

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/366920227>

Comparative analysis of allergenicity and predicted linear epitopes in α and β parvalbumin from turbot (*Scophthalmus maximus*)

Article in *Journal of the Science of Food and Agriculture* · January 2023

DOI: 10.1002/jsfa.12432

CITATION

1

READS

113

10 authors, including:



Yuhao Huang

Ocean University of China

12 PUBLICATIONS 41 CITATIONS

[SEE PROFILE](#)



Zhenxing li

Ocean University of China

135 PUBLICATIONS 2,439 CITATIONS

[SEE PROFILE](#)



Siddanakoppalu N Pramod

Davangere University

55 PUBLICATIONS 990 CITATIONS

[SEE PROFILE](#)



Ziye Zhang

Ocean University of China

40 PUBLICATIONS 1,154 CITATIONS

[SEE PROFILE](#)

Comparative analysis of allergenicity and predicted linear epitopes in α and β parvalbumin from turbot (*Scophthalmus maximus*)

Yuhao Huang,^a Zhenxing Li,^{a*} Yeting Wu,^a Yonghong Li,^{a,b} Siddanakoppalu Pramod,^c Guanzhi Chen,^d Wenjia Zhu,^e Ziye Zhang,^a Hao Wang^a and Hong Lin^a



Abstract

BACKGROUND: Parvalbumin (PV) can be subdivided into two phylogenetic lineages, α PV and β PV. The bony fish β PV is considered a major fish allergen. However, there is no available report on the immunological property and epitope mapping of bony fish α PV.

RESULTS: To characterize the allergenic property of bony fish α PV and investigate the difference in allergenic property of bony fish α PV and β PV, turbot (*Scophthalmus maximus*) α PV and β PV were identified by mass spectrometry and were expressed in *Escherichia coli* system in this study. Spectra analysis and three-dimensional (3D) modeling showed the similar structure between α PV and β PV. However, α PV exhibited lower immunoglobulin E/immunoglobulin G (IgE/IgG) binding capacity than β PV. Three identified β PV epitopes possessed higher IgE reactivity and more hydrophobic residues than three identified α PV epitopes. In addition, less similarity in sequence homology of α PV epitopes was observed with allergen sequences in database.

CONCLUSION: These finding expanded information on fish PV epitopes and substantiated the difference in allergenicity and epitope mapping between fish α PV and β PV, which will improve the epitope-based detection tools of PV and diagnostic of PV induced fish allergy.

© 2023 Society of Chemical Industry.

Supporting information may be found in the online version of this article.

Keywords: parvalbumin; IgE binding; epitope; cross-reactivity; fish allergy

INTRODUCTION

Fish and its products are popular worldwide, but they are also one of the main food sources causing type I allergy with symptoms including urticaria, asthma and atopic dermatitis.^{1,2} Although many other fish allergens such as enolase, aldolase, tropomyosin and vitellogenin have been identified in recent years, parvalbumin (PV) is still the major fish allergen.^{3–5} PV is a small (10–15 kDa) thermal stable calcium-binding protein, which possess typical helix–loop–helix EF-hand structure.⁶ PV could be divided into two distinct phylogenetic lineages, α PV and β PV.⁷ β PV usually contains 108 or less amino acids and its isoelectric point (pI) generally ranges from 3.9 to 4.5, which is considered a major cross-reactive fish allergen.^{8–10} In recent years, α PV also has been reported as a major allergen in cartilaginous fish, which possess long amino acids length (109 or more) and higher pI.¹¹ However, few research has focused on the allergenicity of α PV in bony fish. The difference in allergenicity of bony fish α PV and β PV needs to be further investigated.

Elucidation of immunoglobulin E/immunoglobulin G (IgE/IgG) response is considered as pivotal to assess the immunogenic

potential of protein antigen. Serum IgE/IgG binding capacity is an important index to evaluate antigen triggered allergenicity,

* Correspondence to: Zhenxing Li, College of Food Science and Engineering, Ocean University of China, No.5, Yu Shan Road, Qingdao, Shandong Province, 266003, P. R. China. E-mail: lizhenxing@ouc.edu.cn

a College of Food Science and Engineering, Ocean University of China, Qingdao, P. R. China

b Department of Research and Development, HOB Biotech Group Corp., Ltd, Suzhou, P. R. China

c Department of Studies and Research in Biochemistry, Davangere University, Davangere, India

d Department of Dermatology, Affiliated Hospital of Medical College Qingdao University, Qingdao, P. R. China

e Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, P. R. China

while epitope is the region where antigen interacts with IgE/IgG, which could directly affect the IgE/IgG binding capacity of antigen. Therefore, it is significant to explore the difference of epitope between α PV and β PV. Epitopes of β PV have been identified in several fish, but the research about the identification of fish α PV epitopes and immunogenicity is still not clear.^{12–14} Turbot (*Scophthalmus maximus*), a kind of commercial bony fish in China and Europe, contains two major PV isoforms,¹⁵ which is a proper model for studying the difference in allergenicity and epitope mapping between different PV isoforms.

Therefore, we aimed to compare the IgE/IgG binding capacity of turbot α PV and β PV and to comparatively identify their epitopes in the present study. Natural turbot PV was extracted from fish muscle and identified by mass spectrometry. Similar physico-chemical properties such as molecular weight, pI and salt solubility make it hard to separate natural turbot α PV and β PV. However, recombinant proteins have been broadly prepared as calibrants for natural allergen used in diagnosis and immunotherapy, therefore, we chose the method of expression and purification to obtain turbot α PV and β PV.^{16,17} The IgE/IgG binding capacity and structure of turbot α PV and β PV were analyzed by inhibition Western-blot, inhibition enzyme-linked immunosorbent assay (ELISA) and circular dichroism (CD) spectrum. The identification of epitope was performed by a combination of prediction of immunoinformatic tools and verification of inhibition Western-blot. Besides, we analyzed the amino acid composition of verified epitopes and compared their sequence similarity with other known cross-reactive allergens. These results could help us better comprehend the difference in allergenicity between α PV and β PV and verified epitopes could be utilized in the development of epitope-based detection tools for PV and diagnostic agents to fish allergy.

MATERIALS AND METHODS

Materials and sera

Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody and HRP conjugated goat anti-human IgE antibody was purchased from Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China) and Agrisera AB (Vännäs, Sweden), respectively. Rabbit anti-turbot PV IgG polyclonal antibody was obtained from ProteinGene Biotech Co. (Wuhan, China). Isopropyl- β -D-thiogalactoside (IPTG) and enterokinase were acquired from Solarbio Co. (Beijing, China).

Sera of ten fish allergic patients and two healthy individuals were collected from the Affiliated Hospital of Medical College Qingdao University (Qingdao, China) in accordance with protocol approved by the medical ethics committee (File no. QD-PF-20190618685). The fish crude extract specific IgE of these sera were measured by ImmunoCAP (Thermo Fisher Scientific, Waltham, MA, USA) and details are displayed in Supporting Information Table Table S1.

Purification and mass spectrometry of natural turbot PV

The crude protein and purified natural PV of turbot were prepared as follows. Briefly, turbot was bought from Li Qun supermarket in Qingdao. The crude protein was extracted from the muscle with the 0.1 mol L⁻¹ Tris buffer (pH 8.5) containing 0.1 mmol L⁻¹ DTT and 0.5 mmol L⁻¹ glycine at 4 °C overnight. Then the crude protein was heated at 95 °C for 10 min and further purified by a two-step fractionation with ammonium sulfate (0–60%, 60–100%). The precipitate of 100% saturation fractionation was resuspended and dialyzed with deionized water overnight at 4 °C to obtain purified natural PV.

The separation of different isoforms of natural turbot PV was performed by 4–20% gradient SurePAGE™ precast Gel (Genscript, Piscataway, NJ, USA). The target protein bands were excised from Coomassie Brilliant Blue stained gel. After treatment with 10 mmol L⁻¹ DTT and 55 mmol L⁻¹ iodoacetamide, the bands were digested by trypsin overnight at 37 °C. Digested bands were analyzed using an Q Exactive Mass Spectrometer (Thermo Fisher Scientific) by Beijing Protein Innovation Co., Ltd (Beijing, China).

Production of recombinant turbot α PV and β PV

The coding sequences of turbot α PV (XM_035616246.2) and β PV (XM_035616244.1) were obtained from the National Center for Biotechnology Information (NCBI) and then were synthesized by TSINGKE Biological Technology Co., Ltd (Beijing, China) after optimization. Subsequently, the synthetic sequences were cloned into pET-30a vector between *Bam*H I site and *Xho* I site and then transformed into *Escherichia coli* BL21 (DE3) cells. The expression of recombinant PV was induced by 1 mmol L⁻¹ IPTG at 16 °C for 20 h. After high pressure crushing and centrifugation (8120 × g, 4 °C, 40 min), his-tagged recombinant PV was released from bacteria cells and then purified by Ni-NTA Gravity Column (Sangon Biotech, Shanghai, China). His-tag of recombinant PV was cut-off by enterokinase and then purified further using Ni-NTA Gravity Column to obtain untagged recombinant PV. After purification, protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% SurePAGE™ precast Gel (Genscript).

Structure analysis of turbot α PV and β PV

Recombinant and natural protein solutions were incubated with 5 mmol L⁻¹ EGTA (ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) for 30 min at 37 °C to prepare calcium ion (Ca²⁺) depleted samples. The concentrations of native and Ca²⁺ depleted samples were diluted to 0.3 mg mL⁻¹ by 0.01 mol L⁻¹ phosphate-buffered saline (PBS). Secondary structure analysis was performed using CD on a J-815 CD spectrometer (JASCO, Tokyo, Japan) at room temperature. Bandwidth, scanning speed and wavelength measurement ranges were set to 1.0 nm, 50 nm min⁻¹ and 190–260 nm, respectively. UV-2550 spectrometer (Shimadzu, Kyoto, Japan) was used to measure the ultraviolet (UV) absorption according to a previously reported method.¹⁸ Scanning range and speed were 230–330 nm and 400 nm min⁻¹, respectively.

Inhibition Western-blot analysis of the IgE/IgG binding of recombinant turbot α PV and β PV

SDS-PAGE was run using 4–20% gradient SurePAGE™ precast Gel (Genscript), the separated crude turbot proteins were transferred from gel to polyvinylidene fluoride (PVDF) membrane using iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific). Subsequently, the membrane was blocked with 5% skim milk and allowed to react with human sera (diluted in 1:5) or rabbit IgG polyclonal antibody (diluted in 1:3000) for overnight at 4 °C on Mini-PROTEAN II Multi-screen Apparatus. These primary antibody sources preincubated with 0, 0.01, 0.1 μ g mL⁻¹ (for human sera) or 0, 5, 50, 200 and 400 μ g mL⁻¹ (for IgG polyclonal antibody) recombinant α PV or β PV for 30 min at 37 °C as inhibitor prior to the addition to the membrane. Then, HRP conjugated goat anti-rabbit IgG antibody (diluted in 1:8000) and HRP conjugated goat anti-human IgE antibody (diluted in 1:2000) were used as secondary antibodies to incubate with the membrane at room temperature for 1 h. At last,

the membrane was photographed by ChemiDoc XRS + Imager System (Bio-Rad, Hercules, CA, USA) in signal accumulation mode with the addition of enhanced chemiluminescent substrate (Thermo Fisher Scientific) and the blotting intensity was calculated using Image J software.

Inhibition ELISA analysis of the IgE/IgG binding of recombinant turbot α PV and β PV

ELISA plate was coated with purified natural PV or recombinant α PV or recombinant β PV (10 μ g per well) in bicarbonate buffer (35 mmol L⁻¹ NaHCO₃, 15 mmol L⁻¹ Na₂CO₃, pH 9.6) at 4 °C overnight and then washed for 5 min by 10 mmol L⁻¹ phosphate-buffered saline (pH 7.4) with 0.05% Tween 20 (PBST) three times and the plate was blocked with 5% skim milk for 2 h at 37 °C. Then, pooled human sera (diluted in 1:10) which was preincubated with 0, 0.01, 0.1 μ g mL⁻¹ of purified natural PV or recombinant α PV or recombinant β PV for 30 min at 37 °C or rabbit IgG polyclonal antibody (diluted in 1:8000) which was preincubated with 0, 0.1, 1, 10, 50, 100, 200, 400 μ g mL⁻¹ purified natural PV or recombinant α PV or recombinant β PV for 30 min at 37 °C were added into the wells as source of primary antibody and incubated for 1.5 h at 37 °C. Subsequently, washed and 100 μ L HRP conjugated goat anti-rabbit IgG antibody with dilution of 1:16000 or HRP conjugated goat anti-human IgE antibody with dilution of 1:2000 was added into corresponding well and incubated for 1 h at 37 °C. Finally, washed and added with HRP substrate (3,3',5,5'-tetramethylbenzidine) for development. The reaction was stopped by 50 μ L 2 mol L⁻¹ sulfuric acid and the measurement of absorbance was read at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA).

Epitope prediction and peptides synthesis

Linear B cell epitopes of turbot α PV and β PV were predicted through the aid of three immunoinformatic computational tools: DNASTar Protean system (DNASTar Inc., Madison, WI, USA), Bepipred Linear Epitope Prediction 2.0 (<http://tools.iedb.org/bcell/>) and ABCpred Server (https://webs.iitd.edu.in/raghava/abcpred/ABC_submission.html). Hydrophilicity, flexibility, accessibility, and antigenicity were set as main primary parameters of epitope prediction.^{19,20} The criteria for selection of potential epitopes predicted by three immunoinformatic computational tools was performed as follows: (1) fusion the overlapping potential epitopes predicted by DNASTar Protean and IEDB, for example, the fusion result of potential epitope A (position 13–20, predicted by DNASTar Protean) and potential epitope B (position 17–25, predicted by IEDB) is the fusion potential epitope (position 13–25); (2) extend the fusion potential epitope if the fusion potential epitope is completely covered by the potential epitopes predicted by ABCpred Prediction, unless the antigenicity of amino acids adjacent to epitopes are < 0; (3) shorten the fusion potential epitope if the fusion potential epitope is not completely covered by the potential epitopes predicted by ABCpred Prediction. Finally, selected potential epitope peptide with a purity of 95% was synthesized by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China) using standard Fmoc solid phase peptide synthesis and was characterized by high-performance liquid chromatography and mass spectrometry.

Inhibition Western-blot analysis of epitope peptides

Purified natural turbot PV was transferred to PVDF membrane from gel after SDS-PAGE at 140 V for 30 min using 4–20% gradient SurePAGE™ precast Gel. The membrane was blocked with

5% skim milk at room temperature for 2 h and then cut into squares. The human sera (diluted in 1:5) preincubated with 5 mg mL⁻¹ epitope peptide or 2 mg mL⁻¹ natural PV for 30 min at 37 °C were used as primary antibody and allowed to react with membrane squares overnight at 4 °C. Then, HRP conjugated goat anti-human IgE antibody (diluted in 1:2000) were used as secondary antibodies and were incubated with the membrane at room temperature for 1 h. Subsequently, membrane squares were photographed by ChemiDoc XRS + Imager System and the blotting intensity was analyzed using Image J software. Further, the observed results of IgE/IgG reactivity was scored 0–3 based on the reduction in the degree of blotting intensity as negative reactivity (0–20% reduction, score 0); light reactivity (20–50% reduction, score 1); weak reactivity (50–80% reduction, score 2); strong reactivity (80–100% reduction, score 3).²¹

Molecular modeling and epitope mapping

The three-dimensional (3D) models of turbot α PV and β PV were created by homologous modeling of SWISS-MODEL program (<https://swissmodel.expasy.org/>) using carp PV (1b8r.1.A) as template.²² The epitopes verified by inhibition Western-blot were located on 3D models with different color by PyMOL software (Schrodinger, New York, USA).

Similar sequences matching in known allergen

For matching similar sequences of verified epitopes in known allergen, peptide similarity search program (https://fermi.utmb.edu/SDAP/sdap_pps.html) of Structural Database of Allergenic Proteins (SDAP) was utilized. Briefly, the sequence of verified epitopes was submitted to the program and the property distance (PD) of all region in the sequence of known allergen in SDAP, that are the same length as the verified epitopes, was calculated. According to previous research, region in sequences of known allergen with PD lower than 8.0 was considered as similar sequence of epitopes and two identical sequences have a PD of 0.²³

RESULTS

Preparation and identification of natural turbot PV

Natural turbot PV contained two prominent 12 kDa bands (Fig. 1, lane 8). Band with higher molecular weight was marked as PV I, while lower molecular weight band was marked as PV II. As shown in Fig. 2, the peptide mass fingerprinting of PV I matched turbot α PV (XP_035472139.1) with sequence coverage of 89% while low molecular weight PV II matched turbot β PV (XP_035472137.1) with sequence coverage of 88%.

Expression and purification of recombinant turbot α PV and β PV

As analysis of SDS-PAGE, a prominent band around 17 kDa (Fig. 1, lane 1) and a prominent band around 15 kDa (Fig. 1, lane 4) was observed in the supernatant of broken bacteria cell containing expression vector, but not in the supernatant of broken bacteria cell containing control vector (Fig. 1, lane 7). This confirms the induction and expression of soluble recombinant proteins by the vector inserted recombinant bacterial cells. The molecular weight of recombinant protein bands (17 kDa and 15 kDa) was higher than their natural form (Fig. 1, lane 8) because there was a translation of the sequence including his-tag between the starting codon ATG and *Bam*H I site. After purification by Ni-NTA Gravity Column and treatment with enterokinase, untagged

recombinant α PV (Fig. 1, lane 3) and untagged recombinant β PV (Fig. 1, lane 6) showed similar molecular weight to their natural form (Fig. 1, lane 8). These results illustrated that untagged recombinant turbot α PV and β PV were successfully prepared and purified from *E. coli* system.

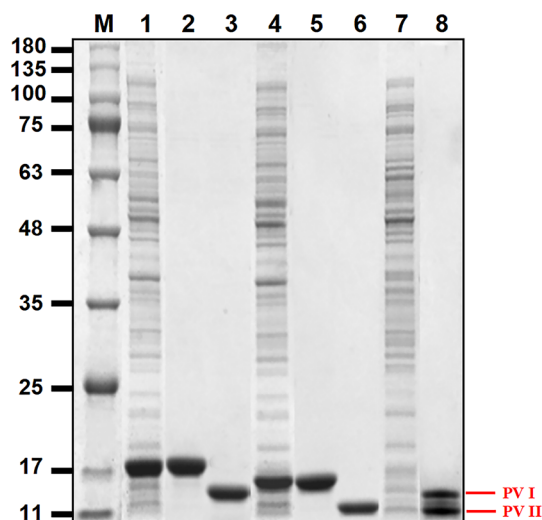
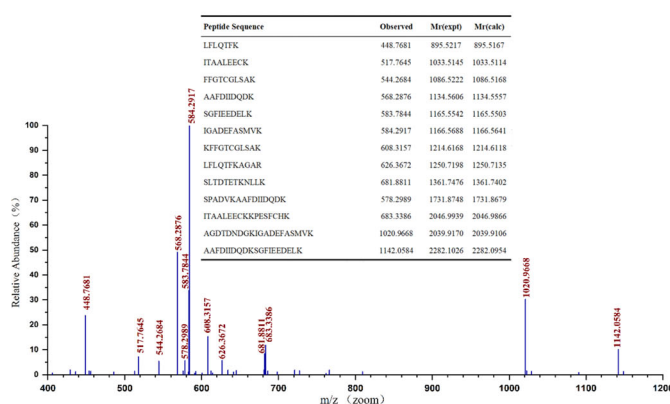
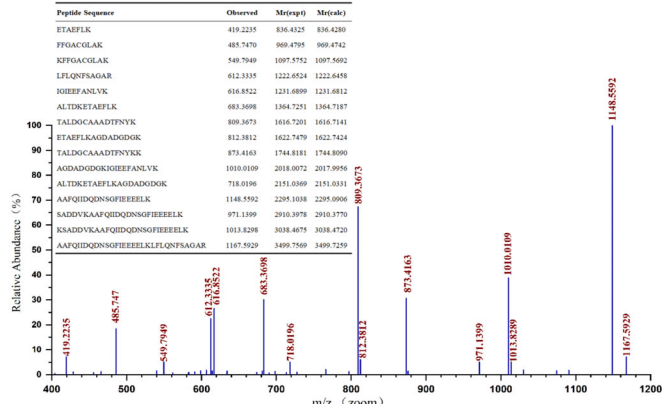


Figure 1. SDS-PAGE of purification of turbot natural PV, recombinant α PV and recombinant β PV. Lane M: marker, lane 1: supernatant of broken cell of *Escherichia coli* expressing recombinant turbot α PV, lane 2: purified recombinant turbot α PV with his-tag, lane 3: purified recombinant α PV without his-tag, lane 4: supernatant of broken cell of *E. coli* expressing recombinant turbot β PV, lane 5: purified recombinant β PV with his-tag, lane 6: purified recombinant β PV without his-tag, lane 7: supernatant of broken cell of *E. coli* containing control vector, lane 8: purified natural turbot PV (PV I and PV II).



SEQ:
MAFGVLDDA KITAALAECK KPESFCHKKF FGTCGLSAKS
PADVKAAFDI IDQDKSGFIE EDELKFLFQT FKAGARSLTD
TETKNLLKAG DTNDGKIGA DEFASMVKV

(A)



SEQ:
MSFAGLNAAE IKTALDCAA ADTFNYKKKF GACGLAKKSA
DDVKAADFQI DQDNGSGFIE EELKFLQNF SAGARALTDK
ETAFLKAGD ADGDKIGIE EFANLVKE

(B)

Isoforms	Matched protein	Accession number	Sequence coverage	Score	Mass	pI
PV I	parvalbumin alpha [<i>Scophthalmus maximus</i>]	XP_035472139.1	89%	4265	11978	4.98
PV II	parvalbumin beta [<i>Scophthalmus maximus</i>]	XP_035472137.1	88%	8301	11630	4.50

(C)

Figure 2. Identification of natural turbot PVs by mass spectrometry. Peptide mass fingerprinting of (A) turbot PV I and (B) turbot PV II. Peptide matched sequence was shown in bold red. (C) Mass spectrometry indicate that PV I was turbot α PV and PV II was turbot β PV.

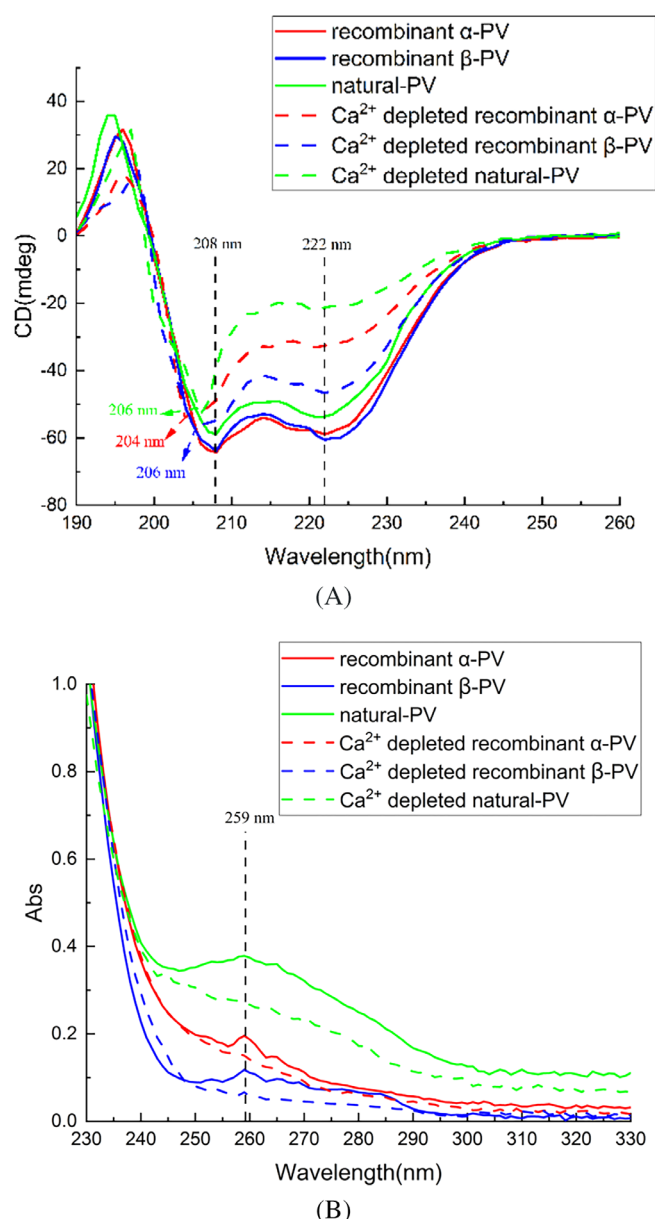


Figure 3. Structure analysis of purified turbot natural PV, recombinant α PV and recombinant β PV in the presence of Ca^{2+} and EGTA treated Ca^{2+} depletion samples. (A) CD spectra indicating the change at negative peaks 208 and 222 nm for both PVs. (b) UV absorption spectra indicate change in UV absorption for both at 259 nm in the absence of calcium.

β PV are major IgE binding proteins in the turbot. However, the inhibition intensity of β PV, measured as reduction binding in comparison with uninhibited control (S1: 68.2%, S2: 80.1%, S3: 100%, S5: 89.6%, S7: 92.4%, S8: 78.1%) was higher than it appeared for α PV (S1: 36.5%, S2: 51.4%, S3: 54.2%, S5: 22.6%, S7: 87.3%, S8: 40.4%) at the inhibition concentration of $1 \mu\text{g mL}^{-1}$ (Fig. 4(C–H)). The inhibition with recombinant β PV was also appeared comparatively more with rabbit IgG binding to natural PV in crude extract than α PV with relatively same concentration (Fig. 4(B)). Interestingly, a 36 kDa was recognized by serum S9 (Fig. 4(I)). Besides, no band was recognized by health sera pool (Fig. 4(J)) which was used as non-atopic control.

In inhibition ELISA, sera pool of ten patients with defined case history to fish allergy (S1–S10 in Table Table S1) and rabbit

polyclonal IgG were used as source of primary antibody. Both recombinant and natural PVs show inhibition of the IgE/IgG binding to natural PV and inhibition increased with increasing concentration of inhibitor. The inhibition rates with recombinant PV appeared lower than natural PV when protein concentration was higher than $0.1 \mu\text{g mL}^{-1}$ (Fig. 5(A,B)). The IgE binding inhibition rate of β PV was 24.6–71.3%, which was higher than α PV with the same concentration (Fig. 5(A)). For IgG binding, the inhibition rate of β PV was 26.4–71.9%, which was also higher than α PV with the same inhibitor concentration (Fig. 5(B)). These results were consistent and resemble the observation made in inhibition of IgG/IgE Western blot. These findings indicated that IgE/IgG binding capacity of β PV was higher than α PV. During the mutual inhibition studies, the rabbit IgG binding to α PV was reduced by 44.5% at $400 \mu\text{g mL}^{-1}$ β PV and IgG binding to β PV was decreased 34.2% at $400 \mu\text{g mL}^{-1}$ α PV (Fig. 5(C)), which revealed the possible cross-reactivity between α PV and β PV due to high structural similarity and sharing of few epitopes.

Prediction and identification of epitopes of turbot α PV and β PV

The possible epitopes for the turbot PVs were predicted using computational tools. As shown in Table 1, the potential epitopes of turbot α PV and β PV were predicted by three immunoinformatic tools including DNASTar Protean system, Bepipred Linear Epitope Prediction 2.0 and ABCpred Server. Subsequently, by screening the overlapping regions of the prediction results of the three immunoinformatic tools, five epitope peptides of α PV (A-1 to A-5) and five epitope peptides of β PV (B-1 to B-5) were chosen and synthesized for further identification (Table 2). Among them, B5 (91–104) showed similarity (53.3%) with the linear IgE epitope (GDKGIGVDEFGAMIKE) of Gad m 1.²⁴ Besides, B-2 (34–45) and A-3 (51–65) were similar with proposed linear-type IgE epitopes region 33–44 (VGLDAFSADELK) and 49–64 (IADEKKEGFIEDELK) of Gad c 1, respectively.²⁵

Inhibition Western blot was carried out for the verification of epitopes of turbot α PV and β PV. The epitope peptides which could inhibit the binding of purified natural PV to IgE from eight selected patients' sera (S1, S2, S3, S5, S6, S7, S8, S10) possessed IgE binding capacity to corresponding sera. As shown in Fig. 6 (A), 2 mg mL^{-1} natural PV was set as positive control, which almost totally inhibited the IgE binding of all eight patient sera to natural PV on the membrane. Peptide A-1, A-3, A-4, B-2, B-4 and B-5 showed IgE binding to > 4 of the eight selected patients' sera whereas peptide A-2, A-5, B-1 and B-3 showed IgE binding to less patients' sera (Fig. 6(B)). To further analyze the IgE binding capacity of epitope peptides, IgE reactivity intensity of peptide to each serum was graded into four levels with corresponding score (0–3) as previously described.²⁰ After calculation, the total IgE reactivity intensity scores of peptides A-1, A-3, A-4, B-2, B-4 and B-5 were higher than the mean scores (11.1) of ten peptides (Fig. 6(B)). Based on the frequency and intensity of IgE reactivity, three α PV major IgE epitopes and three β PV major IgE epitopes were identified as follows: A-1: α PV (17–28), A-3: α PV (51–65), A-4: α PV (73–86), B-2: β PV (34–45), B-4: β PV (72–83) and B-5: β PV (91–104). For IgG binding capacity, peptides A-1, A-3, B-3, B-4 and B-5 had IgG reactivity with rabbit anti-turbot IgG polyclonal antibody and the IgG reactivity intensity of A-1, A-3, B-4 and B-5 were higher, revealing two major α PV IgG epitopes (A-1 and A-3) and two major β PV IgG epitopes (B-4 and B-5) (Fig. 6(B)). Interestingly, the major IgG epitopes of α PV and β PV were also identified as their major IgE epitopes.

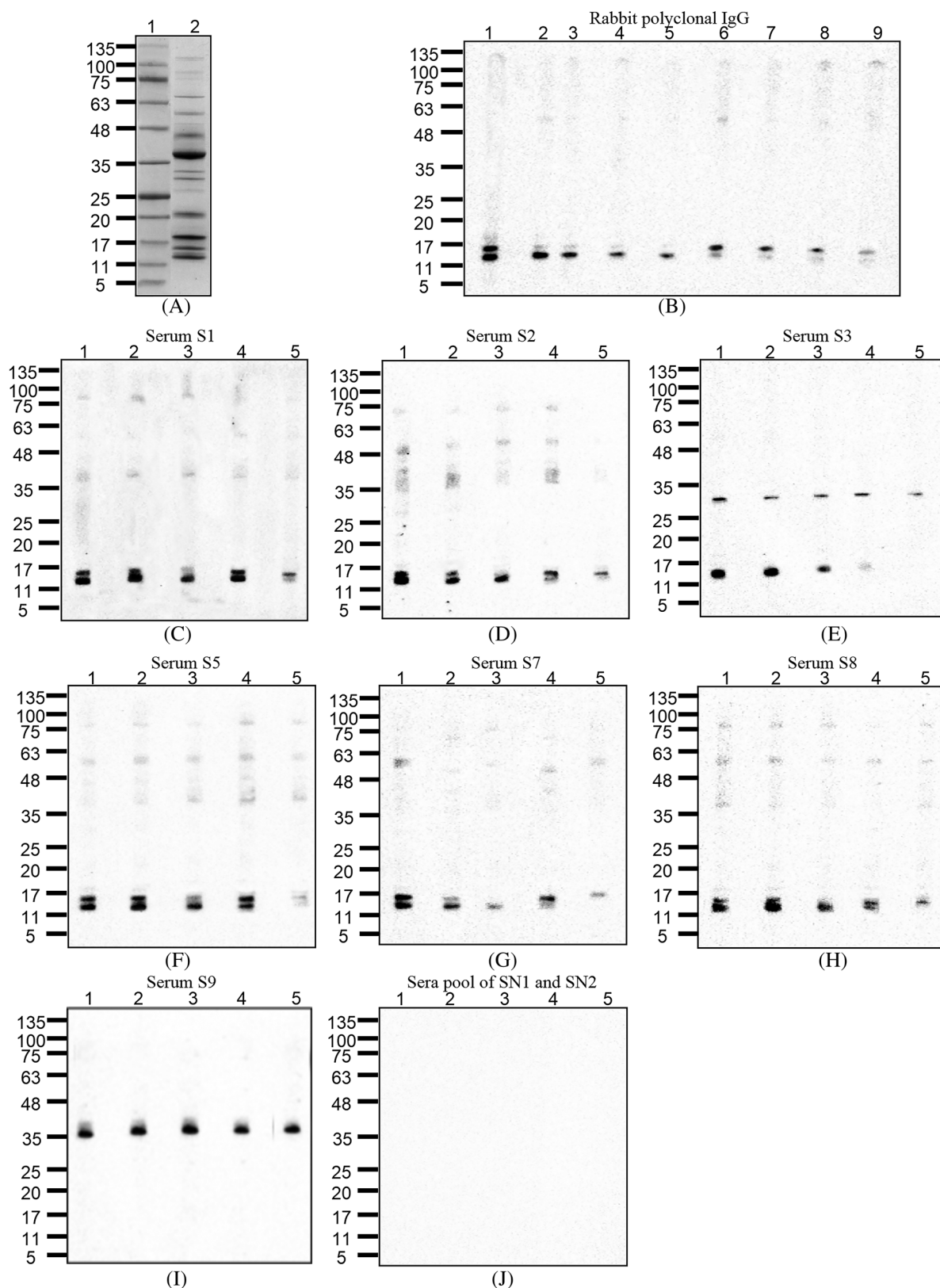
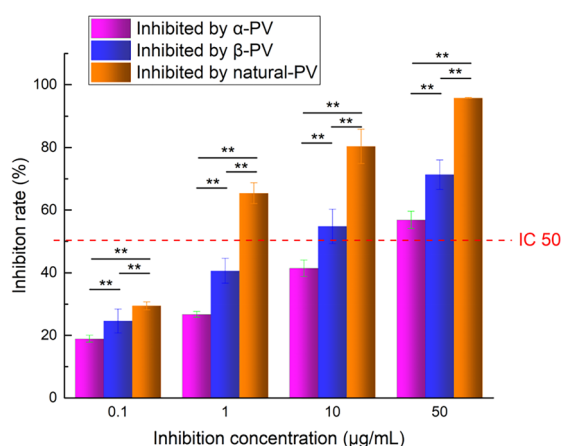
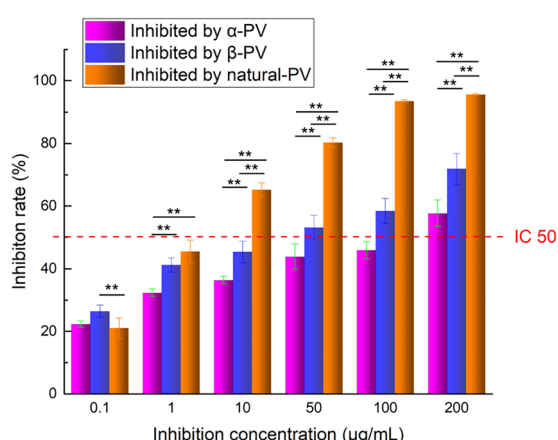


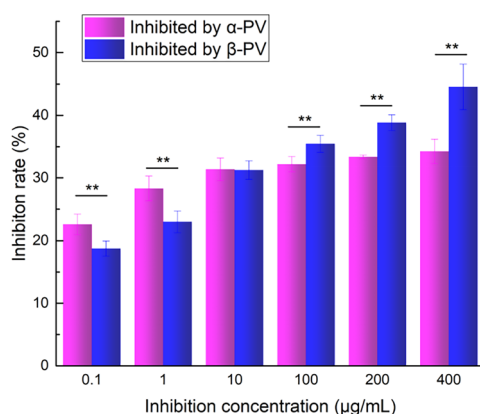
Figure 4. Comparison of IgG/IgE binding capacity of recombinant turbot α PV and β PV by inhibition Western blot. Rabbit polyclonal IgG and fish-allergic patient sera (S1, S2, S3, S5, S7, S8, S9 in Supporting Information Table Table S1) IgE binding to the PV in crude turbot extract and the inhibition of binding by recombinant turbot α PV and β PV. The IgG/IgE binding capacity of recombinant turbot α PV and β PV was represented by the degree of inhibition. (A) SDS-PAGE of crude turbot extract: lane 1: marker, lane 2: crude turbot extract. (B) Binding of rabbit IgG to crude turbot extract and inhibition by recombinant α PV and β PV. Lane 1: IgG binding to crude turbot extract, lanes 2–5: IgG binding to crude turbot extract inhibited by 5, 50, 200 and 400 $\mu\text{g mL}^{-1}$ α PV, lanes 6–9: IgG binding to crude turbot extract inhibited by 5, 50, 200 and 400 $\mu\text{g mL}^{-1}$ β PV. (C–J) Binding of human sera (S1, S2, S3, S5, S7, S8, S9 and sera pool of SN1 and SN2) IgE to turbot crude extract and inhibition by recombinant α PV and β PV, sera pool of SN1 and SN2 was set as control pool sera: lane 1: IgE binding to crude turbot extract, lanes 2 and 3: IgE binding to crude turbot extract inhibited by 0.1 and 1 $\mu\text{g mL}^{-1}$ α PV, Lanes 4 and 5: IgE binding to crude turbot extract inhibited by 0.1 and 1 $\mu\text{g mL}^{-1}$ β PV.



(A) Inhibition of patient serum IgE binding to purified natural parvalbumin



(B) Inhibition of rabbit polyclonal IgG binding to purified natural parvalbumin



(C) Inhibition of rabbit polyclonal IgG binding to recombinant α PV or β PV

Figure 5. Comparison of IgG/IgE binding capacity of recombinant turbot α PV and β PV by inhibition ELISA using rabbit IgG polyclonal antibody and fish-allergic sera pool (S1–S10 in Supporting Information Table S1). The percent inhibition represented the IgG/IgE binding capacity of recombinant turbot α PV and β PV. (A) Inhibition of patient serum IgE binding to purified natural PV by natural PV, recombinant α PV and recombinant β PV. (B) Inhibition of rabbit polyclonal IgG binding to purified natural PV inhibited by natural PV, recombinant α PV and recombinant β PV. (C) Inhibition of rabbit polyclonal IgG binding to recombinant α PV by recombinant β PV and inhibition of rabbit polyclonal IgG binding to recombinant β PV by recombinant α PV. Data shown as the mean $\pm$ standard deviation ($n = 3$). ** represent $P < 0.01$.

Table 1. Potential epitopes of α PV and β PV predicted by immunoinformatic tools

Allergen	Tools	Position of predicted epitopes
α PV	DNAstar	18–24, 36–44, 51–58, 60–65, 78–86, 90–97
	Protean	
	IEDB	5–10, 21–26, 36–42, 52–62, 73–81, 92–104
	ABCpred	1–14, 10–23, 21–34, 32–45, 41–54, 46–59, 61–74, 68–81, 75–88, 84–97
β PV	DNAstar	23–29, 37–43, 51–57, 58–64, 77–83, 92–98
	Protean	
	IEDB	19–24, 35–41, 52–61, 72–80, 91–104
	ABCpred	5–18, 17–30, 25–38, 30–43, 38–51, 44–57, 58–71, 63–76, 75–88, 80–93

Mapping of identified epitopes on the 3D model

As shown in Fig. 7, three major α PV epitopes (A-1, A-3 and A-4) and three major β PV epitopes (B-1, B-4 and B-5) were mapped on the 3D model of α PV and β PV, respectively. Most amino acids of the epitopes were on the surface of α PV or β PV, which corresponded to the prediction by immunoinformatic tools. It should be noticed that all six epitopes were mainly located on the loop regions between α -helices. Besides, α PV epitope A-3 and β PV epitope B-5 were in Ca^{2+} binding region.

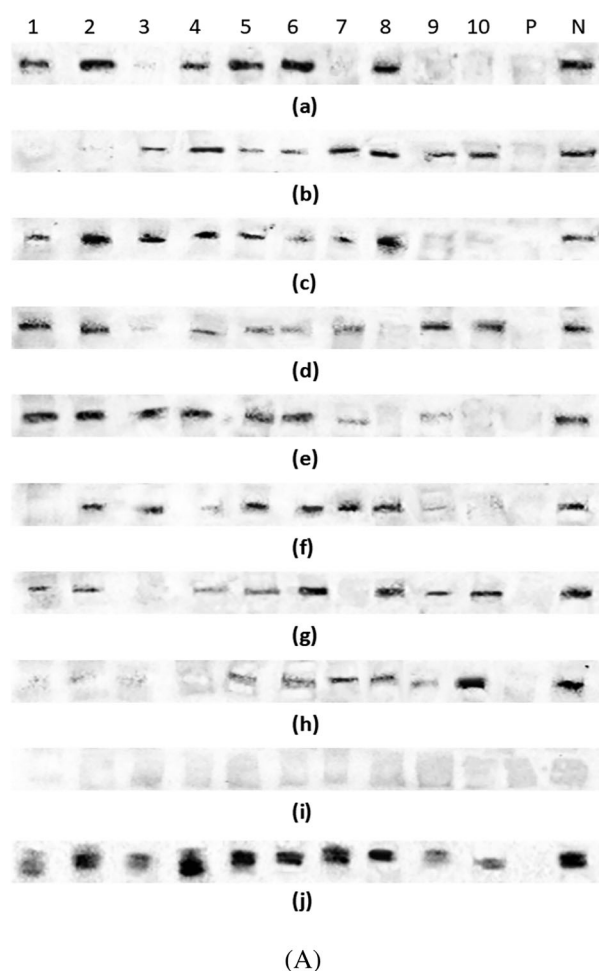
Amino acid composition analysis and similar sequences searching of verified epitopes

The amino acid composition of turbot α PV and β PV and their epitopes were compared. The amino acid sequences of turbot α PV and β PV showed similarity with 64.81% (Fig. 8(A)), corresponding to the low sequence identity ($< 50\%$ sequence identity between α PV from leopard shark or thornback ray and β PV from cod, carp, or salmon) between fish α PV and β PV.²⁶ It is worth mentioning that tyrosine (Tyr) was only present in β PV while proline (Pro) and histidine (His) were only present in α PV (Fig. 8(B)). In terms of epitope, β PV epitopes contained more phenylalanine (Phe), alanine (Ala), glycine (Gly), asparagine (Asn) and Tyr than α PV epitopes (Fig. 8(B)). The contents of glutamic acid (Glu), valine (Val), isoleucine (Ile), glutamine (Gln), arginine (Arg) in β PV epitopes were similar with that in α PV epitopes whereas contents of other amino acid in β PV epitopes were less than that in α PV epitopes (Fig. 8(B)). In general, the frequency of occurrence of hydrophobic residues of α PV epitopes (20.18%) was lower than that of β PV (25.92%).

Similar sequences searching of verified epitopes was performed to analyze their possible cross-reactivity due to sharing of amino acid identity in other known allergen sequences. Epitopes A-3, A-4, B-2 and B-4 had similar sequence (PD value < 8.0 , amino acid identity $> 33.3\%$) in 35, 25, 35 and 30 known allergens sequences in database, respectively (Fig. 8(C)). This represented comparable possible cross-reactivity with these epitopes. The details of similar sequences are shown in Table S2. Interestingly, epitope A-1 had similar sequences in only three PV sequences and the PD of these similar sequences was over seven (Table S2), which suggested that A-1 was the specific epitope of turbot α PV. By contrast, 75 allergen sequences in database contained similar sequence of epitope B-5, which indicated that B-5 should be the major cross-reactivity epitope of turbot β PV (Fig. 8(C)).

Table 2. Predicted epitopes finally synthesized by Fmoc solid phase peptide synthesis

Allergen	Epitope name	Sequence	Position
α PV	A-1	EECKKPESFCHK	17–28
	A-2	LSAKSPADVK	36–45
	A-3	IDQDKSGFIEEDELK	51–65
	A-4	AGARSLTDTETKNL	73–86
	A-5	GDTDNDGKIGAD	90–101
β PV	B-1	AAADTFNYKKFF	19–30
	B-2	GLAKKSADDVKA	34–45
	B-3	DQDNSGFIEEEELK	51–64
	B-4	AGARALTDKETA	72–83
	B-5	ADGDGKIGIEEFAN	91–104



Sera No.	Epitope peptide No.										PN
	A-1	A-2	A-3	A-4	A-5	B-1	B-2	B-3	B-4	B-5	
S1											4
S2											5
S3											5
S4											6
S5											4
S6											4
S7											7
S8											9
SN											0
Positive subjects number	5	3	5	5	4	4	6	3	7	5	
> 50% subjects	+		+	+			+		+	+	
Intensity score	12	8	14	12	8	6	13	8	17	13	
> Mean score	+		+	+			+		+	+	
IgG											

Figure 6. IgG/IgE binding verification of ten synthesized allergic epitope peptides of both PVs (A-1 to A-5 and B-1 to B-5) by inhibition Western-blot analysis to assess the difference in binding efficiency of turbot α PV and β PV. (A) Inhibition of eight patient sera (S1, S2, S3, S5, S6, S7, S8, S10) IgE (a–h), healthy human sera (SN1 and SN2) pool (i) and rabbit IgG polyclonal antibody (j). Lanes 1–5 represents A-1 to A-5 of α PV epitopes whereas lanes 6–10 represents B-1 to B-5 of β PV epitopes. Binding to natural turbot PV inhibited by 5 mg mL^{−1} ten epitope peptides (1–10), 2 mg mL^{−1} natural turbot parvalbumin (P) and 0.01 mol L^{−1} PBS (N). (B) Reactivity intensity analysis of ten epitope peptides by Image J software. The reactivity was scored in 0–3 levels based on the reduction in the degree of blotting intensity binding due to inhibitor: □ negative reactivity (score 0), □ light reactivity (score 1), ■ weak reactivity (score 2), ■ strong reactivity (score 3).

DISCUSSION

There are two distinct phylogenetic lineages of PV in fish, namely α PV and β PV.⁵ Several fish species were reported to possess > 1

PV isoforms.^{27–30} According to previously reported studies, bony fish β PV was known to be a major fish allergen and more allergic than cartilaginous α PV.²⁶ However, the difference in the

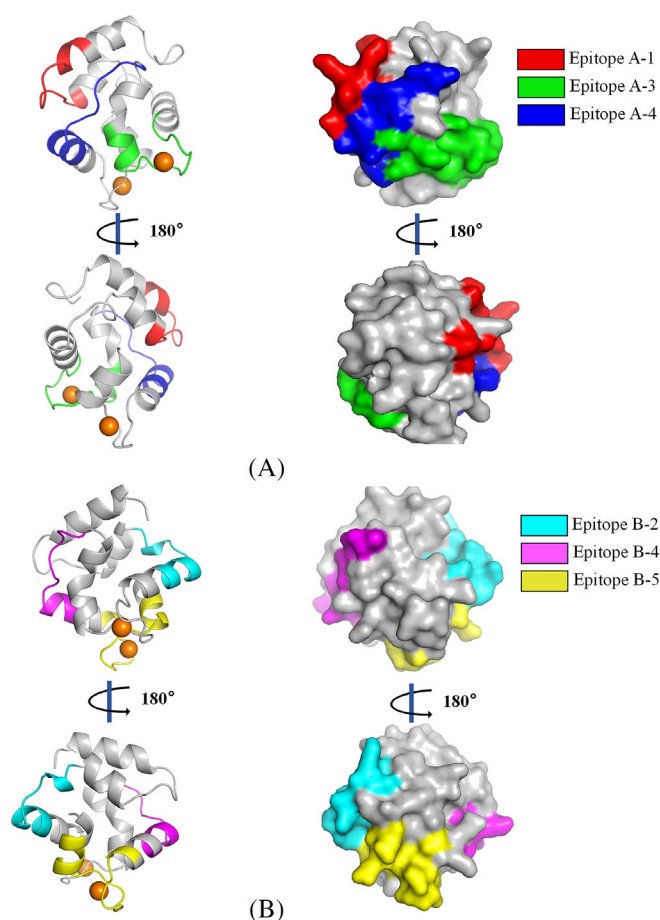


Figure 7. Homologous modeling of turbot α PV and β PV and mapping of their epitopes. (A) A 3D model of turbot α PV using PV from carp (1b8r.1.A) as a template, epitope A-1, A-3 and A-4 highlighted in red, green and blue, respectively. (B) A 3D model of turbot β PV using PV from carp (1b8r.1.A) as a template, epitope B-2, B-4 and B-5 highlighted in light blue, pink and yellow, respectively. The orange spheres represent calcium atoms.

immunological and allergic properties between α PV and β PV in bony fish was still not well explained. It may be due to the difficulty in separating the two natural isoforms (α PV and β PV) as they share very similar physicochemical properties. Turbot α PV and β PV was expressed in *E. coli* system with his-tag and was removed later to make them closer to their natural forms.³¹ Although the sequence similarity between turbot α PV and β PV was only 64.81%, results of CD and UV showed similar secondary and tertiary structures between recombinant α PV and β PV. Besides, the 3D model of α PV also appeared very similar to that of β PV, where both represent and share six α -helical regions in the 3D structure. Similar secondary and tertiary structures between natural and recombinant turbot PVs indicated that recombinant α PV and β PV were well folded in *E. coli* system. Both PVs required Ca^{2+} for the maintenance of the native conformation and it was revealed with spectral studies that recombinant α PV and β PV both depends on Ca^{2+} and depletion of Ca^{2+} led to more drastic changes to the secondary structure of both α PV and β PV.³²

To compare and reveal the difference in the IgE/IgG binding capacity of α PV and β PV, inhibition Western blot and inhibition ELISA were performed. Both human sera IgE and rabbit polyclonal IgG showed binding to two 12 kDa bands of natural PV in turbot crude extract and were subsequently inhibited by both

recombinant α PV and β PV at $1 \mu\text{g mL}^{-1}$ concentration with a variable degree. This revealed that α PV also possessed IgE/IgG binding capacity and may cross react with β PV. Mutual inhibition between α PV and β PV in inhibition ELISA confirmed their IgG cross-reactivity. Higher inhibition degree was observed for β PV when used at similar concentration in both inhibition Western blot and inhibition ELISA for both IgE and IgG binding capacity than that of α PV. The observed results indicate that β PV will be more allergenic than α PV in turbot. Interestingly, only a single band at 12 kDa was recognized by serum S3 and it was almost completely inhibited by $0.1 \mu\text{g mL}^{-1}$ recombinant β PV whereas $0.1 \mu\text{g mL}^{-1}$ recombinant α PV showed no obvious inhibition. This finding suggested that the recognized band was β PV and α PV was not identified by serum S3, which further supported that β PV was more allergenic. Serum S9 only showed binding to a 36 kDa protein band. Based on its molecular weight, the IgE positive band could be tropomyosin, which has been reported as an allergen in other fish species.³³

Considering the low sequence similarity and high 3D structural homogeneity of α PV and β PV, the difference in amino acid sequence and positioning of epitopes may differentiate the primary structure of α PV and β PV epitopes. The variability in epitope peptide sequence may be responsible for the observed difference in IgG and IgE binding ability between α PV and β PV. Therefore, the epitopes of α PV and β PV were predicted by three immunoinformatic tools and verified by inhibition Western blot. Three α PV epitopes and three β PV epitopes were finally identified which were commonly represented in all three epitope prediction tools. The loop region of the PV representing most of the identified epitopes was reported to be more flexible than α -helix region and could form cap structure in PV to cover the hydrophobic surface of adjacent domains according to previous studies, which also indicated increased accessibility to the region.³⁴ This may explain why all six epitopes are located on the loop region of the PV proteins. Ca^{2+} binding region are generally conserved in fish PV and have been identified as IgE epitopes in Gad c 1, Gad m 1 and Cyp c 1.01.^{24,25,35} In the present study, peptide A-3 is in Ca^{2+} binding region of α PV and peptide B-5 is in Ca^{2+} binding region of β PV which were also verified as IgE and IgG epitopes of turbot PVs. However, peptide B-3 and peptide A-5 were not identified as IgE epitopes, which indicated that Lys-55, Asp-61 in α PV and Ala-91, Gly-93, Glu-100, Glu-101, Ala-103, Asn-104 in β PV should be crucial to the epitope of A-3 and B-5, respectively. These amino acids in the epitope structure may contribute to the interaction with IgE by hydrogen bonds and electrostatic attraction. It was reported that Ala, serine (Ser), Asn, Gly and lysine (Lys) frequently occurred in IgE binding sites of most allergic epitopes.³⁶ Nine of the twelve amino acids in β PV epitope B-4 matched to α PV epitope A-4, which showed high similarity between these two epitopes and thereby explained the possibility of IgE cross-reactivity due to sharing of this epitope between α PV and β PV. Although no similar major IgG epitopes between α PV and β PV were found, weak IgG binding was observed for epitope peptide B-3 which was highly similar to the major IgG epitope A-3. This accounts for the predicted possible IgG cross-reactivity between two isoforms of PV. In general, the IgE reactivity intensity score of the β PV epitopes was higher than that of the α PV epitopes, which indicated higher IgE binding capacity of β PV epitopes and thereby could directly lead to the stronger IgE binding capacity of β PV. Hydrophobic residues which can function as weak interface hubs to promote IgE binding ability of protein, were reported to account for 60% of the amino acids represented in the epitopes of β -globulin.^{37,38} In

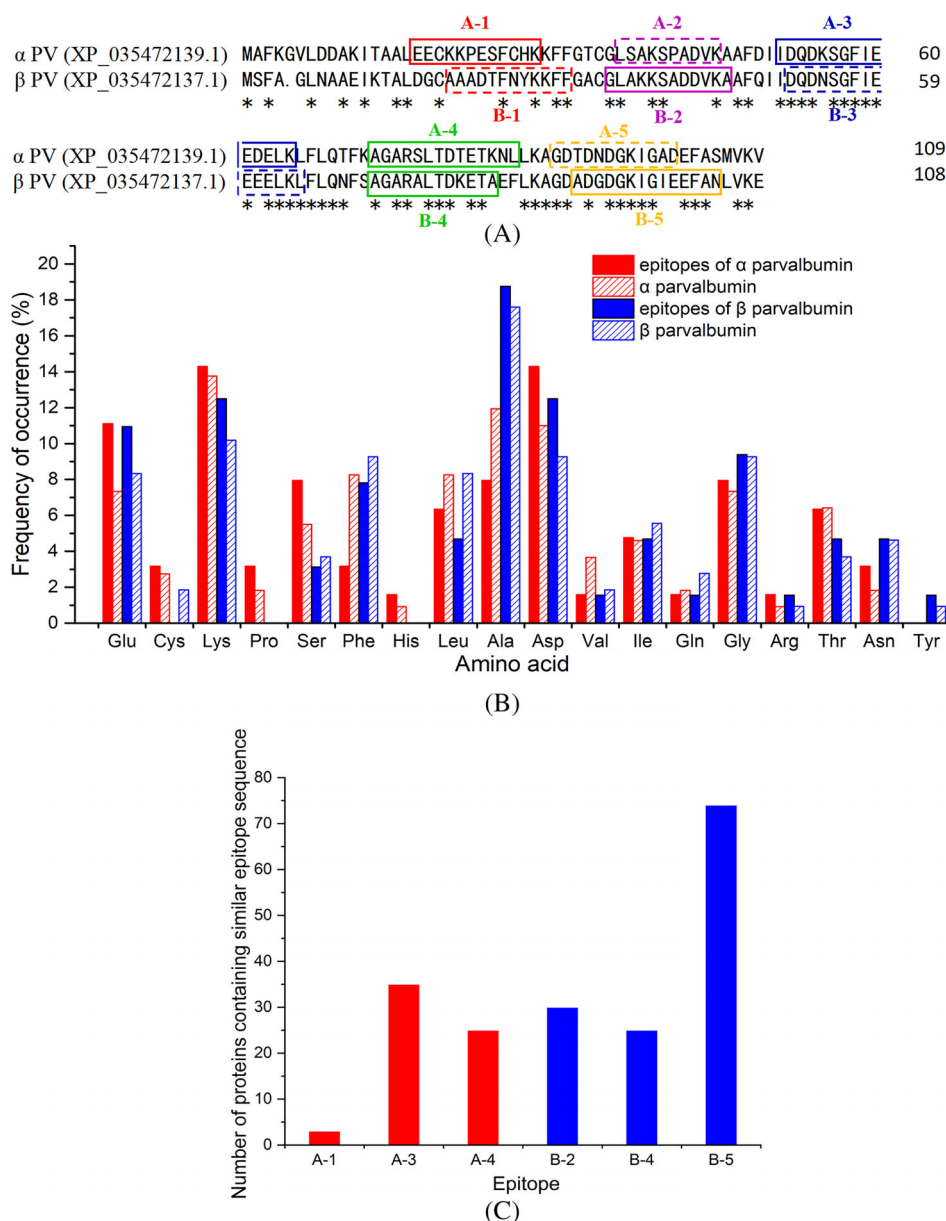


Figure 8. Sequence alignment of turbot α PV and β PV and sequence analysis of verified epitope. (A) Sequence alignment of turbot α PV and β PV. Ten predicted peptides were marked with box in different colors (red, pink, blue, green and yellow) box. Peptides marked with solid line box were identified as epitopes whereas peptides marked with dash box were not identified as epitopes. (B) Amino acid composition analysis of turbot α PV, turbot β PV and their epitopes. (C) Number of similar sequences of turbot α PV epitopes and β PV epitopes searched in SDAP allergen database.

the present study, β PV epitopes possessed more hydrophobic residues such as Phe and Ala than α PV epitopes, which may be responsible for the higher IgE reactivity intensity score of β PV epitopes. In contrast, both α PV and β PV had two major IgG epitopes, the presence of an extra weak IgG binding epitope peptide B-3 in β PV could be responsible for the observed higher IgG binding capacity of β PV. PD between two peptide sequences represent the sequence similarity of two peptide sequences. Allergens with similar sequences in equivalent epitope areas might be cross-reactive.^{23,39} Based on the search results in SDAP, the number of the allergen sequences in the allergen database which contained similar sequence of β PV epitopes, especially B-5, was higher than that of α PV epitopes, which suggested that β PV epitopes have

more potential as cross-reactive epitope and further illustrated that β PV was more likely to cross-react with other allergens. It is significant to mention that turbot α PV specific epitope A-1 was found matching similar sequences in only three allergen sequences in the database. The presence of epitope A-1 further revealed the difference between α PV epitopes and β PV epitopes. The study reveals that β PV epitopes exhibit high IgE binding capacity and have high epitope sequence similarity with predominant allergen in the database, indicating the possible increased allergic cross reactivity as compared to α PV epitopes. It is noticed that the epitopes analyzed in this study are linear, the influence of other potential conformational epitopes should be further explored in the future.

CONCLUSIONS

In conclusion, turbot recombinant α PV showed lower IgE/IgG binding capacity than recombinant β PV, however it exhibited similarity with secondary and 3D structure with β PV. It is observed that due to the presence of more hydrophobic residues, the IgE reactivity intensity of three identified β PV epitopes was higher than that of three identified α PV epitopes. This variation could be the cause for the lower IgE binding capacity of α PV among turbot allergic subjects as compared to β PV. Besides, epitope A-1 (EECKKPESFCHK) matching similar sequences in only three allergen sequences in SDAP database, could be a specific turbot α PV epitope. The present report in this study unravels the difference in immunogenic characteristic of IgE epitope peptides of turbot PVs. Additionally, it provides information on the allergic potential of α PV and β PV epitopes and explains the difference of IgE binding capacity indicating location variations and amino acid composition among the predicted epitopes between α PV and β PV. These findings will be useful for the improvement of detection tools for fish PV and development of specific diagnosis to allergy induced by fish PV.

ACKNOWLEDGEMENTS

This study was supported by the Fundamental Research Funds for National Natural Science Foundation of China (31871719) and the Fundamental Research Funds for the Central Universities (202161065).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

- Fu L, Wang C, Zhu Y and Wang Y, Seafood allergy: occurrence, mechanisms and measures. *Trends Food Sci Technol* **88**:80–92 (2019).
- Kuehn A, Swoboda I, Arumugam K, Hilger C and Hentges F, Fish allergens at a glance: variable allergenicity of parvalbumins, the major fish allergens. *Front Immunol* **5**:179 (2014).
- Kuehn A, Hilger C, Lehnert-Weber C, Codreanu-Morel F, Morisset M, Metz-Favre C *et al.*, Identification of enolases and aldolases as important fish allergens in cod, salmon and tuna: component resolved diagnosis using parvalbumin and the new allergens. *Clin Exp Allergy* **43**:811–822 (2013).
- Keshavarz B, Rao Q, Jiang X and Hsieh Y-HP, Immunochemical analysis of pepsin-digested fish tropomyosin. *Food Control* **118**:107427 (2020).
- Dijkema D, Emons JAM, Van de Ven A and Elberink J, Fish allergy: fishing for novel diagnostic and therapeutic options. *Clin Rev Allergy Immunol* **62**:64–71 (2020).
- Henzl MT, Tanner JJ and Tan A, Solution structures of chicken parvalbumin 3 in the Ca^{2+} -free and Ca^{2+} -bound states. *Proteins* **79**:752–764 (2011).
- Jenkins JA, Breiteneder H and Mills EN, Evolutionary distance from human homologs reflects allergenicity of animal food proteins. *J Allergy Clin Immunol* **120**:1399–1405 (2007).
- Stephen JN, Sharp MF, Ruethers T, Taki A, Campbell DE and Lopata AL, Allergenicity of bony and cartilaginous fish - molecular and immunological properties. *Clin Exp Allergy* **47**:300–312 (2017).
- Van Do T, Elsayed S, Florvaag E, Hordvik I and Endresen C, Allergy to fish parvalbumins: studies on the cross-reactivity of allergens from 9 commonly consumed fish. *J Allergy Clin Immunol* **116**:1314–1320 (2005).
- Van Do T, Hordvik I, Endresen C and Elsayed S, The major allergen (parvalbumin) of codfish is encoded by at least two isotypic genes: cDNA cloning, expression and antibody binding of the recombinant allergens. *Mol Immunol* **39**:595–602 (2003).
- Yang RQ, Chen YL, Chen F, Wang H, Zhang Q, Liu GM *et al.*, Purification, characterization, and crystal structure of Parvalbumins, the major allergens in *Mustelus griseus*. *J Agric Food Chem* **66**:8150–8159 (2018).
- Carrera M, Gonzalez-Fernandez A, Magadan S, Mateos J, Pedros L, Medina I *et al.*, Molecular characterization of B-cell epitopes for the major fish allergen, parvalbumin, by shotgun proteomics, protein-based bioinformatics and IgE-reactive approaches. *J Proteomics* **200**:123–133 (2019).
- Perez-Gordo M, Lin J, Bardina L, Pastor-Vargas C, Cases B, Vivanco F *et al.*, Epitope mapping of Atlantic salmon major allergen by peptide microarray immunoassay. *Int Arch Allergy Immunol* **157**:31–40 (2012).
- Sharp MF, Taki AC, Ruethers T, Stephen JN, Daly NL, Lopata AL *et al.*, IgE and IgG4 epitopes revealed on the major fish allergen Lat c 1. *Mol Immunol* **131**:155–163 (2021).
- Luo C, Guo Y, Li Z, Ahmed I, Pramod SN, Gao X *et al.*, Lipid emulsion enhances fish allergen parvalbumin's resistance to in vitro digestion and IgG/IgE binding capacity. *Food Chem* **302**:125333 (2020).
- Albrecht M, Alessandri S, Conti A, Reuter A, Lauer I, Vieths S *et al.*, High level expression, purification and physico- and immunochemical characterisation of recombinant pen a 1: a major allergen of shrimp. *Mol Nutr Food Res* **52**:S186–S195 (2008).
- Ma Y, Griesmeier U, Susani M, Radauer C, Briza P, Erler A *et al.*, Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp. *Mol Nutr Food Res* **52**:S196–S207 (2008).
- Xu LL, Lin H, Li ZX, Ahmed I, Pramod SN, Lin H *et al.*, Influence of non-thermal extraction technique and allergenicity characteristics of tropomyosin from fish (*Larimichthys crocea*) in comparison with shrimp (*Litopenaeus vannamei*) and clam (*Ruditapes philippinarum*). *Food Chem* **309**:125575 (2020).
- Hopp TP and Woods KR, Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci Belarus-Agrar Ser* **78**:3824–3828 (1981).
- Zhang Z, Li XM, Xiao H, Nowak-Wegrzyn A and Zhou P, IgE-binding epitope mapping of tropomyosin allergen (Exo m 1) from *Exopalaemon modestus*, the freshwater Siberian prawn. *Food Chem* **309**:125603 (2020).
- Ayuso R, Lehrer SB and Reese G, Identification of continuous, allergenic regions of the major shrimp allergen pen a 1 (tropomyosin). *Int Arch Allergy Immunol* **127**:27–37 (2002).
- Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R *et al.*, SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* **46**:W296–W303 (2018).
- Nesbit JB, Schein CH, Braun BA, Gipson SAY, Cheng H, Hurlburt BK *et al.*, Epitopes with similar physicochemical properties contribute to cross reactivity between peanut and tree nuts. *Mol Immunol* **122**:223–231 (2020).
- Perez-Gordo M, Pastor-Vargas C, Lin J, Bardina L, Cases B, Ibanez MD *et al.*, Epitope mapping of the major allergen from Atlantic cod in Spanish population reveals different IgE-binding patterns. *Mol Nutr Food Res* **57**:1283–1290 (2013).
- Elsayed S and Apold J, Immunochemical analysis of cod fish allergen M: locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* **38**:449–459 (1983).
- Kalic T, Morel-Codreanu F, Radauer C, Ruethers T, Taki AC, Swoboda I *et al.*, Patients allergic to fish tolerate ray based on the low Allergenicity of its Parvalbumin. *J Allergy Clin Immunol Pract* **7**:500–508 e511 (2019).
- Cai QF, Liu GM, Li T, Hara K, Wang XC, Su WJ *et al.*, Purification and characterization of parvalbumins, the major allergens in red stingray (*Dasyatis akajei*). *J Agric Food Chem* **58**:12964–12969 (2010).
- Li Z, You J, Luo Y and Wu J, Purification and characterization of parvalbumin isotypes from grass carp (*Ctenopharyngodon idella*). *J Agric Food Chem* **62**:6212–6218 (2014).
- Liu GM, Wang N, Cai QF, Li T, Sun LC, Su WJ *et al.*, Purification and characterization of parvalbumins from silver carp (*Hypophthalmichthys molitrix*). *J Sci Food Agric* **90**:1034–1040 (2010).

- 30 Guo F-F, Kubota H and Shiomi K, Purification, immunological properties and molecular cloning of two allergenic parvalbumins from the crimson sea bream, *Evynnis japonica*. *Food Chem* **132**:835–840 (2012).
- 31 Vologzhannikova AA, Khorn PA, Kazakov AS, Permyakov EA, Uversky VN and Permyakov SE, Effects of his-tags on physical properties of parvalbumins. *Cell Calcium* **77**:1–7 (2019).
- 32 Costa J, Villa C, Verhoeckx K, Cirkovic-Velickovic T, Schrama D, Roncada P *et al.*, Are physicochemical properties shaping the allergenic potency of animal allergens? *Clin Rev Allergy Immunol* **62**:1–36 (2021). <https://doi.org/10.1007/s12016-020-08826-1>.
- 33 Gonzalez-Fernandez J, Alguacil-Guillen M, Cuellar C and Daschner A, Possible allergenic role of tropomyosin in patients with adverse reactions after fish intake. *Immunol Invest* **47**:416–429 (2018).
- 34 Swoboda I, Bugajska-Schretter A, Linhart B, Verdino P, Keller W, Schulmeister U *et al.*, A recombinant hypoallergenic parvalbumin mutant for immunotherapy of IgE-mediated fish allergy. *J Immunol* **178**:6290–6296 (2007).
- 35 Untersmayr E, Szalai K, Riemer AB, Hemmer W, Swoboda I, Hantusch B *et al.*, Mimotopes identify conformational epitopes on parvalbumin, the major fish allergen. *Mol Immunol* **43**:1454–1461 (2006).
- 36 Oezguen N, Zhou B, Negi SS, Ivanciuc O, Schein CH, Labesse G *et al.*, Comprehensive 3D-modeling of allergenic proteins and amino acid composition of potential conformational IgE epitopes. *Mol Immunol* **45**:3740–3747 (2008).
- 37 Wolff N, Yannai S, Karin N, Levy Y, Reifen R, Dalal I *et al.*, Identification and characterization of linear B-cell epitopes of beta-globulin, a major allergen of sesame seeds. *J Allergy Clin Immunol* **114**:1151–1158 (2004).
- 38 Brinda KV and Vishveshwara S, Oligomeric protein structure networks: insights into protein-protein interactions. *BMC Bioinform* **6**:296 (2005).
- 39 Maleki SJ, Teuber SS, Cheng H, Chen D, Comstock SS, Ruan S *et al.*, Computationally predicted IgE epitopes of walnut allergens contribute to cross-reactivity with peanuts. *Allergy* **66**:1522–1529 (2011).