus monodon* allergen [11], whereas hydrophobic residues occurred to be the most common for IgE binding in milk allergen α_{s1}-casein [27] and peanut allergen *Ara h* 1 [7]. At the meanwhile, homologous substitutions in the center of *Cra g* 1 epitopes seemed to have the same effects as those found in the peripheral parts, this was also observed for the peanut allergen *Ara h* 2 [6]. The predominant effect of the homologous substitutions on major epitopes was to decrease affinity of oyster-specific IgE antibodies, however, this manner did not abolish binding completely, implying a more complicated recognition pattern between *Cra g* 1 epitopes and specific IgE than we expected. Future work will be directed towards constructing recombinant, native and mutated oyster tropomyosins and studying differences in allergenicity through cell and animal models.

3.4. Location of the IgE-binding epitopes on the three-dimensional structure of *Cra g* 1

A homology model of *Cra g* 1 was developed. The best template structure was tropomyosin from *Sus scrofa*, which was deposited on a Protein Data Bank as 1C1G. The model of *Cra g* 1 three-dimensional structure is similar to those of other homologous molecules that have been solved, 5 major epitopes and the most critical amino acids at positions 44, 45, 69, 77, 100, 102, 103, 107, 141, 144, 209, 211 and 213 have been labeled (Fig. 6).

Tropomyosins are coiled-coil dimers, which made up of parallel α-helical molecules that associated with actin in muscle and non-muscle cells. As for oyster tropomyosin, it is a stretched and very flexible protein, hydrophilic regions occupy almost 95% of the whole sequence. Five immunodominant linear epitopes are evenly distributed on the surface of the molecule, containing approximately 60 residues. Hence, we speculated that the 5 epitopes, particularly 13 most critical amino acids exposed on the surface, could interact with serum IgE through hydrogen bonds and electrostatic attraction. Mutations that altered polarity (T44 V, S45A, S102A, Q107A, L144Q and N211A) and charges (T69E, T77K, E103Q and Q213K) of critical residues in the identified epitopes could partially breakdown the interactions between IgE and *Cra g* 1, resulting in a more efficient and obvious reduction of IgE-binding. Besides, although there are several enzyme cleavage sites throughout the protein, the formation of polymers by intra- or intermolecular crosslinking during cooking could protect the molecule from digestion and allow passage through small intestine [34], which could further explain the extreme allergenicity of oyster tropomyosin. In order to expound the molecular mechanism of interactions between *Cra g* 1 and oyster-specific IgE, the next steps would try to crystallize and resolve the complex of two proteins.

Till now, the only therapeutic option of managing food allergy is avoidance. However, oyster, as a ubiquitous food, is inevitably consumed, leading to frequent occurrence of severe clinical symptoms observed in patients with oyster hypersensitivity. To our knowledge, we have, for the first time, identified the five immunodominant epitopes and amino acids essential to IgE binding and illustrated the positional distribution of these epitopes on *Cra g* 1 molecule. Our experimental results not only provide a better understanding of shellfish allergy but also have significance in clinical diagnosis and immunotherapy.

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References

[1] R. Kumar, Food allergy in bronchial asthma: diagnostic modalities, Indian J. Allergy Asthma Immunol. (2014) 27.
[2] M. Toomanian, A novel model of sensitization and oral tolerance to peanut protein, Immunology 113 (2010) 293–303.
[3] F. Rance, X. Grandmottet, H. Grandjean, Prevalence and main characteristics of school children diagnosed with food allergies in France, Clin. Exp. Allergy 35 (2005) 167–172.
[4] I. Gonzalez Galan, J.M. Garcia Menaya, G. Jimenez Ferrera, G. Gonzalez Mateos, Anaphylactic shock to oysters and white fish with generalized urticaria to prawns and white fish, Allergol. Immunopathol. 30 (2002) 300–303.
[5] R. Castillo, J. Delgado, J. Quiralte, C. Blanco, T. Carillo, Food hypersensitivity among adult patients: epidemiological and clinical aspects, Allergol. Immunopathol. 24 (1996) 93–97.
[6] J.S. Stanley, N. King, A.W. Burks, S.K. Huang, H. Sampson, G. Cockrell, et al., Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2, Arch. Biochem. Biophys. 342 (1997) 244–253.
[7] D.S. Shin, C.M. Compadre, S.J. Maleki, R.A. Kopper, H. Sampson, S.K. Huang, et al., Biochemical and structural analysis of the IgE binding sites on ara h1, an abundant and highly allergenic peanut protein, J. Biol. Chem. 273 (1998) 13753–13759.
[8] R.M. Helm, G. Cockrell, C. Connaughton, C.M. West, E. Herman, H.A. Sampson, et al., Mutational analysis of the IgE-binding epitopes of P34/Gly m Bd 30K, J. Allergy Clin. Immunol. 105 (2000) 378–384.
[9] G. Ball, M.J. Shelton, B.J. Walsh, D.J. Hill, C.S. Hosking, M.E. Howden, A major continuous allergenic epitope of bovine beta-lactoglobulin recognized by human IgE binding, Clin. Exp. Allergy 24 (1994) 758–764.
[10] R. Ayuso, S.B. Lehrer, G. Reese, Identification of continuous, allergenic regions of the major shrimp allergen pen a 1 (tropomyosin), Int. Arch. Allergy Immunol. 127 (2002) 27–37.
[11] L.N. Zheng, H. Lin, R. Pawar, Z.X. Li, M.H. Li, Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools, Food Chem. Toxicol. 49 (2011) 2954–2960.
[12] H.R. Myrset, B. Barletta, G. Di Felice, E. Egaas, M.M. Dooper, Structural and immunological characterization of recombinant Pan b 1, a major allergen of northern shrimp, *Pandalus borealis*, Int. Arch. Allergy Immunol. 160 (2013) 221–232.
[13] Y.L. Liang, M.J. Cao, W.J. Su, L.J. Zhang, Y.Y. Huang, G.M. Liu, Identification and characterization of the major allergen of Chinese mitten crab (*Eriocheir sinensis*), Food Chem. 111 (2008) 998–1003.
[14] M. Ishikawa, Y. Nagashima, K. Shiomi, Identification of the oyster allergen crag 2 as tropomyosin, Fish. Sci. 64 (1998) 854–855.
[15] K. Shiomi, Y. Sato, S. Hamamoto, H. Mita, K. Shimakura, Sarcoplasmic calcium-binding protein: identification as a new allergen of the black tiger shrimp *Penaeus monodon*, Int. Arch. Allergy Immunol. 146 (2008) 91–98.
[16] R. Ayuso, G. Grishina, L. Bardina, T. Carrillo, C. Blanco, M.D. Ibáñez, et al., Myosin light chain is a novel shrimp allergen, lit v 3, J. Allergy Clin. Immunol. 122 (2008) 795–802.
[17] K.D. Garcia-Orozco, E. Aispuro-Hernandez, G. Yepiz-Plascencia, A.M. Calderon-dela-Barca, R.R. Sotelo-Mundo, Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*, Int. Arch. Allergy Immunol. 144 (2007) 23–28.
[18] Y. Shen, M.J. Cao, Q.F. Cai, W.J. Su, H.L. Yu, W.W. Ruan, et al., Purification, cloning, expression and immunological analysis of *Scylla serrata* arginine kinase, the crab allergen, J. Sci. Food Agric. 91 (2011) 1326–1335.
[19] A.L. Lopata, S.B. Lehrer, New insights into seafood allergy, Curr. Opin. Allergy Clin. Immunol. 9 (2009) 270–277.
[20] G. Reese, J. Viebranz, S.M. Leong-Kee, M. Plante, I. Lauer, S. Randow, et al., Reduced allergenic potency of VR9-1, a mutant of the major shrimp allergen pen a 1 (tropomyosin), J. Immunol. 175 (2005) 8354–8364.
[21] M. Ishikawa, K. Shimakura, Y. Nagashima, K. Shiomi, Isolation and properties of allergenic proteins in the oyster, Fish. Sci. 63 (2008) 610–614.
[22] H. Bian, J.F. Reidhaar-Olson, J. Hammer, The use of bioinformatics for identifying class II-restricted T-cell epitopes, Methods 29 (2003) 299–309.
[23] N. Salimi, W. Fleri, B. Peters, A. Sette, Design and utilization of epitopebased databases and predictive tools, Immunogenetics 62 (2010) 185–196.
[24] J. Söllner, B. Mayer, Machine learning approaches for prediction of linear B cell epitopes on proteins, J. Mol. Recognit. 19 (2006) 200–208.
[25] R. Ayuso, S.B. Lehrer, L. Tanaka, M.D. Ibanez, C. Pascual, W. Burks, et al., IgE antibody response to vertebrate meat proteins including tropomyosin, Ann. Allergy Asthma Immunol. 83 (1999) 399–405.
[26] X. Yang, X. Yu, An introduction to epitope prediction methods and software, Rev. Med. Virol. 19 (2009) 77–96.

[27] R.R. Cocco, K.M. Järvinen, H.A. Sampson, K. Beyer, Mutational analysis of major, sequential IgE-binding epitopes in alpha s1-casein, a major cow's milk allergen, J. Allergy Clin. Immunol. 112 (2003) 433–437.
[28] J. Wang, Y. Wang, Y. Pan, D. Chen, Z. Liu, L. Feng, et al., Preparation of a generic monoclonal antibody and development of a highly sensitive indirect competitive ELISA for the detection of phenothiazines in animal feed, Food Chem. 221 (2017) 1004–1013.
[29] X.L. Feng, S.Y. Lu, D. Liu, L. Li, X.Z. Wu, J. Song, et al., Direct competitive immunosorbent assay for detection of MEHP in human urine, Chemosphere 92 (2013) 150–155.
[30] X.Y. Meng, Y.S. Li, Y. Zhou, Y.Y. Zhang, L. Yang, B. Qiao, et al., An enzyme-linked immunosorbent assay for detection of pyrene and related polycyclic aromatic hydrocarbons, Anal. Biochem. 473 (2015) 1–6.
[31] D.H. Beezhold, V.L. Hickey, J.E. Slater, G.L. Sussman, Human IgE-binding epitopes of the latex allergen Hev b 5, J. Allergy Clin. Immunol. 103 (1999) 1166–1172.
[32] M. Ishikawa, M. Ishida, K. Shimakura, Y. Nagashima, K. Shiomi, Purification and IgE-binding epitopes of a major allergen in the gastropod *Turbo cornutus*, Biosci. Biotechnol. Biochem. 62 (1998) 1337–1343.
[33] R.M. Helm, G. Cockrell, C. Connaughton, H.A. Sampson, G.A. Bannon, V. Beilinson, et al., A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modeling, Int. Arch. Allergy Immunol. 123 (2000) 213–219.
[34] L. Fang, G. Li, R. Gu, M. Cai, J. Lu, Influence of thermal treatment on the characteristics of major oyster allergen Cra g 1 (tropomyosin), J. Sci. Food Agric. 98 (2018) 5322–5328.