



Hypoallergenic mutants of the major oyster allergen Cra g 1 alleviate oyster tropomyosin allergenic potency

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ARTICLE INFO

Article history:

Received 16 June 2020

Received in revised form 30 July 2020

Accepted 31 July 2020

Available online 03 August 2020

Keywords:

Cra g 1

Hypoallergen

IgE

RBL-2H3 cells

Mediator

ABSTRACT

Design of hypoallergen with low IgE reactivity is desirable for allergen-specific immunotherapy. Despite oyster tropomyosin (Cra g 1) is considered as the major allergen, no immunotherapy is available now. In the current research, we generated hypoallergens of Cra g 1 and evaluated their allergenicity. Four hypoallergenic derivatives were constructed by epitope deletion or site-directed mutagenesis on grounds of the identified epitopes. They showed obvious reduction in reactivity towards IgE from oyster-allergic patients and Cra g 1-sensitized BN rats, as well as significant decrease in degranulation and secretion of allergic mediators including histamine, IL-4, IL-6 and TNF- α . In addition, to further investigate the molecular mechanism, we examined the effects of these variants on Fc ϵ RI-dependent signalling pathway in IgE-challenged RBL-2H3 cells. We found that the hypoallergenic mutants were able to attenuate Fc ϵ RI-mediated signaling cascades in tested cells. These results indicate that the hypoallergenic molecules have ideal characteristics and offer a promising new strategy in clinical immunotherapy for shellfish-allergic subjects.

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1. Introduction

Food allergy is a life-threatening disorder, which seriously impacts on the normal life of allergic patients [1]. Oyster is popular seafood in the world with high nutritional value [2]. With an increase in oyster production and consumption, the comprehensive studies of oyster-related allergic reactions are necessary [3]. 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 [4–7]. Among them, tropomyosin is the major allergen with a frequency of 80% in shellfish-allergic subjects [8]. Oyster tropomyosin, named Cra g 1, forms coiled-coil dimer and causes type I allergic reactions mediated by IgE [9].

Although avoidance of allergic foods is currently the most effective treatments to prevent allergies, allergen-specific immunotherapy is the main strategy to modify the diseases [10,11]. Natural allergens have allergic side-effects during clinical application, so hypoallergens with low IgE reactivity are needed [12,13]. Reese et al. introduced 12 point mutations into the identified epitopes of the shrimp allergen Pen a 1 through homologous substitution to generate hypoallergenic mutant [12]. Christine et al. also applied the same concept to construct

hypoallergenic proteins of shrimp allergen Met e 1 by point mutations or epitopes deletion [13]. However, the hypoallergenic derivative of Cra g 1 has not been reported so far. In order to design safer immunotherapy regimens for oyster allergic patients, molecular characterization of Cra g 1 IgE-binding epitopes is essential. Our previous experiments have already demonstrated five major epitopes and key amino acids (underlined amino acids) in each epitope of the protein (epitope 1, TSLQKK; epitope 2, TKLEEAAKTASEAEQEL; epitope 3, LEEDMERSEERLQTA; epitope 4, NNASEERTDVL; epitope 5, VQNDQASQREDSYEET) [14]. Furthermore, tropomyosin is highly conserved protein in invertebrate and vertebrate, and vertebrate tropomyosins are regarded as non-allergenic molecules [8,15]. We tried to produce hypoallergenic variants of Cra g 1 by substitution with vertebrate homologous sequences in IgE-binding epitopes identified, or by deleting these epitopes. Such mutated proteins are expected to reduce allergenicity as much as possible and will have significance for oyster allergy treatments.

Mast cells activation mediated by the high-affinity IgE receptor (Fc ϵ RI) acts as central role in allergic inflammatory reactions [16]. Mast cells signal transduction is initiated following aggregation of Fc ϵ RI at the cell surface. Recent studies suggested that Fc ϵ RI-dependent degranulation was regulated through PI3K and PLC γ signalling pathways [17,18]. After stimulation with antigen, Fc ϵ RI interacts with cytosolic adaptor molecules including Lyn, Syk, Fyn, resulting in activation of downstream LAT, PI3K and PLC γ [19]. Ultimately, these signalling cascades cause cell release of β -hexosaminidase that is a

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marker of degranulation and secretion of other chemokines and cytokines such as histamine, IL-4, IL-6 and TNF- α [19]. The inflammatory mediators are now thought to contribute to immediate symptoms of IgE-mediated allergic responses. Hence, one of the aims of this study was to investigate whether the hypoallergenic derivatives weakened Fc ϵ RI-dependent signalling pathway in IgE-sensitized RBL-2H3 cells.

In this communication, we attempted to construct four hypoallergenic oyster tropomyosins according to the five major IgE-binding epitopes identified, and to evaluate the allergic potential of them. These results may be applied in clinical immunotherapy for oyster-allergic subjects and provide valuable insights for management of other related allergic diseases.

2. Materials and methods

2.1. Subjects' sera

Serum samples from 15 oyster-allergic subjects were purchased from PlasmaLab International (USA). All 15 subjects had a clinical history of at least two or more symptoms within 1 h of oyster intake, including pruritus, allergic rhinitis, gastrointestinal disease and diarrhea. A serum pool from these patients was made by mixing equal aliquots of IgE. The specific IgE reactivity to purified oyster tropomyosin was characterized by immunoblot analysis. Negative control sera were obtained from 3 subjects without adverse reactions after ingestion of shellfish. All sera were stored at -80°C until used. All experiments were approved by medical ethics committee at the China National Research Institute of Food and Fermentation Industries (Approval number: 2020-0005).

2.2. Animal sensitization and tissue staining

Female Brown Norwegian rats (BN rats) had no abnormal reactions after one week of feeding. They were randomly divided into two groups (12 rats in each group): Cra g 1 sensitization group and PBS control group. Negative serum was obtained before immunization. The experimental group was sensitized by intragastric administration combined with intraperitoneal injection (Fig. 1), and blood samples were collected 4 h after the immunization to determine antibody levels. The same operation was carried out in the control group. A serum pool from sensitized rats was made by mixing equal aliquots of serum IgE. Finally, small intestinal tissue was taken for hematoxylin-eosin staining to observe pathological changes and toluidine blue staining to observe the degranulation of jejunal mast cells on day 42. The experiments were approved by medical ethics committee at the China National Research Institute of Food and Fermentation Industries.

2.3. Design of hypoallergenic oyster tropomyosins

Previous experiments have identified five major IgE-binding epitopes and key amino acids in the epitopes of Cra g 1 protein [14]. Because vertebrate tropomyosins have been regarded as non-allergenic proteins, we compared Cra g 1 with vertebrate muscle tropomyosins of Atlantic salmon (GenBank accession number NP_001117128.1), rabbit (GenBank accession number AAK77199) and human (GenBank accession number AAB59509.1). Five IgE-binding epitopes in Cra g 1 were partly converted into the homologous sequence of fish, rabbit or human tropomyosins and total 32, 34 and 13 point mutations were introduced to generate mutants CG2, CG3 and CG4, respectively. A truncated mutation, named CG1, was also constructed by deleting the identified epitopes (Fig. 2A and B). The amino acid sequences of CG1-4 were reverse translated using MEGA 5.0 and the corresponding encoding sequences were commercially synthesized (Genscript Co., Ltd., China).

2.4. Purification of recombinant wild type and mutant oyster tropomyosins

The gene was cloned into pET21(a) vector and verified by sequencing, then the recombinant plasmid was transformed into BL21 (DE3), plated on ampicillin-containing plates, and cultured overnight at 37°C . Single colonies were inoculated into 20 mL of ampicillin-containing LB medium overnight at 37°C with shaking. 10 mL of overnight culture was transferred to 1 L fresh LB medium and grown at 37°C . When the $\text{OD}_{600\text{ nm}}$ reached 0.6, 0.5 mM IPTG was added to induce expression. After culturing at 18°C for 18 h, the cells were harvested by centrifugation, resuspended in solution (20 mmol L^{-1} Tris-HCl, pH 8.0, 100 mmol L^{-1} NaCl) and ultrasonicated. The protein solution after centrifugation was loaded onto a Ni^{2+} charged Hi-Trap chelating HP column of an ÄKTA-fast protein liquid chromatography (FPLC) system. After washing the column, the bound protein was eluted with a linear 20–500 mmol L^{-1} gradient of imidazole in the same buffer. To further purify the protein, the concentrated elution was applied onto a gel-filtration Hi-load Superdex-75 column equilibrated with a buffer containing 50 mmol L^{-1} Tris-HCl, pH 7.5, 100 mmol L^{-1} NaCl and 1 mmol L^{-1} DTT. All purification procedures described here were performed at 4°C . Protein was analyzed on 15% SDS-PAGE and stored at -80°C until used.

2.5. Conformational analysis of wild type protein and hypoallergenic mutants

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 by SWISS-MODEL online program (<https://>

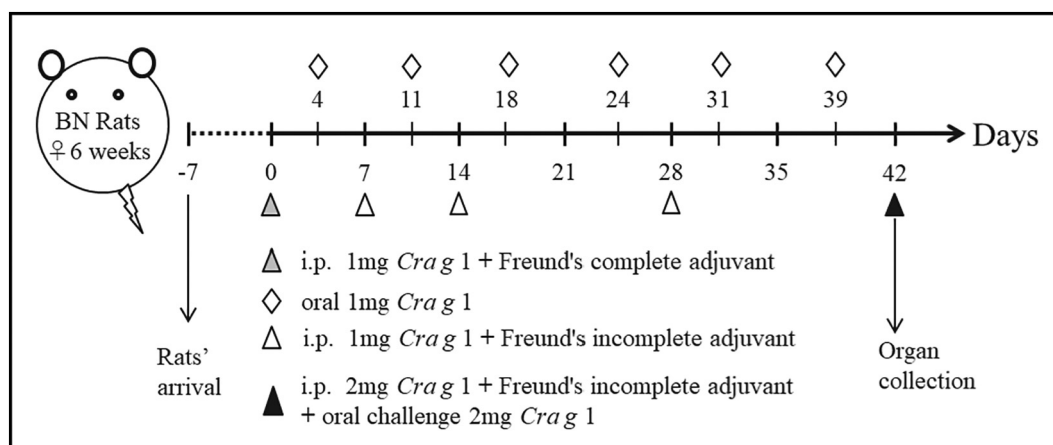
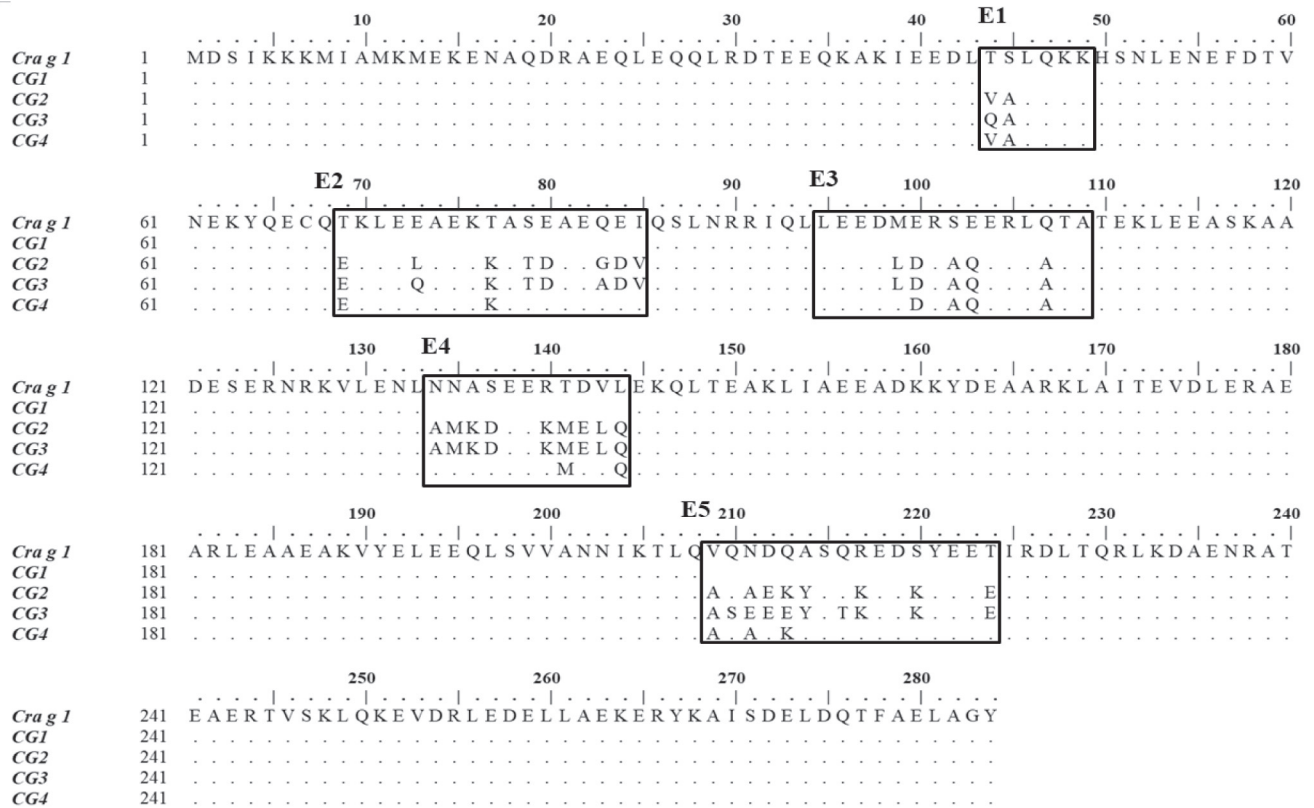
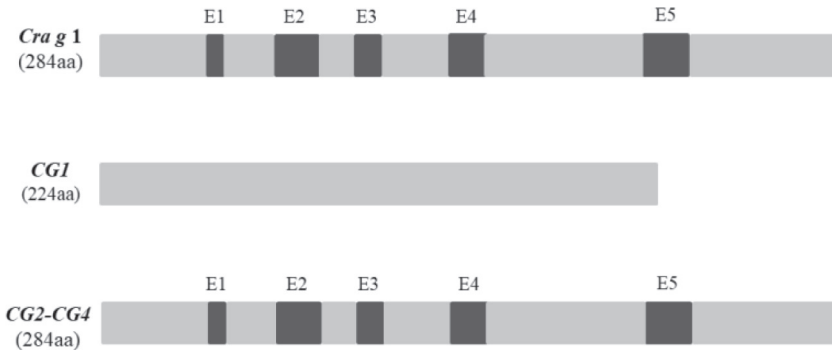


Fig. 1. Sensitization process in BN rats. The 'i.p.' stands for intraperitoneal injection.

A



B



C

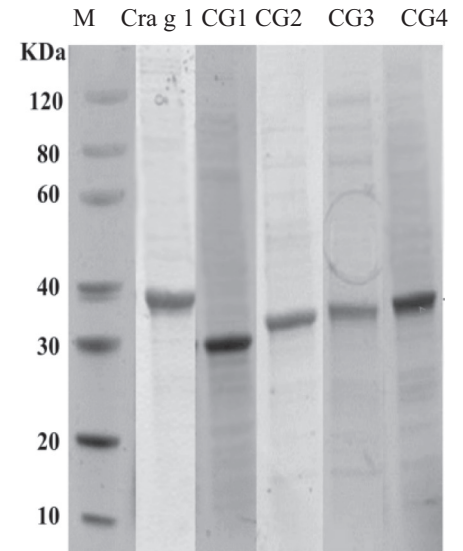
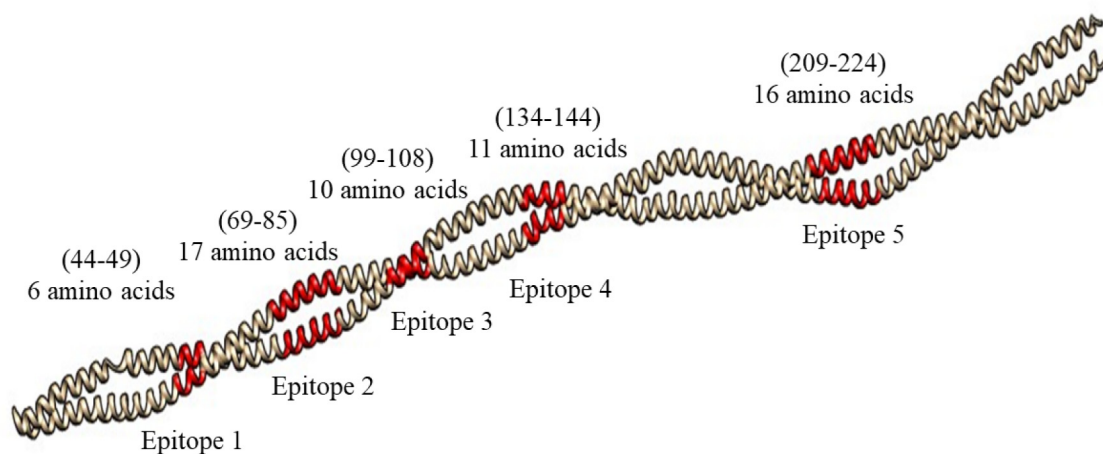


Fig. 2. Design of four hypoallergenic tropomyosin mutants. (A) Location of the five epitopes in tropomyosin. The epitopes designated as E1–E5 are shown in boxes and the location of the amino acid residues that are converted in CG2, CG3 and CG4 are also shown as one letter amino acid code. CG1 was constructed by deleting five epitopes. Identical residues are shown as ‘.’. (B) Schematic representation of Cra g 1 and CG1–CG4. Epitopes E1 to E5 in Cra g 1 and CG2–CG4 are represented as black boxes. CG1 is a truncated protein without identified epitopes. (C) SDS-PAGE of the purified Cra g 1, CG1, CG2, CG3 and CG4. M, molecular mass marker.

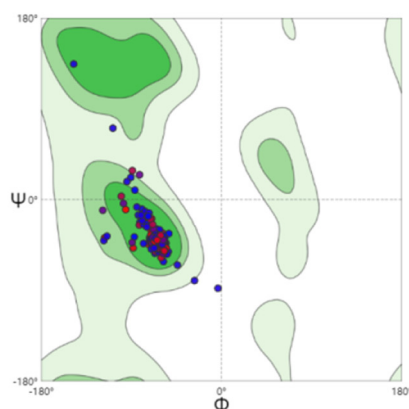
swissmodel.expasy.org/interactive), and the model quality was assessed by Ramachandran Diagram. Image of Cra g 1 and positional distribution of each IgE-binding epitope was drawn by Pymol.

The CD spectrum of protein sample (0.5 mg mL^{-1}) was measured with a Jasco J-810 Spectropolarimeter (Jasco Inc., USA) and the average of three spectra was taken as the final data. For wavelength analysis,

A



B



C

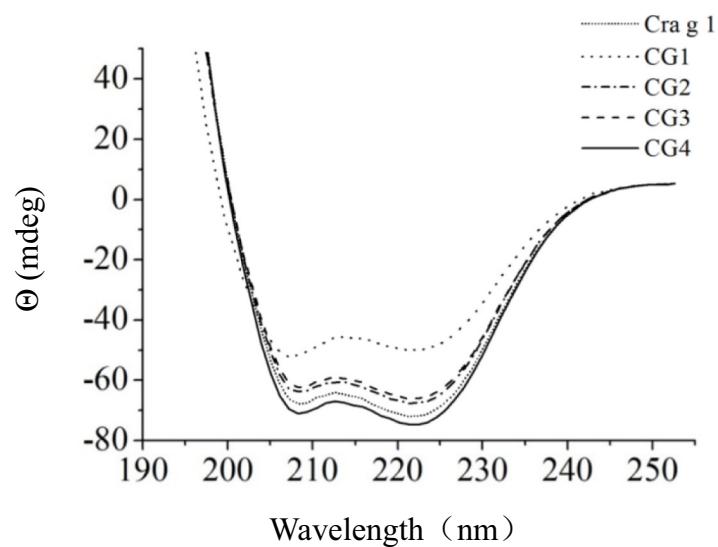


Fig. 3. Conformational analysis of wild type and hypoallergenic mutant proteins. (A) The three-dimensional structure of Cra g 1 based on homology simulation is drawn by cartoon image. Five major IgE-binding epitopes are labeled. (B) The Ramachandran Diagram of Cra g 1 model. A plot of the ψ and ϕ angles for the amino acids in Cra g 1 three-dimensional structure model is shown. Each dot within boxes represents an amino acid. (C) The CD spectra of Cra g 1 and its mutants. (D) Surface hydrophobicity of Cra g 1 and its mutants. * $P < 0.05$ vs. Cra g 1, ** $P < 0.01$ vs. Cra g 1. Data are presented as the mean $\pm$ SD ($n = 3$).

Fig. 3 (continued).

samples were scanned with step increments of 0.2 nm and a band width of 1.0 nm. The spectral range was 195–260 nm and the scanning speed was 200 nm min⁻¹. The baseline spectrum of buffer was subtracted from each spectrum and the resultant values were converted to molar ellipticity (θ) using the Jasco software. The hydrophobic surface was monitored by ANS-binding fluorescence using an F-4600 fluorescence spectrophotometer (Hitachi Inc., Japan). 1.0 mL of different samples was mixed with 40 μ mol L⁻¹ ANS for 30 min at 25 °C prior to measurement. Following reaction, F-4600 was used to measure the relative fluorescence intensities of solution with an excitation wavelength of 370 nm and emission wavelength of 490 nm. The surface hydrophobicity was determined as the initial slope of the curve plot *versus* protein concentration. The experiment was performed in triplicate and data were given as the mean.

2.6. Direct ELISA

The samples were diluted to 5 μ g mL⁻¹ with CBS solution, 100 μ L of each sample was taken to a 96-well plate (Costar Inc., USA) and coated at 4 °C overnight. After washing 4 times with Tween 20/PBS (PBS-T), 350 μ L of 5% skimmed milk powder was added and incubated at 37 °C for 1 h. Washed again, added 100 μ L of pooled allergic sera that diluted (1:50) in 2% skimmed milk powder and incubated for 3 h at room temperature. After washing, 100 μ L of peroxidase-labeled goat anti-human/rat IgE antibody (1:5000, Cell Signaling Technology, Inc., USA) was added to wells and then plates were incubated at 25 °C for 1 h. After washing 5 times with PBS-T and 3 times with PBS, 200 μ L of TMB solution was added to wells for 30 min, 50 μ L of 2 M H₂SO₄ was used to stop the reaction. Next, the O.D. values were measured with a spectrophotometer (Dynex Technologies Inc., USA) at 450 nm. The same procedure was performed with negative sera to determine the extent of non-specific binding, which was subtracted from test data. ELISA was performed in triplicate and the average values were taken as the final data.

2.7. Cell culture and sensitization

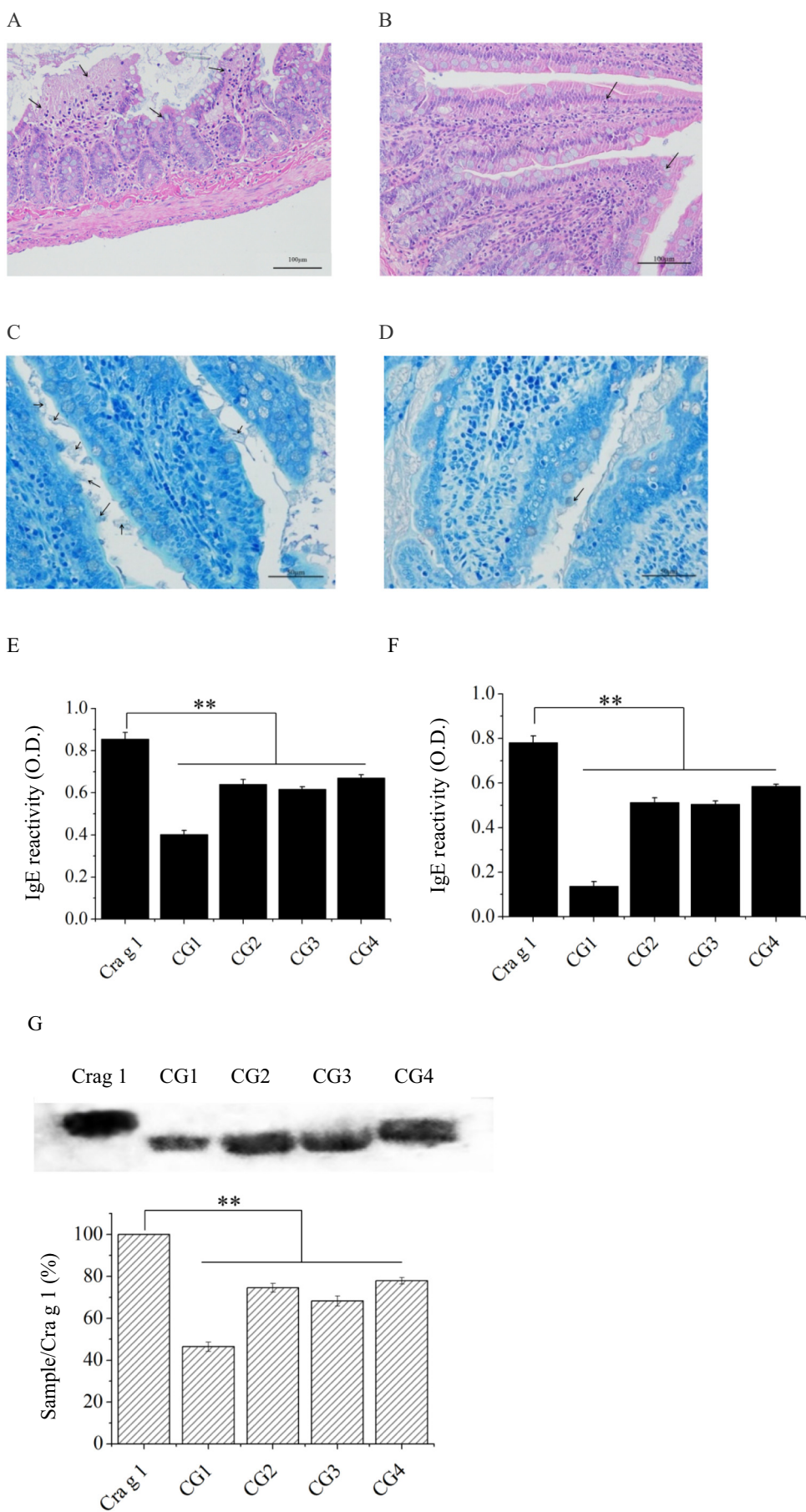
The rat cell line, RBL-2H3, was obtained from the Chinese Academy of Sciences, grown in DMEM (containing 10% FBS, 100 μ g mL⁻¹ streptomycin and 100 U mL⁻¹ penicillin) and incubated at 37 °C with a 5% CO₂/95% air atmosphere. The cells were cultured at stationary phase for 24 h, passively sensitized with rat serum IgE overnight and further washed 5 times with Tyrode's buffer.

2.8. β -Hexosaminidase and histamine release assay

After stimulated with Cra g 1 or its mutants in Tyrode's buffer for 45 min, IgE-sensitized cells were lysed with 1% Triton-X100 prior to removing supernatant. 50 μ L of cell supernatant and 50 μ L of 1.0 mM 4-phenylnitro-D-acetyl- β -glucosamine (PNAG) in 0.1 M citrate buffer were mixed in 96-well plates and incubated at 37 °C for 1 h. The total β -hexosaminidase activity was quantified by measuring the O.D. values of hydrolyzed PNAG in a spectrophotometer (Dynex Technologies Inc., USA) at 405 nm. For the control, IgE-sensitized cells without antigen stimulation were performed the same procedure. The calculation formula for the release rate of β -hexosaminidase is as follows: β -hexosaminidase release rate (%) = (sample O.D. value – control O.D. value) / (total enzyme release O.D. value – control O.D. value). To determine histamine content, sensitized RBL-2H3 cells were treated with antigen or Tyrode's buffer (control) at 37 °C for 30 min, then histamine release level was measured using spectrophotometer (Dynex Technologies Inc., USA) at 450 nm according to the manufacturer's instruction (Immuno-Biological Laboratories Inc., USA). All the experiments were performed in triplicate and data were given as the mean.

2.9. Real time PCR analysis

Total RNA from treated cells was prepared with Trizol reagent (TransGen Biotech Co., Ltd., China), reverse transcribed to produce cDNA with All-In-One RT Mix kit (TransGen Biotech Co., Ltd., China), and PCR products were monitored by SYBR Green Master Mix kit (TransGen Biotech Co., Ltd., China). The real time PCR was performed in triplicate using PikoReal 96 system (Thermo Scientific, USA). In the study, real time PCR primer sequences were: 5'-TTCCAGGGTGCTTCGC AAAT-3' (sense) and 5'-CACCGAGAACCCAGACTTG-3' (antisense) for IL-4; 5'-CTGGTCTTCTGGAGTCCGT-3' (sense) and 5'-TGGTCCTTAGC CACTCCTTCT-3' (antisense) for IL-6, 5'-CATCCGTTCTCTACCCAGCC-3' (sense) and 5'-CCCAGAGCCACAATTCCTT-3' (antisense) for TNF- α , 5'-TGAAGACTCAACCAGTTCCTGA-3' (sense) and 5'-ACAAGTCGTCT GGGTGGATG-3' (antisense) for Lyn, 5'-GGCGGAAGACGGTTAAGGAA-3' (sense) and 5'-TAAGCACACAACAGCGGACT-3' (antisense) for Syk, 5'-ATTTCATGTGTGTGGCGGC-3' (sense) and 5'-CACACAACGGTCATCC ACCA-3' (antisense) for IP₃R, 5'-GACAGTGCCAGCTCTGATGAAAT-3' (sense) and 5'-CTGGGGCCTCTGTGAATAGG-3' (antisense) for Gab2, 5'-TCGGACTGTCTTCTTTCACAA-3' (sense) and 5'-GACCCCGTCCATCA TGTGTT-3' (antisense) for PKC and 5'-GCAGGAGTACGATGAGTCCG-3'



(sense) and 5'-ACGCAGCTCAGTAACAGTCC-3' (antisense) for β -actin. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative mRNA level, C_t is the threshold cycle while ΔC_t is calculated from test C_t - β -actin C_t . β -Actin was used as the internal control.

2.10. Detection of allergic mediators

IgE-sensitized RBL-2H3 cells were grown in 96-well plates (1.5×10^5 cell/well) overnight and treated with antigen or Tyrode's buffer (control) for 8 h. The supernatant was harvested and determined the contents of IL-4, IL-6 and TNF- α at specific time points by ELISA kits according to the instructions (Biological Technology Co., Ltd., China). Meanwhile, nuclear and cytoplasmic proteins of IgE-sensitized cells with or without antigen stimulation for 8 h were extracted to acquire NF- κ B p65, PLC- γ and phosphorylated PLC- γ . These mediators were quantified by Western blot analysis using corresponding antibodies (Cell Signaling Technology, Inc., USA). For evaluating intracellular calcium level, cells were cultured overnight in 48-well plates (1×10^6 cell/well) and sensitized, incubated with Fluo-3AM fluorescent probe (Solibao Technology Co., Ltd., China) for 30 min prior to stimulating with Cra g 1, mutants or Tyrode's buffer (control). The intracellular Ca^{2+} fluorescence was measured by flow cytometry (Sony, Japan). All experiments were performed in triplicate and the average values were taken as the final data.

2.11. Western blotting

The equal amounts of protein (20 μ g) were subjected to SDS-PAGE and transferred onto PVDF membranes. After blocking, the membranes were incubated with the targeted primary antibody and then with HRP-labeled secondary antibody. Immunoreactive proteins were visualized by ECL Western blot detection system (Clin Co., Ltd., China), and protein level was compared to control.

2.12. Statistical analyze

Statistical analysis was performed by one-way ANOVA analysis of variance using Originpro 8.0 (OriginLab Corp., USA). All data are presented as the mean values $\pm$ standard deviation (SD). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Purification of recombinant wild type and mutant oyster tropomyosins

For further research, the recombinant wild type Cra g 1 and its four hypoallergenic derivatives based on the epitope data were constructed by epitope deletion and site-directed mutagenesis. Fig. 2A and B illustrated the positions of five major IgE-binding regions and corresponding amino acid sequence changes in mutant CG1-4. Approximately 6–10 mg of soluble recombinant protein was obtained from 1 l of *E. coli* culture. Compared with the Cra g 1 band, the deletion mutant CG1 showed 30 kDa while the site-directed mutations CG2, CG3 and CG4 had molecular weights about 37 kDa (Fig. 2C).

3.2. Conformational analysis of wild type and hypoallergenic mutant proteins

A homology-based model of Cra g 1 three-dimensional structure was developed using the best template from *Sus scrofa* (PDB code

1C1G) (Fig. 3A). The quality of the model was assessed by the Ramachandran Diagram to reflect the conformational rationality. Most of the backbone ψ and ϕ torsion angles for non-glycine residues lay within the allowed regions (Fig. 3B), demonstrating Cra g 1 model was reasonable. Additionally, the Cra g 1 three-dimensional structure is similar to those of other homologous molecules that have been solved, and five major epitopes were even distribution on the outer surface of allergenic molecule (Fig. 3A).

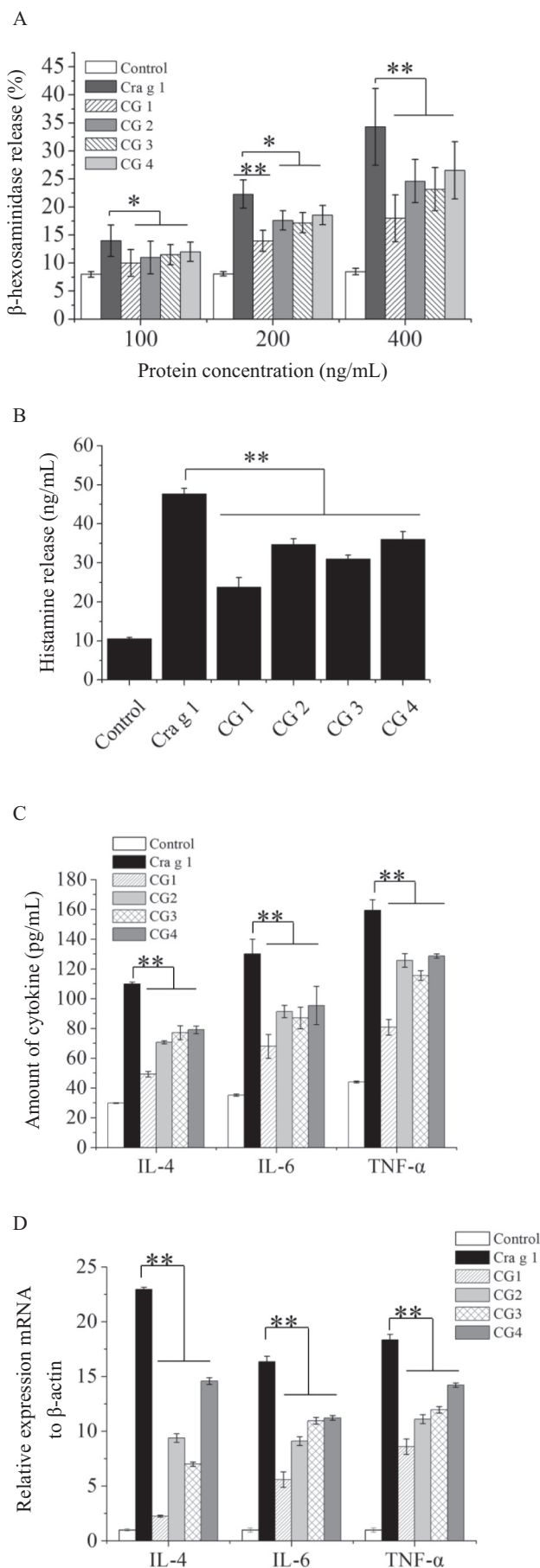
In order to analyze the structural changes of mutant proteins, it is allowed to monitor folding changes using CD spectroscopy. The CD spectrum has a peptide bond absorbing signal in the far-UV region and reflects the contents of the regular secondary structure feature of protein. According to the CD results, Cra g 1 has two negative peaks near 208 nm and 222 nm, and the molar ellipticity 222/208 is greater than 1. These data indicated that it was a typical α -helical protein and formed a double-stranded coiled-coil structure, which were in accordance with the homology-based model of Cra g 1 tertiary structure that we generated. The CD signal of the deletion epitope mutation CG1 was significantly changed, the signal absorption peak was obviously weakened and showed a slight red shift, while other three site-directed mutations had little change compared to the wild type protein (Fig. 3C). Moreover, the surface hydrophobicity of the proteins was determined through ANS-binding fluorescence. The hydrophobic fluorescence intensity of Cra g 1 and CG1-4 was calculated and the results were shown in Fig. 3D. Compared with Cra g 1 protein, the surface hydrophobicity of CG1 was markedly reduced by about 50% ($P < 0.01$) but other three mutant proteins were increased by 19%–25% ($P < 0.05$).

3.3. Immunoreactivity of Cra g 1 and hypoallergenic mutations

BN rats developed a series of allergic symptoms after sensitization, showing an increase of scratching around the nose, ears, head and abdomen, swelling around the eyes and mouth, even shivering and convulsions. Previous studies indicated that food allergens could infiltrate the tissues such as lung, oesophagus and small intestine [20]. Herein, we attempted to observe pathological changes of jejunum before and after sensitization. In the experimental group, we found that the villi epithelium fell off, the small blood vessels were congested and a large number of inflammatory cells were infiltrated relative to control group (Fig. 4A and B). The degranulation of small intestinal mast cells was also analyzed by toluidine blue staining. The jejunum mast cells aggregated and degranulated with ruptured cell membrane and diffuse shape after administration of Cra g 1 as compared to control group (Fig. 4C and D). Meanwhile, owing to Cra g 1 can allow body to engender specific IgE, we further examined Cra g 1 specific antibody level. During the test period, the level of specific IgE reached the highest point on the day 42 following allergen repeated challenge (data not shown), then the sera from rats were separated and used for subsequent experiments.

In vitro immune experiments were performed by pooled sera from oyster-allergic subjects and Cra g 1-immunized rats to illustrate the immunoreactivity of tropomyosin mutants. The IgE-binding ability of CG1-4 towards patients' sera reduced by 52.84%, 24.97%, 27.71% and 21.35% compared to Cra g 1, respectively ($P < 0.01$), whereas that to rats' sera decreased by 82.56%, 28.31%, 25.20% and 35.52%, respectively ($P < 0.01$) (Fig. 4E and F). Interestingly, the IgE reactivity of mutants when tested with patients' sera was significantly higher than that of mutants when tested with rats' sera. At the same time, we found that the results of Western blot analysis were consistent with ELISA data and CG1-4 were effectively able to decrease affinity of IgE ($P < 0.01$)

Fig. 4. *In vivo* and *in vitro* immunoreactivity of Cra g 1 and hypoallergenic mutants. Hematoxylin-eosin staining of jejunum, Cra g 1-sensitized group (A) and control group (B). Toluidine blue staining of jejunum, Cra g 1-sensitized group (C) and control group (D). Reactivity of Cra g 1 and hypoallergenic mutants to IgE from oyster allergy patients (E) ($n = 15$) and Cra g 1-sensitized rats (F) ($n = 12$) in ELISA. (G) Western blot analysis of Cra g 1 and hypoallergenic mutants using the pooled rat sera. ** $P < 0.01$ vs. Cra g 1. Data represent the mean $\pm$ SD ($n = 3$).



(Fig. 4G). These findings clearly indicated that the four mutant proteins had obvious reduction in their IgE-binding ability, especially CG1.

3.4. Effects of hypoallergenic mutations on mediators release in IgE-sensitized RBL-2H3 cells

RBL-2H3 cells that have phenotypic characteristics of mucosal mast cells are generally used as a good model for studying allergenic potential [21]. After sensitization, cells release β -hexosaminidase and histamine that are markers of degranulation and can effectively reflect the degree of cell allergy. Hence, we examined the effects of Cra g 1 and its derivatives on β -hexosaminidase and histamine release. CG1-4, particularly CG1, remarkably inhibited degranulation in IgE-sensitized cells at different concentrations ($P < 0.05$) (Fig. 5A). In addition, the four mutants also had the inhibitory effect of histamine release when compared to wild type form in antigen-induced RBL-2H3 cells ($P < 0.01$) (Fig. 5B), but no statistical significance was found among the CG2-4. Moreover, various cytokines, for instance IL-4, IL-6 and TNF- α , play an important role during allergic response. IL-4 is Th2 cell related cytokine, which promotes native T cells to form allergic Th2 cells [22]. IL-6 and TNF- α , as pro-inflammatory mediators, contribute to the development of allergic inflammation through NF- κ B signalling pathway in activated mast cells [23]. Therefore, we explored the effects of Cra g 1 and its mutants on the production of these cytokines. The results shown in Fig. 5C implied that CG1-4 could reduce the levels of IL-4, IL-6 and TNF- α secretion ($P < 0.01$). The expression levels of IL-4, IL-6 and TNF- α mRNA were further tested. CG1-4 suppressed mRNA expression of the three cytokines ($P < 0.01$), which was in agreement with ELISA results (Fig. 5D). Altogether, the four mutant proteins could markedly inhibit mediators release in IgE-sensitized RBL-2H3 cells.

3.5. Effects of hypoallergenic mutations on Fc ϵ RI-mediated signalling pathway in IgE-sensitized RBL-2H3 cells

Activation of Fc ϵ RI-mediated signalling pathway is considered to be an important event in antigen-induced allergic response [19,24]. Thus, we performed further research focusing on the effects of the variants on this signalling pathway. The mRNA levels of key factors including Lyn, Syk, IP $_3$ R, Gab2 and PKC and protein concentrations of PLC γ , phosphorylated PLC γ and NF- κ B p65 after treatment with antigen were examined in IgE-challenged RBL-2H3 cells. Our results implied that the four hypoallergenic proteins obviously down-regulated mRNA expression of above mentioned signal molecules in tested cells ($P < 0.05$) (Fig. 6A, B, C, D and E), and they also brought the reduction of phosphorylated PLC γ and NF- κ B p65 levels ($P < 0.01$) (Fig. 6F and G). Besides, it is largely known that calcium acts as key factor to involve in degranulation and mediators secretion, although calcium signal induced by IP $_3$ is transient [24,25]. We tried to analyze whether the mutants affected calcium mobilization in sensitized cells. From Fig. 6H, these mutants suppressed intracellular calcium signal ($P < 0.05$), however, there was no significant difference among them. These data suggested that the mutant proteins may relieve allergic response through down-regulation of Fc ϵ RI-dependent signaling activation in IgE-challenged RBL-2H3 cells.

4. Discussion

Food allergy belongs to type I allergic reaction mediated by IgE, which affects up to 10% children and adults [26]. Shellfish allergy is one of the most common types among all food allergies. Tropomyosin

Fig. 5. Effects of hypoallergenic mutants on mediators release in IgE-sensitized RBL-2H3 cells. The release levels of (A) β -hexosaminidase and (B) histamine were measured by commercial kits. (C) The concentrations of IL-4, IL-6 and TNF- α were assessed by ELISA kits. (D) IL-4, IL-6 and TNF- α mRNA expression levels were determined by real time PCR. * $P < 0.05$ vs. Cra g 1, ** $P < 0.01$ vs. Cra g 1. Data are presented as the mean $\pm$ SD ($n = 3$).

has been identified as shellfish pan-allergen, owing to its high amino acid sequence homology between the crustaceans and mollusks [8]. Currently, food avoidance is a major treatment for shellfish-allergic subjects, however, allergen-specific immunotherapy is also a good candidate strategy for clinical management of this disorder. It is necessary

to develop hypoallergenic derivatives with low IgE reactivity that may blunt allergic reactions, because native allergens are apt to cause allergic side-effects in the period of treatment.

Our previous studies have already identified five major IgE-binding epitopes and crucial amino acids within each epitope of Cra g 1 using

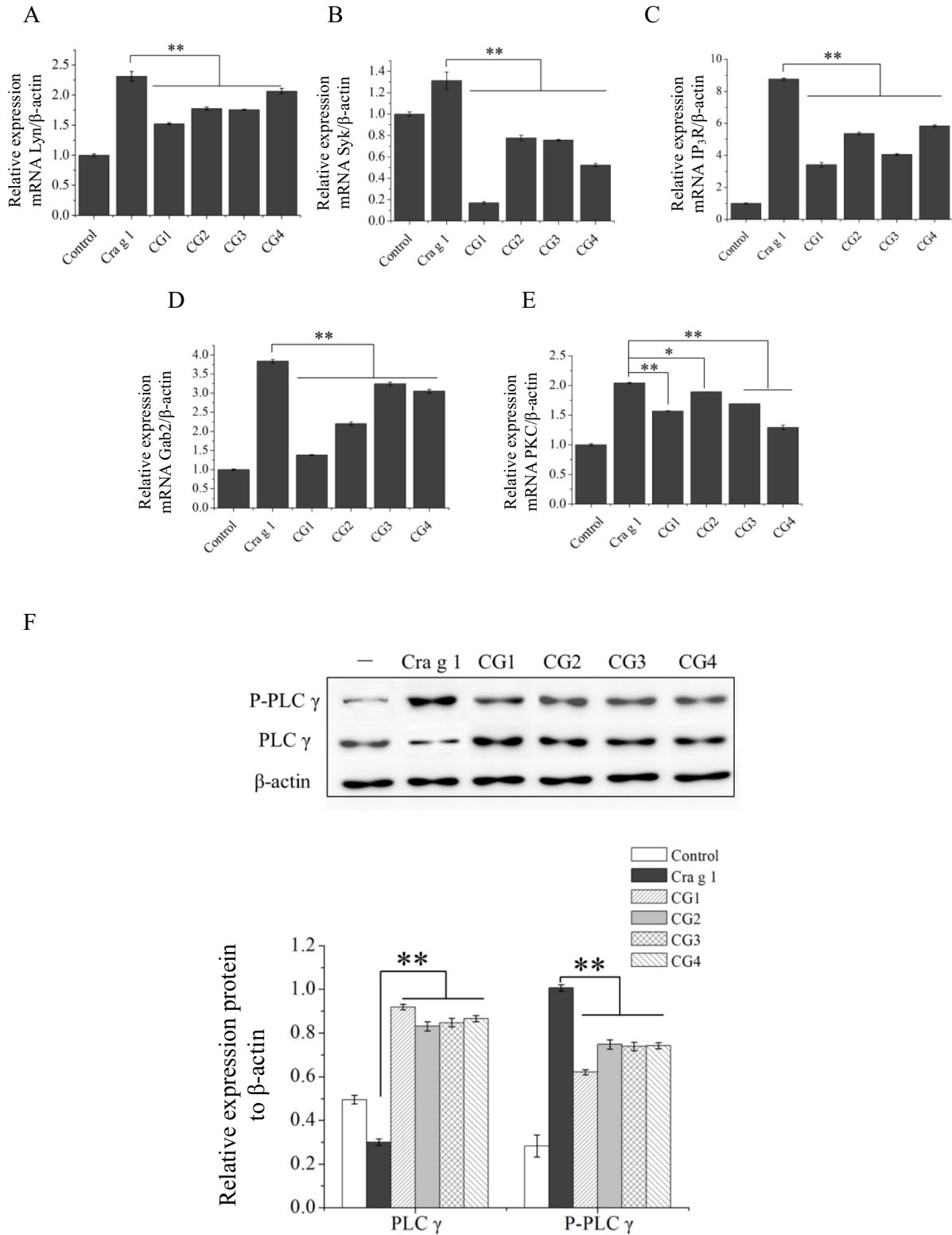
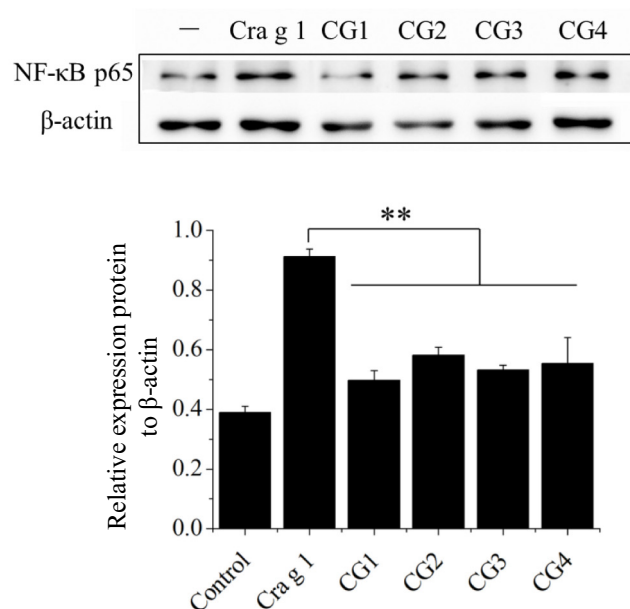


Fig. 6. Effects of hypoallergenic mutants on Fc ϵ RI-mediated signalling pathway in IgE-sensitized RBL-2H3 cells. The mRNA expression levels were analyzed for (A) Lyn, (B) Syk, (C) IP₃R, (D) Gab2 and (E) PKC by real time PCR. (F) PLC γ and (G) NF- κ B p65 activation were evaluated by Western blotting using commercial antibodies. (H) The intracellular Ca²⁺ fluorescence was measured by flow cytometry using Fluo-3 AM fluorescent probe. * P < 0.05 vs. Cra g 1, ** P < 0.01 vs. Cra g 1. Data represent the mean $\pm$ SD (n = 3).

G



H

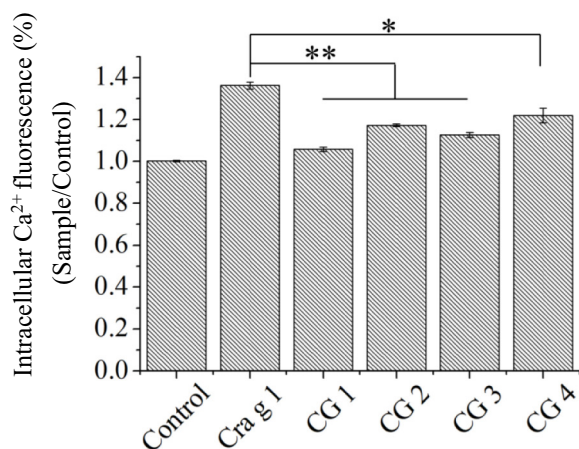


Fig. 6 (continued).

to Cra g 1 was a stretched and very flexible protein. The phenomenon agreed well with previous reports in the analysis of critical amino acids of shrimp allergen and cow's milk allergen [29,30]. At the meanwhile, based on the epitope data, hypoallergen CG1, a truncated variant of only 224 amino acid residues, was produced by removing all the IgE-binding epitopes and hoped to reduce allergenicity as much as possible. However, deleting epitopes would break the three-dimensional structure of Cra g 1 whereas homologous substitution may retain conformation of the protein (Fig. 3C). According to the ANS-binding fluorescence assay, we speculated that CG2-4 were exposed more hydrophobic regions on the surface of the protein by homologous substitutions, resulting in several antigenic epitopes were buried inside the molecule (Fig. 3D). As we expected, *in vitro* immune experiments suggested that these variants significantly decreased affinity of specific IgE when tested with the pooled sera from oyster allergic patients or Cra g 1-sensitized rats (Fig. 4E, F and G). This reduction was more pronounced using rat sera than human sera, which was in agreement with previous studies [12,13]. However, it was worth noting that the derivatives produced by deletion or substitutions did not abrogate binding completely, illustrating some minor epitopes that have not been identified might exist. Moreover, animal models of food allergy are necessary to test new vaccines for treating humans and to better understand the mechanisms of sensitization. Due to the epitopes that were relevant for human IgE binding were also major binding sites for rat IgE (Fig. 4E and F), we deduced that the rat model might be used to develop Cra g 1 mutants for future immunotherapy.

In our view, mast cells tests are more meaningful to evaluate allergic potency. RBL-2H3 cells, having phenotypic characteristics of mast cells, are a convenient method to study type I allergic reactions and allergenic potential of certain substances by measuring release of related chemokines and cytokines. The four hypoallergenic variants displayed a drastically reduced allergenic potency in β -hexosaminidase and histamine release assays (Fig. 5A and B), which enable us to speculate that the mutant proteins may be vaccines for clinical management of allergy. Additionally, various cytokines also contribute to allergic disorder progression. In particular, IL-4 is an important Th2-cytokine. It can promote IgE production, facilitate native T cells to form allergic Th2 cells, and up-regulate levels of cell surface adhesion molecules such as ICAM-1 and VCAM-1. In presence of high concentration of IL-4, specific IgE accelerates Fc ϵ RI aggregation on the surface of mast cells [31,32]. Furthermore, IL-6 and TNF- α are pro-inflammatory mediators secreted by activated mast cells, contributing to allergic inflammatory disorders. They participate in the development of inflammation through NF- κ B signalling pathway in response to IgE challenge [33]. Our results suggested that CG1-4 suppressed antigen-induced gene expression and secretion of IL-4, IL-6, and TNF- α in IgE-sensitized RBL-2H3 cells (Fig. 5C and D). Taken together, the hypoallergenic proteins we constructed may be safer immunotherapy regimens to reduce the potential risk of allergic side-effects during treatment through inhibition of mediators release in activated mast cells.

To evaluate the molecular mechanism of effects of Cra g 1 and its mutants on mediators production, we examined the effects of these proteins on Fc ϵ RI-mediated signalling pathway in IgE-sensitized RBL-2H3 cells. Generally, in response to antigen stimuli, a series of signaling events is triggered following Fc ϵ RI aggregation in the plasma membrane. Once antigen-IgE binds to microdomains of Fc ϵ RI, Lyn and Syk, the main Src-family kinases, are recruited to high-affinity docking sites of Fc ϵ RI and phosphorylated, leading to tyrosine phosphorylation of downstream LAT and PLC γ . Activated PLC γ induces calcium mobilization, activates PKC, and then triggers NF- κ B transduction pathway by controlling IKK activation and subsequent I κ B- α degradation [19]. These signaling cascades finally result in degranulation and mediators secretion. Alternatively, another Src-family kinase, Fyn, also involves in mast cell activation in a LAT-independent manner. When phosphorylated, Fyn directly activates adaptor molecule Gab2, resulting in phosphorylation of downstream PI3K which is associated with hydrolysis of

immunoinformatics tools and ELISA with the pooled sera [14]. Because Cra g 1 crystal structure has not been characterized, we constructed its tertiary structure through molecular modeling. Remarkably, the immunodominant epitopes were almost located on the outer surface of the molecule, which may easily to bind specific IgE (Fig. 3A). This could explain why Cra g 1 exhibited the extreme allergenicity. Food allergies are the interaction of IgE to specific epitopes of food proteins. This may allow us to design hypoallergenic oyster tropomyosin that the critical amino acids in each epitope were converted into the homologous sequence of vertebrate tropomyosin, because no reports showed that vertebrate tropomyosins were allergenic molecules. So, we generated hypoallergen CG2, CG3 and CG4 by replacing 32, 34 and 13 key amino acid residues within five major IgE-binding epitopes with homologous sequences of salmon, rabbit or human (Fig. 2A). Unlike Gly m Bd 30 K (soybean allergen) and Ara h1 (peanut allergen) that the single-site amino acid substitution could reduce or eliminate IgE-binding ability [27,28], more than two crucial residues homologous substitutions were introduced into each Cra g 1 epitope to inhibit IgE reactivity due

PI(4,5)P₂ in the cell membrane to generate IP₃ and DAG [34]. Our results indicated that the four variants were able to dramatically attenuate antigen-induced FcεRI-dependent signaling activation through down-regulating the levels of Lyn, Syk, IP₃R, Gab2, PKC, calcium ion, phosphorylated PLCγ and NF-κB p65 in IgE-challenged RBL-2H3 cells (Fig. 6). Thus, the variants might be used for immunotherapy of oyster-allergic patients. Besides, MAPK signalling cascades also play a key role in degranulation, secretion, activation and migration of mast cells [35]. Future work is directed towards expounding the effects of Cra g 1 and its mutants on MAPK signalling pathway in sensitized RBL-2H3 cells.

In summary, hypoallergen with low IgE reactivity is desirable for allergen-specific immunotherapy. We designed four oyster tropomyosin hypoallergens CG1–4 on grounds of the identified epitopes. The results demonstrated marked decrease in the immunoreactivity, degranulation, cytokines secretion and FcεRI-mediated signaling activation of the hypoallergenic mutants compared to the wild type allergen Cra g 1. This work provides a promising new strategy for treating shellfish-allergic diseases and has great significance in clinical immunotherapy.

Author statement

Guoming Li, Jun Lu and Jiangtao Zhang all participated in the design of all experiments. Under supervision by Guoming Li and Jun Lu, Jiangtao Zhang performed sample preparation and data analysis. Jiangtao Zhang, Wenying liu, Ruixue zhang, Xiaohan Zhao, Lei Fang all participated in the BN rat animal experiment. Jiangtao Zhang, Ruixue Zhang and Wenyingliu all participated in the RBL-2H3 cell experiment. Guoming Li, Jiangtao Zhang, Xiuyuan Qin and Ruizeng Gu all participated in writing and editing the article. All authors read and contributed to the manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the General Program of National Natural Science Foundation of China (No. 31671963), Cultivation Projects of the China National Research Institute of Food and Fermentation Industries (No. 2020GNT505) and the National Key Research and Development Program of China (No. 2016YFD0400604).

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