

Identification of Allergenic Epitopes and Critical Amino Acids of Major Allergens in Chinese Shrimp (*Penaeus chinensis*) by Immunoinformatics Coupled with Competitive-Binding Strategy

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ABSTRACT: Chinese shrimp (*Penaeus chinensis*) is widely cultured and consumed in Asia but is also a major food allergen locally. Although they may be the foundation for preventing and treating allergies, the allergenic epitopes of the major allergens tropomyosin (TM) and arginine kinase (AK) in *Penaeus chinensis* have not been identified. Here, we applied Immunoinfo-CB (immunoinformatics coupled with competitive-binding strategy) to address the point. Potential allergenic epitopes of TM and AK were predicted by multiple immunoinformatics tools, followed by validating with inhibitory dot-blot assay, indirect competition ELISA, and mast cell degranulation assay. Furthermore, critical amino acids in allergenic epitopes were also identified by Immunoinfo-CB. Our findings provide new insight into allergenic epitopes and critical amino acids of TM and AK responsible for the anaphylactic response. The Immunoinfo-CB therefore offers promises for characterization of IgE-binding epitopes that might be used as new targets for immunotherapy of food allergy.

KEYWORDS: *Penaeus chinensis*, allergen, epitope, critical amino acids, Immunoinfo-CB

INTRODUCTION

Food allergy, an abnormal (immunological) response to certain food components, has emerged as a common public health concern both in developed and developing countries, where it has been estimated to affect around 2% of the adult population and up to 8% of children.¹ Fish and shellfish, as highly consumed foods in recent years, represent a valuable source of proteins for the general population.² Unfortunately, two of the main elicitors of food allergies worldwide are suggested to be fish and crustaceans.^{3,4} Among crustaceans, shrimps are the most predominant cause of allergic reactions affecting patients with life-long lasting symptoms and thus have been more extensively studied.⁵ Tropomyosin (TM) and arginine kinase (AK) have been identified as the major allergens of crustaceans by previous studies.^{6,7} The amino acid sequences of allergenic TM in different 12 shrimp species and AK in 9 species can be retrieved in the National Center for Biotechnology Information (NCBI) library. Two crystal structures of the Pacific whiteleg shrimp (*Litopenaeus vannamei*) AK, one in a binary complex with arginine (LvAK-Arg) and a ternary transition state analogue complex (TSAC), have been determined by the X-ray method;⁸ however, it can only be deduced that TM is a coiled-coil protein formed by two parallel α -helices with a molecular weight (MW) of approximately 36 kDa. Importantly, identification of antigenic epitopes is necessary for characterization of cross-reactivity, allergenicity, immunogenicity, and the inhibitory potential of allergens and thus better understanding of recognition and interaction mechanisms in the complex allergic reactions. So far, TM epitopes have been identified in some shrimp species, including *Litopenaeus vannamei*,⁹ *Penaeus monodon*,¹⁰ *Farfantepenaeus aztecus*,¹¹ and *Penaeus indicus*,¹² but AK epitopes study only in *Litopenaeus vannamei*.¹³ Chinese shrimp (*Penaeus chinensis*), which is

common in Asian diets, has higher consumption worldwide with the globalization of trade. Nevertheless, the epitopes of allergenic TM and AK in *Penaeus chinensis* have not been known. Clarifying the allergenic epitopes of shrimp proteins among different species provides more evidence for study of the relationship between protein structure and allergenicity.

Generally, some classic methods such as overlapping synthetic peptides,¹⁴ phage display,¹⁵ mass spectrometry,¹⁶ surface plasmon resonance,¹⁷ protein chip and nuclear magnetic resonance mapping¹⁸ can be employed to identify the allergenic epitopes. However, these methods are costly, time-consuming and also need more experienced practitioners to perform. Immunoinformatics, a newly developed branch of bioinformatics, has already become a familiar and useful tool for selecting epitopes from immunological proteins, which can lead to the targeted synthesis of potential epitope candidates by efficient prediction.^{19,20} After computational prediction of epitopes, the confirmatory experiments can be carried out based on the competitive binding assays of antigen-epitope peptides. In addition, further prediction and identification of critical amino acids in target epitopes can also be analyzed by the immunoinformatics coupled with competitive-binding strategy (Immunoinfo-CB).

In previous studies, the critical amino acids in epitopes mostly appeared as charged or aromatic amino acids, being in conjunction with nearby amino acids to affect the folding and hydrophilic of proteins or direct binding ability to IgE.^{21,22} When introducing a mutated critical amino acid, the stability of the epitope may

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alter and the binding ability to IgE probably reduces.²³ On the basis of the prediction of physicochemical properties, relative frequency, and conservation^{10,24–26} of different amino acids in the target epitopes, the critical amino acids can also be characterized by our established Immunoinfo-CB method.

The present work aims to characterize the IgE binding epitopes and critical amino acids of two major allergens, TM and AK, in Chinese shrimp (*Penaeus chinensis*). First, the potential allergenic epitopes were predicted by immunoinformatic analysis on the hydrophobicity, accessibility, antigenicity, and plasticity of the secondary structure of TM and AK, respectively, and then synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis method. The inhibitory dot-blot assay, icELISA (indirect competition ELISA) and LAD2 cell degranulation assay were employed to detect the binding affinity and antigenicity of the allergenic epitopes. Furthermore, the critical amino acids of the confirmed allergenic epitopes were also identified by the Immunoinfo-CB method.

MATERIALS AND METHODS

Human Sera and Ethics Statement. Serum samples of 30 subjects with confirmed clinical history of allergic responses to *Penaeus chinensis* were obtained as a normal routine procedure during the allergic disease diagnostic workup from the second affiliated hospital of Zhejiang University School of Medicine (Hangzhou, Zhejiang, China). Aliquots of these sera were stored at -80°C until further use. This procedure as well as the whole study were approved by Zhejiang Gongshang University Ethics Review Committee and Ethics Committee of Zhejiang University as it is part of a routine procedure where no additional consent is required by law.

Immunoinformatics Prediction. The amino acid sequences of TM (Pen c 1; GenBank, ADA70137.1) and AK (Pen c 2; GenBank, AAV83993.1) in *Penaeus chinensis* were obtained from Entrez protein database of NCBI (<http://www.ncbi.nlm.nih.gov/entrez>). To predict allergenic IgE linear epitopes, the complete sequences of TM and AK were analyzed using five immunoinformatics based computational approaches including DNASTar (DNAS- TAR, Madison, WI), AntheProt (Institute of Biology and Chemistry of Proteins, France), BepiPred 1.0 (<http://www.cbs.dtu.dk/services/BepiPred/>), ABCpred (<http://www.imtech.res.in/raghava/abcpred/dataset.html>), and Immunomedicine Group (<http://imed.med.ucm.es/Tools/antigenic.pl>). In the DNASTar Protean system and AntheProt 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 was performed by the methods of Hopp and Woods²⁷ and Kyte and Doolittle.²⁸ Moreover, the properties of flexibility, surface accessibility, and antigenicity were analyzed by the strategies of Karplus and Schulz,²⁹ Emini et al.,³⁰ and Jameson and Wolf,³¹ respectively. Peptide regions with high hydrophilicity, flexibility, surface accessibility, and antigenic index were chosen as candidate epitopes. On the other hand, BepiPred 1.0, ABCpred and Immunomedicine Group predict allergenic epitopes based on combination of physicochemical properties of amino acids (such as the secondary structure, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity). The amino acid sequences were put into these three tools, and the prediction were performed with default parameters. Finally, the results of the five immunoinformatics tools were combined, and allergenic epitopes predicted by no less than three tools were considered as candidates.

Peptides and Proteins. Peptides were synthesized using standard Fmoc solid phase peptide synthesis by GL Biochem Ltd. (Shanghai, China). The purity of the peptides was greater than 95% as assessed by high-performance liquid chromatography (HPLC). The molecular mass of the synthesized peptides was confirmed by electrospray ionization-mass spectrometry (ESI-MS). The peptides were stored at -20°C until used.

Codon optimized *Penaeus chinensis* TM and AK genes were directly synthesized and cloned into a prokaryotic expression vector, then the

recombinant TM and AK proteins were expressed and purified from *E. coli* by GenScript (NJ). KLH (Keyhole limpet hemocyanin) was obtained from Sigma (MO), and goat antihuman IgE serum from Novus Biologicals (CO).

Inhibitory Dot-Blot Assay. Inhibitory dot-blot assay was performed to determine the binding of IgE with the potential allergenic epitope peptides. Nitrocellulose membranes (0.45 μm pore size) were immersed in double-distilled water for 15 min. When the membranes were damp-dry, purified TM or AK samples dissolved in carbonate buffered saline (CBS, pH 9.6) were spotted on the membranes and left to dry at room temperature (4 μg for each dot) and blocked for 2 h in 3% BSA/PBST. Then the membranes were incubated with human sera (1:25) for 1.5 h at 37°C , which had been preincubated with indicated peptide (2 mg/mL with 2 mg/mL KLH) for 1 h at 37°C , followed by further incubating with HRP-labeled goat antihuman IgE serum (1:5000) for 1 h at 37°C . The membranes were then incubated in ECL reagent (Thermo Fisher Scientific Inc., MA) for 1 min and detected by Alpha Protein imager (ProteinSimple, CA). The membranes were washed for 5 min with PBST three times between each step and all dilutions were made in 2.5% BSA/PBS.

Indirect Competition Enzyme-Linked Immunosorbent Assay (icELISA). For peptide icELISA, 100 μL $\times$ 0.5 mg/mL of TM or AK protein were coated on 96-well plates in 0.05 M carbonate buffer overnight at 4°C . After blocking with 3% bovine serum albumin (BSA)/0.01 M pH 7.4 phosphate-buffered saline (PBS) for 2 h at 37°C , the plates were incubated with individual serum samples (1:50) and 100 μL of indicated peptide (2 mg/mL with 2 mg/mL KLH) for 1.5 h at 37°C . Meanwhile, negative control (KLH and serum), positive control (TM or AK and serum), and blank control (serum only) were also performed. Thereafter, the plates were incubated with HRP goat antihuman IgE serum (1:5000) for 1.5 h at 37°C . The plates were then developed with TMB substrate reagent set (BD Biosciences) in a dark environment for 15 min at 37°C and terminated by 2 M H_2SO_4 . Absorbance was measured at 450 nm by Versa max Microplate reader (Molecular Devices, Sunnyvale). The plates were washed for 1 min with PBST (0.5% Tween 20 in PBS) three times between each step and all dilutions were made in 3% BSA/PBS. The ability to bind Shrimp allergic-IgE antibody was determined using the equation: inhibition rate = $[1 - \text{OD}_{\text{inhibitor}}/\text{OD}_{\text{blank}}] \times 100\%$.

LAD2 Cell Degranulation Assay. LAD2 (Laboratory of Allergic Diseases 2) cells (ATCC) were cultured in RPMI 1640 medium (GIBCO, Los Angeles, CA), supplemented with 9% (v/v) fetal bovine serum, and 1% (v/v) penicillin–streptomycin at 37°C with 5% CO_2 . For degranulation assay, cells were seeded in 10 cm dishes with 1×10^6 cells/mL and incubated for 2 h with shrimp allergenic sera (1:100). After centrifuged at 900g for 5 min at room temperature, cells were washed and resuspended in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.6 mM glucose, 1.8 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4% BSA, pH 7.4) and stimulated for 30 min with indicated peptide and KLH at 37°C /5% CO_2 . Stimulation with KLH alone was performed as negative control and that with C48/80 (Sigma, St. Louis, MO) as positive control. β -Hexosaminidase released into the supernatants and in total cell lysates was quantified by the hydrolysis of *p*-nitrophenyl *N*-acetyl-*b*-*D*-glucosamide (Sigma, St. Louis, MO) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37°C . The reaction was terminated by Tris-HCl (1 M, pH 9.0) and measured by absorbance at 405 nm. The percentage of β -hexosaminidase released was calculated as a percentage of the total content.³² Cell-free supernatants were isolated and analyzed for the release of histamine and tryptase using Human HIS ELISA Kit (BIM, San Francisco, CA) and Human Tryptase (MCT) ELISA Kit (BIM, San Francisco, CA).

Identification of Critical Amino Acids in Allergenic Epitopes.

The amino acid composition and frequency of occurrence in whole protein (TM or AK), all potential allergenic epitopes and the selected allergenic epitope were calculated using BioEdit (Department of Microbiology, North Carolina State University, Raleigh, NC),¹⁰ and the amino acids with the highest frequency were chosen. Sequences of TM and AK in all the allergenic foods in Structural Database of Allergenic Proteins (SDAP) (<http://Fermi.utmb.edu/SDAP/index.html>) were also investigated (Table 1), and the high conservative amino acids were identified by Vector NTI (Invitrogen Corp., CA). The common

Table 2. Allergenic Epitopes Predicted by Each Immunoinformatics Tools

	software	epitope position
tropomyosin	Immunomedicine Group	46–64, 73–86, 100–108, 131–145, 151–167, 177–183, 185–192, 232–245
	BepiPred	16–43, 55–59, 76–85, 99–108, 112–128, 131–147, 158–164, 176–188, 209–223, 236–241, 243–259, 261–276
	ABCpred	10–25, 19–34, 27–42, 38–53, 44–59, 58–73, 75–90, 82–97, 93–108, 100–115, 106–121, 117–132, 132–147, 153–168, 162–177, 169–184, 187–206, 209–224, 215–230, 221–236, 232–247, 248–263, 263–282
	DNAstar	12–25, 50–65, 70–85, 87–105, 111–128, 130–147, 150–167, 185–203, 220–235, 245–280
	AntheProt	8–25, 43–65, 73–85, 87–105, 105–125, 179–192, 187–202, 221–239, 243–260, 263–280
arginine kinase	Immunomedicine Group	19–37, 46–71, 73–93, 105–113, 115–127, 135–141, 150–160, 177–187, 193–203, 216–222, 238–251, 257–273, 277–290, 296–308
	BepiPred	19–20, 40–45, 58–63, 66–74, 94–106, 112–119, 130–137, 139–151, 153–154, 161–165, 170–176, 203–213, 275–278, 295–299, 309–324
	ABCpred	3–9, 14–30, 27–43, 37–53, 61–77, 69–85, 82–98, 91–107, 100–116, 116–131, 123–143, 139–155, 145–161, 151–167, 164–179, 179–195, 185–211, 203–219, 209–225, 216–231, 228–244, 234–250, 243–259, 250–266, 258–274, 273–289, 282–298, 290–306, 301–317, 309–325, 320–336, 330–346, 340–356
	DNAstar	22–33, 37–47, 55–66, 94–107, 122–135, 140–154, 159–169, 172–184, 222–231, 250–265, 287–298, 337–347, 350–356
	AntheProt	4–45, 51–63, 65–72, 91–107, 121–137, 137–147, 149–166, 249–267, 287–335, 337–347, 350–356

Table 3. Allergenic Epitopes Predicted by Combined Immunoinformatics Tools

	peptide	sequence	position	length (no. of amino acids)
tropomyosin	T-01	KLEKDNAMDRADTL	12–25	14
	T-02	LEQQNKKEANNRAEKSE	25–40	16
	T-03	QKRMQQLENDLDQV	47–60	14
	T-04	EKDKALSNAEGEV	73–85	13
	T-05	ALNRRIQLEEDLERSEER	87–105	19
	T-06	KLAEASQAADSESRMRK	112–128	17
	T-07	ENRSLSDDEERMDALENQ	131–147	17
	T-08	AEEADRKYDEVAR	155–167	13
	T-09	ESKIVELEELRVVGNN	187–203	17
	T-10	YKEQIKTLTNKLKAAEARA	221–239	19
	T-11	ERSVQKLQKEVDRLEDE	243–259	17
	T-12	EKEKYKSITDELDTQTFSE	263–280	18
arginine kinase	A-01	AATDCKSLLKKYLTK	19–33	15
	A-02	QSGVENLD SGVGIYAPDA	55–72	18
	A-03	VGFKQTDKHPNKDFGDV	91–107	17
	A-04	TRVRCGRSMEGYP	123–135	13
	A-05	LTEDQYKEMESKVSS	140–154	15
	A-06	SLEGELKGTYP	158–169	12
	A-07	VNEIEKRIPFSHHDRLG	250–266	17
	A-08	KLPKLAANRDKLEE	286–299	14
	A-09	GGIYDISNKRMRGL	320–333	14
	A-10	QAVKEMQDGILELIKMEKEM	337–356	20

predicted by immunoinformatics tools, an inhibitory dot-blot assay was performed based on the competitive interaction between potential epitope peptides and purified whole allergen protein on the IgE from specific allergic sera. As shown in Figure 2, compared with the negative control (carrier protein KLH only), strong inhibition was evident with positive control (purified TM or AK protein itself), and the peptides presented different degree of inhibition among the 30 sera. T-06, T-07, T-012, and A-01, A-05 were critical to IgE binding, as they can inhibit the reactions between the allergen protein and all the 30 sera (Figure 2). In contrast, T-02, T-04 and A-03, A-06, A-10 displayed less IgE binding activity, indicating these regions were not overlapped with the epitopes of *Penaeus chinensis*.

Validation of Allergenic Epitopes by icELISA. In addition to the semiquantitative analysis by inhibitory dot-blot assay, based on the similar mechanism, we further quantified the allergenicity of the potential allergenic epitopes by icELISA. Compared with

the negative control KLH, most allergenic epitopes showed significant effects ($P < 0.01$) on inhibiting the interaction between specific allergic sera and allergen protein, suggesting them to be allergenic. Consistent with dot-blot results, several epitopes including T-02, T-04, A-03, A-06, and A-10 did not show inhibitory effects, we considered these epitopes to be false positive results from immunoinformatics prediction. Overall, by icELISA, 10 peptides of TM (T-01, T-03, T-05, T-06, T-07, T-08, T-09, T-10, T-11, and T-12) and seven peptides of AK (A-01, A-02, A-04, A-05, A-07, A-08, A-09) were verified as allergenic epitopes (Figure 3).

Validation of Allergenic Epitopes by Mast Cell Degranulation Assay. Mast cells play a central role in allergic and inflammatory disorders by degranulation that releases inflammatory mediators, so the mast cell degranulation assay is usually employed to measure the allergenicity of potential allergens or epitopes. Compared with competitive-binding (CB) methods (i.e., inhibitory dot-blot assay and icELISA), mast cell based

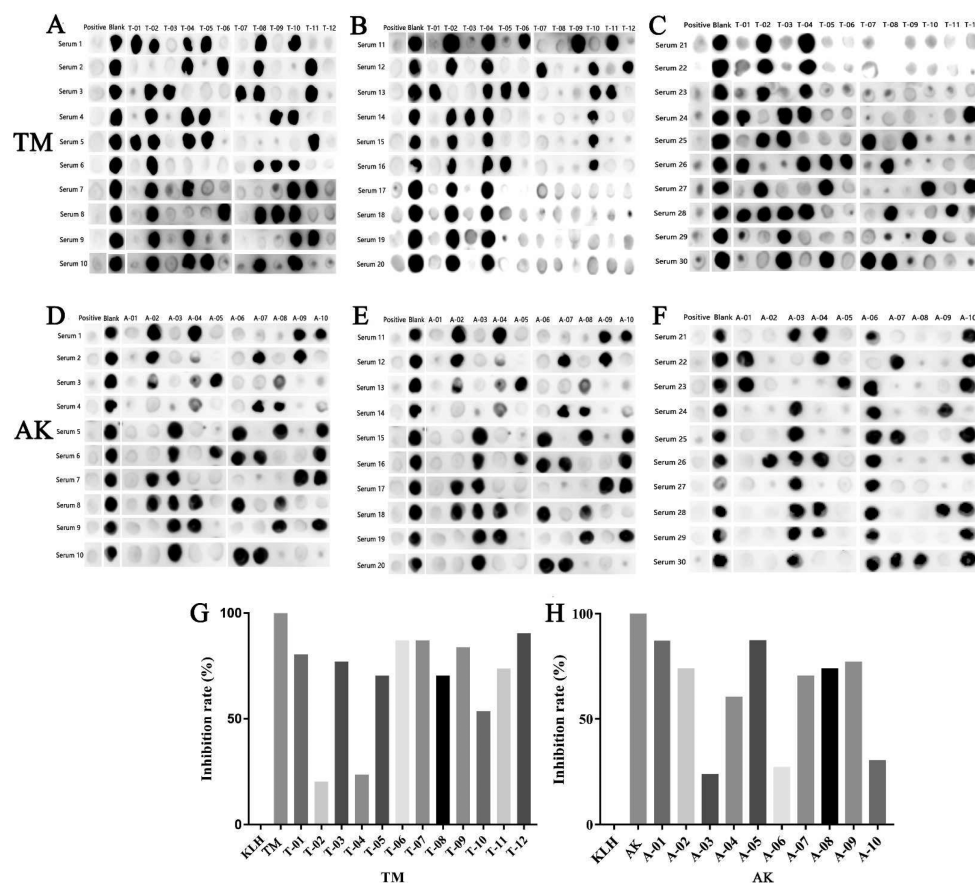


Figure 2. Validation of allergenic epitopes by inhibitory dot-blot assay. (A–C) Allergenicity of potential allergenic epitopes of TM (T-01 to T-12) were measured by inhibitory dot-blot assay with individual shrimp-allergic sera. (D–F) Allergenicity of potential allergenic epitopes of AK (A-01 to A-10) were measured by inhibitory dot-blot assay with individual shrimp-allergic sera. (G) Summarized inhibitory dot-blot assay results of potential TM allergenic epitopes. (H) Summarized inhibitory dot-blot assay results of potential AK allergenic epitopes. KLH: carrier protein Keyhole limpet hemocyanin, negative control.

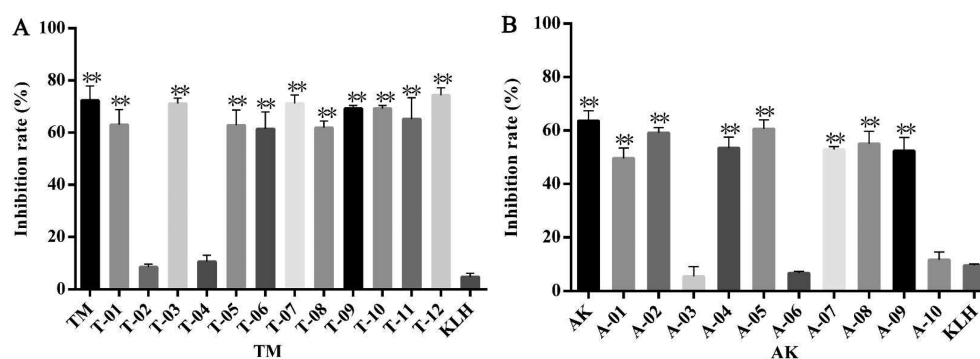


Figure 3. Validation of allergenic epitopes by icELISA. (A) Allergenicity of potential allergenic epitopes of TM (T-01 to T-12) were measured by icELISA. (B) Allergenicity of potential allergenic epitopes of AK (A-01 to A-10) were measured by icELISA. KLH: carrier protein Keyhole limpet hemocyanin, negative control. ** $p < 0.01$ compared with negative control.

degranulation assay is a more straightforward method and has more biological relevance. To test whether potential allergenic peptides of AK and TM could induce human mast cell LAD2 degranulation, we determined three biomarkers (histamine, β -hexosaminidase enzyme, and tryptase) of LAD2 cells. C48/80 was used as a positive control because it is a potent activator to increase intracellular Ca^{2+} concentration, leading to degranulation in mast cells. In total, 12 TM peptides (T-01 to T-12) and 10 AK peptides (A-01 to A-10) were screened for their ability to induce human mast cell degranulation using that assay. Following stimulation of LAD2 cells with identical concentrations of each

peptide, biomarkers, especially the release of β -hexosaminidase, showed that not all peptides were capable of inducing LAD2 degranulation. Compared with negative control (KLH), peptides that show a significant difference ($p < 0.01$) were considered to be allergenic epitopes. Overall, peptides T-01, T-03, T-05, T-06, T-07, T-08, T-09, T-10, T-11, T-12 of TM and A-01, A-02, A-04, A-05, A-07, A-08, A-09 of AK induced significant degranulation (Figure 4). The results of the three biomarkers showed a high degree of consistency, and the results were also consistent with CB results, reflecting the robustness of these determination approaches and the reliability of the results.

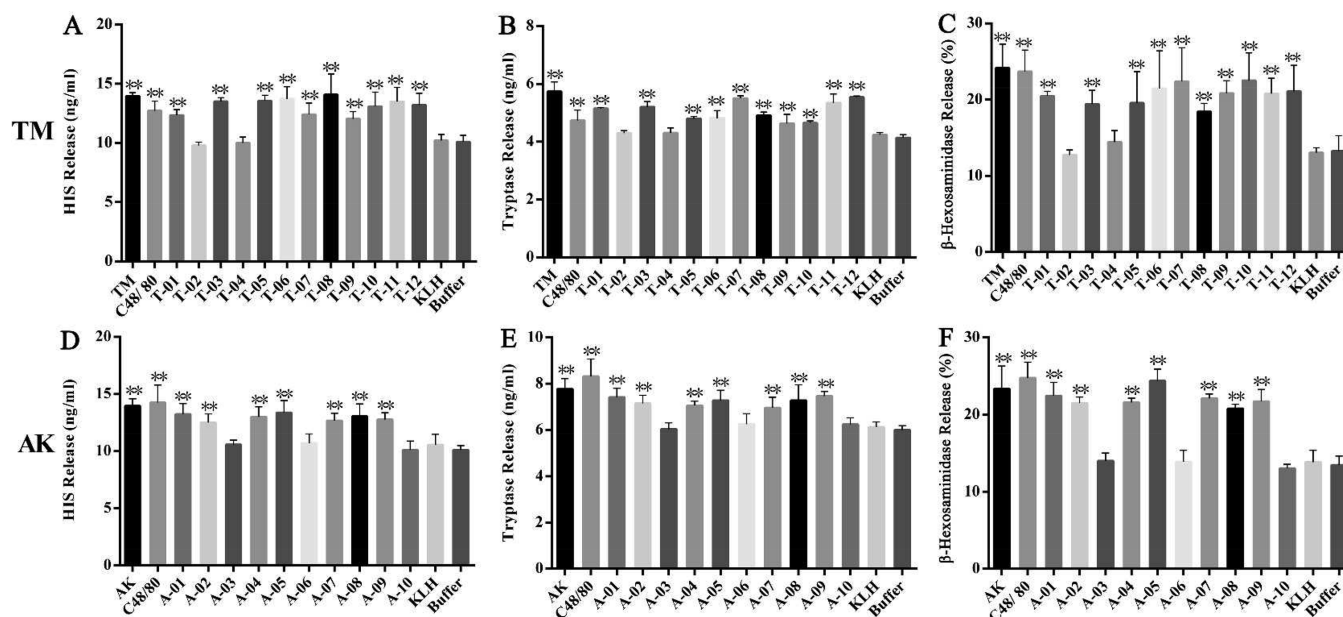


Figure 4. Validation of allergenic epitopes by mast cell degranulation assay. LAD2 cell degranulation induced by potential allergenic epitopes of TM (T-01 to T-12) were measured by the release of histamine (A), tryptase (B), and β -hexosaminidase (C). LAD2 cell degranulation induced by potential allergenic epitopes of AK (A-01 to A-10) were measured by the release of histamine (D), tryptase (E), and β -hexosaminidase (F). KLH: carrier protein Keyhole limpet hemocyanin, negative control. C48/80: positive control. ** $p < 0.01$ compared with negative control.

Table 4. Mutated Peptides for Critical Amino Acids Validation

	original sequence	mutated point	mutated sequence	mutant name
tropomyosin	QKRMQQLENDLDQV	E-G (54)	QKRMQQLENDLDQV	T-03-E
		D-G (58)	QKRMQQLENDLAQV	T-03-D
	ENRSLSDDEERMDALENQ	E-G (131, 139)	ANRSLSDAERMDALGNQ	T-07-E
		D-G (142)	ENRSLSDDEERMAALENQ	T-07-D
		R-G (140)	ENRSLSDDEERMDALENQ	T-07-R
		Q-G (147)	ENRSLSDDEERMDALENA	T-07-Q
	EKEKYKSITDELDTQTFSE	E-G (263, 265)	AKAKYKSITDELDTQTFSG	T-12-E
		D-G (272, 275)	EKEKYKSITAELAQTFSE	T-12-D
		Y-G (267)	EKEKAKSITDELDTQTFSE	T-12-Y
arginine kinase	QSGVENLD SGVGIYAPDA	S-G (56, 64)	QAGVENLDGAVGIYAPDA	A-02-S
		L-G (61)	QSGVENADSGVGIYAPDA	A-02-L
		I-G (67)	QSGVENLD SGVGAYAPDA	A-02-I
		D-G (62)	QSGVENLADSGVGIYAPGA	A-02-D
		Y-G (68)	QSGVENLD SGVGIAPDA	A-02-Y
	LTEDQYKEMESKVSS	L-G (140)	ATEDQYKEMESKVSS	A-05-L
		S-G (153, 154)	LTEDQYKEMESKVA	A-05-S
		Y-G (145)	LTEDQAKEMESKVSS	A-05-Y
		K-G (146, 151)	LTEDQYAKEMESAVSS	A-05-K

Identification of Critical Amino Acid Residues for Allergenicity. After identifying the allergenic epitopes, we tried to go further by determining the critical amino acid residues responsible for the allergenicity of the proteins and epitopes and developed a screening method based on Immunoinfo-CB. We first predicted critical amino acid residues by immunoinformatic approaches (see Table 4). Each amino acid frequency in *Penaeus chinensis* allergenic epitopes was calculated using BioEdit software and their conservation across different species was analyzed using Vector NTI software. As shown in Figure 5A,B, amino acids glutamic acid (E), isoleucine (I), arginine (R), glutamine (Q), serine (S), aspartic acid (D), and tyrosine (Y) in TM and leucine (L), isoleucine (I), arginine (R), lysine (K), serine (S), aspartic acid (D), and tyrosine (Y) in AK performed higher frequency occurrence in

allergenic epitopes than in the whole protein. On the other hand, multiple sequence alignment of TM and AK from allergenic foods in Structural Database of Allergenic Proteins (SDAP) and *Penaeus chinensis* with Vector NTI discovered that D, E, K, N in T-03, C, D, E, L, R in T-07, and most amino acids in T-12, A-02 and A-05 were conserved in all the species (Figure 5C,D). Subsequently, amino acids selected in both methods above were regarded as critical amino acid candidates. To validate the importance of the candidates, we changed these amino acids into alanine in the allergenic peptides and determined the allergenicity of the mutant peptides by icELISA with specific sera. Compared with whole protein or wild-type peptides, mutant peptides showed significantly impaired allergenicity ($p < 0.01$). Specifically, only peptides T-03-D, T-07-E, T-07-D, T-12-E, A-02-L, A-02-D, and A-05-L

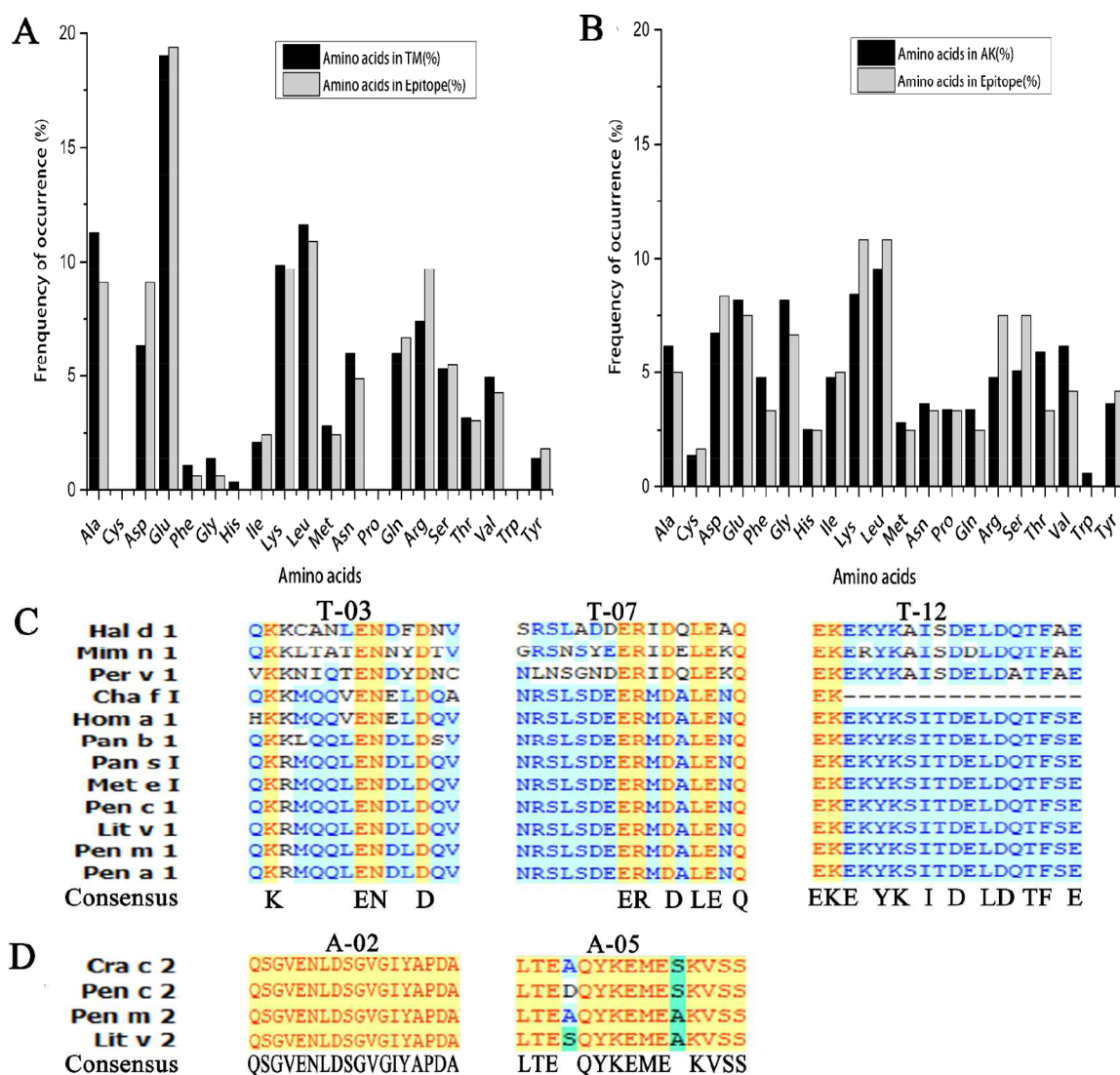


Figure 5. Identification of critical amino acids for allergenicity by immunoinformatics tools. (A) Amino acids frequency of TM whole protein and allergenic epitopes were measured by BioEdit. (B) Amino acids frequency of AK whole protein and allergenic epitopes were measured by BioEdit. (C) Conservative property of amino acids in TM allergenic epitopes across different species were measured by Vector NTI. (D) Conservative property of amino acids in AK allergenic epitopes across different species were measured by Vector NTI.

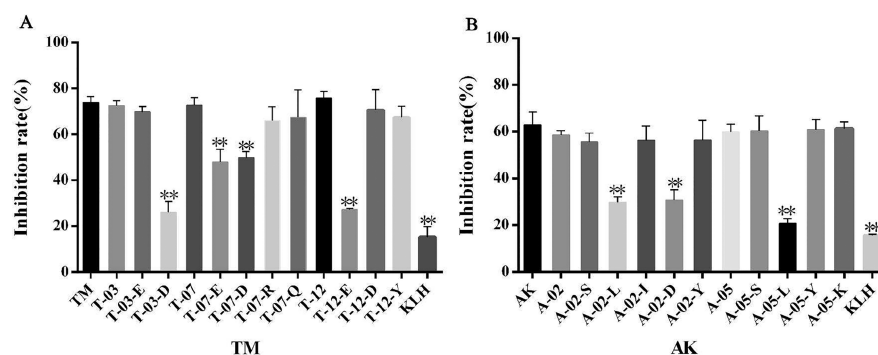


Figure 6. Validation of critical amino acids for allergenicity by icELISA. (A) Allergenicity of potential allergenic epitopes of TM with indicated amino acid mutants were measured by icELISA. (B) Allergenicity of potential allergenic epitopes of AK with indicated amino acid mutants were measured by icELISA. KLH: carrier protein Keyhole limpet hemocyanin, negative control. ** $p < 0.01$ compared with positive control (wild type peptide).

showed greatly impaired allergenicity (Figure 6). Overall, Immunoinfo-CB approach showed that D in T-03, E, D in T-07, E in T-12, L, D in A-02, and L in A-05 were critical amino acids for the allergenicity of *Penaeus chinensis* allergen TM or AK.

DISCUSSION

In recent years, *Penaeus chinensis* allergy has caused serious physical illness along the coast of China where the shrimp is largely consumed. Unfortunately, so far there is no research on

the antigenic epitopes of *Penaeus chinensis*, though relevant studies are required for preventing and treating allergy. In previous studies, synthetic overlapping peptides spanning the whole sequence of the allergen was the most common method, which required the synthesis of a large number of peptides.³³ Nowadays, other new powerful tools like one-bead-one-compound (OBOC) combinatorial peptide library have also been developed to identify allergenic epitopes.³⁴ Although these methods improve the accuracy and throughput of the detection of epitopes, they will spend a lot of time and effort and the cumbersome operation cannot be widely used. Luckily, with the development of immunoinformatics method, the prediction of allergenic epitopes is becoming easier, which further reduces the number of candidate epitopes that need to be synthesized and validated. In the present study, we mainly focused on the allergenic epitopes of TM and AK in *Penaeus chinensis* and predicted 12 and 10 potential antigenic epitopes by the immunoinformatics tools, respectively.

After prediction, we synthesized peptides of these potential antigenic epitopes and further qualitatively validated the allergenicity by ELISA and dot-blot. Because of the short length and high hydrophilicity of the synthesized peptides, they cannot bind well with the general solid carriers such as enzyme plates and blotting membranes. As a result, we applied competitive-binding (CB) methods including iELISA and inhibitory dot-blot assay to overcome these problems, in which the peptides are not immobilized to solid carriers. Dot-blot is one of the most common method for screening epitopes,³⁵ by using inhibitory dot-blot assay, 10 of the 12 TM potential epitopes and 7 of the 10 AK potential epitopes were identified as the major epitopes. The further iELISA analysis obtained a consistent and more precise result.

We termed the method containing immunoinformatics prediction and competitive-binding methods validation as Immunoinfo-CB (immunoinformatics coupled with competitive-binding strategy), which is fast, efficient, and economical for epitope identification.

In addition to competitive-binding methods, we further validated the Immunoinfo-CB results by human mast cells LAD2 degranulation assay, which assessed the direct interaction between specific IgE and allergenic epitope on the cell level, and thus had more biological relevance. The consistent results further confirmed the reliability of the results.

To validate the specificity of our results, we established the 3D structure of *Penaeus chinensis* TM and AK based on the *Sus scrofa* TM sequence and *Litopenaeus vannamei* AK by homology modeling and illustrated the location of allergenic epitopes identified in this study. These allergenic epitopes showed high overlapping ratio with previously reported allergenic TM or AK epitopes including *Met e 1*,³⁶ *Pen a 1*,¹¹ *Pen m 1*,¹⁰ and *Litopenaeus vannamei* arginine kinase,⁹ indicating that our Immunoinfo-CB methods are reliable and accurate. Furthermore, allergenic epitopes in the present study showed higher coverage than any others in previous study, suggesting the high efficiency of our method (Figure 7).

Some amino acid residues play critical roles in affecting the conformation of proteins and epitopes, which is tightly correlated with allergenicity. Therefore, locating the critical amino acids in allergens is important for understanding and controlling protein allergenicity. On the basis the allergenic epitope results, we further determined the critical amino acids by Immunoinfo-CB. We take the lead in software prediction by amino acid frequency and conservation, combining with specific site mutation method validation. The analysis of the frequency and

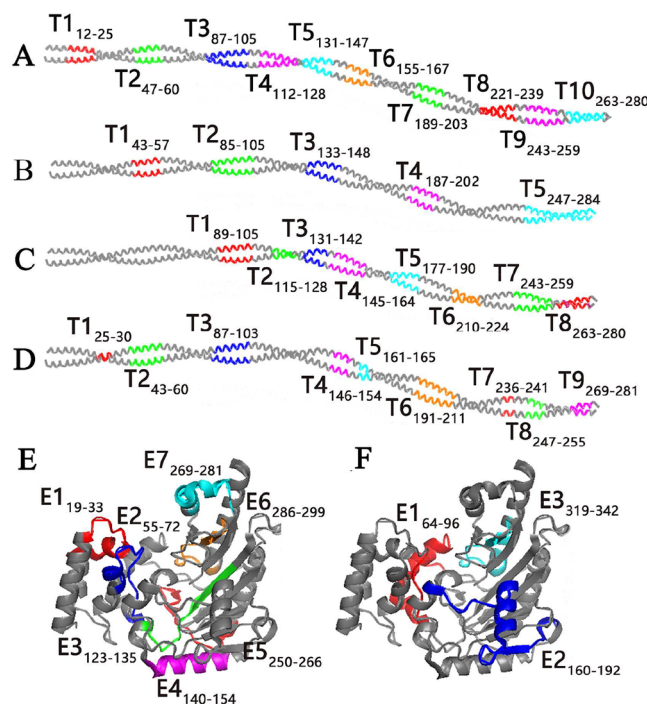


Figure 7. Location and structure of shrimp allergenic epitopes. (A) Position of allergenic epitopes determined in the present study on *Penaeus chinensis* TM protein 3D structure model. (B) Position of major allergenic epitopes on *Pen a 1*. (C) Position of major allergenic epitopes on *Pen m 1*. (D) Position of major allergenic epitopes of *Met e 1*. (E) Position of allergenic epitopes determined in the present study on *Penaeus chinensis* AK protein 3D structure model. (F) Position of major allergenic epitopes of *Lit v 1* AK.

conservativeness of amino acids in the epitope makes the prediction of critical amino acids more accurate, while avoiding the complex operation of individual amino acid mutations.²³ Amino acid analysis demonstrated that D, E, and L were the critical amino acids in epitopes. Leucine (L) as a neutral hydrophobic amino acid, which plays an important role in epitope activity, may constitute specific conformation with other amino acids and maintain the secondary structure. Aspartic acid (D) and glutamic acid (E) are acidic negatively charged amino acids. The negatively charged point may also increase the allergenicity of the peptide. Finally, the potential critical amino acids were verified by iELISA, and the predominant amino acids in each epitope were revealed.

In conclusion, as a regional shrimp species, *Penaeus chinensis* allergy has not been intensively investigated. In recent years, epitopes were predicted and screened in a variety of ways, but the accuracy of prediction and the efficiency of detection were still limited. In the present study, we use immunoinformatics approaches to quickly predict antigen epitopes and optimize screening methods to improve the detection efficiency. Based on this, we revealed allergenic epitopes of major allergens in *Penaeus chinensis*, as well as the critical amino acids within them. This study will advance our knowledge on *Penaeus chinensis* and even all the food allergens, and shed light on the prevention and therapy of shrimp allergy as well as the elimination of allergenicity during food processing. Furthermore, our results provided Immunoinfo-CB as an advanced and effective method for allergenic epitopes and critical amino acids identification, which could be widely applied in allergen investigation and other immunology studies.

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Notes

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ABBREVIATIONS USED

TM, tropomyosin; AK, arginine kinase; KLH, Keyhole limpet hemocyanin; icELISA, indirect competition enzyme-linked immunosorbent assay; Immunoinfo-CB, the immunoinformatics coupled with competitive-binding strategy; CBS, carbonate buffered saline; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LAD2, Laboratory of Allergic Diseases 2

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