

Purification, Characterization, and Crystal Structure of Parvalbumins, the Major Allergens in *Mustelus griseus*

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ABSTRACT: Fish play important roles in human nutrition and health, but also trigger allergic reactions in some population. Parvalbumin (PV) represents the major allergen of fish. While IgE cross-reactivity to PV in various bony fish species has been well characterized, little information is available about allergens in cartilaginous fish. In this study, two shark PV isoforms (named as SPV-I and SPV-II) from *Mustelus griseus* were purified. Their identities were further confirmed by mass spectroscopic analysis. IgE immunoblot analysis showed that sera from fish-allergic patients reacted to both SPV-I and SPV-II, but the majority of sera reacted more intensely to SPV-I than SPV-II. Thermal denaturation monitored by CD spectrum showed that both of the SPV allergens are highly thermostable. SPV-I maintained its IgE-binding capability after heat denaturation, while the IgE-binding capability of SPV-II was reduced. The results of crystal structure showed that SPV-I and SPV-II were similar in their overall tertiary structure, but their amino acid sequences shared lower similarities, indicating that the differences in the IgE-binding capabilities of SPV-I and SPV-II might be due to differential antigen epitopes in these two isoforms.

KEYWORDS: *Mustelus griseus*, parvalbumin, purification, characterization, crystal structure

INTRODUCTION

Increasing production and consumption of fish has led to an increment in adverse reactions of people to fish. Although fish play important roles in human nutrition and health, they are one of the eight major sources responsible for IgE-mediated food allergy.¹ While the main clinical manifestations of allergic reactions to fish include vomiting and diarrhea, the most extreme form of reaction is life-threatening anaphylactic shock.² A Canadian survey among 9667 individuals demonstrated a prevalence of fish allergy of 0.51%.³ In Australia, 5.6% of a large cohort of food-allergic children was identified as having fish allergy. Among them, 16% developed symptoms to fish vapors.⁴ Fish allergy is common, not only in the Western civilization, but also in Asian countries. The population-based study among Filipino, Singaporean, and Thai children established that 2.29, 0.26, and 0.29% of the children suffered from allergic sensitization to fish.⁵ In China, fish allergy prevalence accounts for 0.2%⁶ and 0.32%⁷ of food-allergic children in Chongqing and Hong Kong, respectively. Although many food allergies developed during childhood are frequently outgrown, allergy to fish often remains persistent, even in adulthood.

Several fish allergens have been well characterized, such as the hormone vitellogenin identified from Beluga caviar⁸ and gelatin (type I collagen) from tuna and cod skin.⁹ The allergenicity of isinglass derived from fish swim bladder used for filtering beer has also been investigated, demonstrating that the gelatin content of isinglass is harmless to fish allergic subjects.² Other fish allergens such as beta-enolase, aldolase, and aldehyde phosphate dehydrogenase have been simultaneously identified.¹⁰ Furthermore,

in tilapia-sensitized patients, tropomyosin (major allergen in allergy to shellfish) was identified as a fish allergen.¹¹ Interestingly, this tilapia allergen showed high homology to human tropomyosin and was suggested to be involved as a causative agent in the autoimmune reaction of inflammatory bowel disease.¹²

As a major fish allergen, PV was first identified in Baltic cod (*Gadus morhua*) in 1969 and named as Gad c 1.¹³ It is an acidic, sarcoplasmic, 10–15 kDa protein that is extremely resistant to heat,¹⁴ proteolytic and chemical degradation¹⁵ and sensitizes up to 90% of fish allergic patients.^{16,17} PV is involved in the muscle relaxation and contraction cycle as well as signal transduction.^{18,19} Two distinct phylogenetic lineages of PV, namely α and β lineages, exist in different fish species. The α - and β -lineages differ slightly in length, with α -PV usually consisting of 109 or more amino acids while β -PV generally have 108 or fewer amino acids.²⁰ The α -lineage of this protein is predominant in muscle tissue of cartilaginous fish (*Chondrichthyes*) with isoelectric point (pI) generally above 5.0,^{21,22} while β -PV is abundant in muscle tissue of bony fish (*Osteichthyes*) with pI ranging from 3.9 to 4.5.^{23,24} Both protein lineages are similar structurally and physiologically, while the amino acid homology is only 47–54% identity.²⁵ Prior to this study, it is not clear whether both lineages of PVs are present in cartilaginous fish, due to the lack of biochemical as well as genomic studies.

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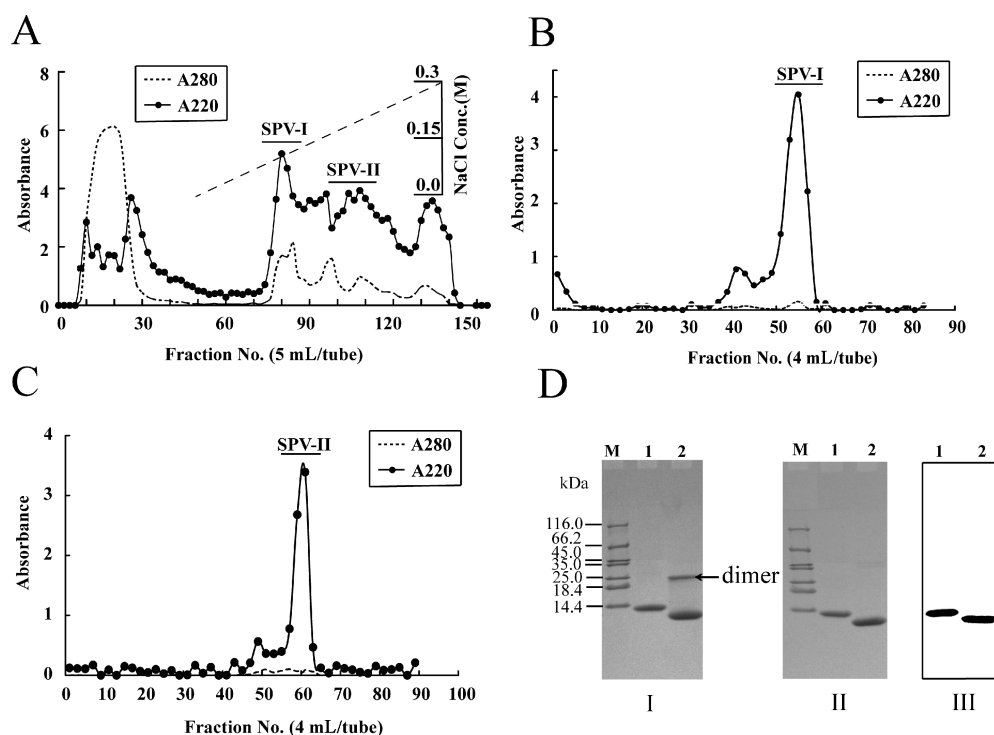


Figure 1. Chromatographic purification of SPVs from *Mustelus griseus*. (A) DEAE-Sepharose chromatography. (B) Sephacryl S-200 gel filtration purification of SPV-I. (C) Sephacryl S-200 gel filtration purification of SPV-II. (D) Tricine-SDS-PAGE and Western blot analysis of purified SPVs. Panel I, nonreducing Tricine-SDS-PAGE; panel II, reducing Tricine-SDS-PAGE; and panel III, Western blot analysis of purified SPVs using antiserum carp PV antibody. M, protein marker; 1, SPV-I; 2, SPV-II. The SPV-II dimer was indicated with arrow.

Allergy to bony fish is common and probably increasing worldwide. Bony fish allergens have been identified and characterized in numerous fish species.^{23,24} In contrast, few studies focused on cartilaginous fish allergens despite of its widespread consumption. As a member of the *Mustelus* Linck of the class *Chondrichthyes*, *Mustelus griseus* is one of the most important species in China, especially in the east and southeast coastline.²⁶ However, systematic studies on allergens in *Mustelus griseus* are relatively few. In this study, we purified and characterized both lineages of PV (named as SPV-I and SPV-II) from *Mustelus griseus*. IgE immunoblot analysis showed that sera from fish-allergic patients reacted to both SPV-I and SPV-II, but the majority of sera reacted more intensely to SPV-I than SPV-II. We further compared biochemical properties such as thermal and pH stabilities of both allergens. Finally, we determined the crystal structures of both SPV-I and SPV-II. Structural comparison showed that they had highly conserved folding, despite considerably different amino acids composition. Therefore, our study represents the first report on the characterization and comparison of PVs of two lineages in fish.

MATERIALS AND METHODS

Raw Material. Fresh *Mustelus griseus* with a body weight of about 1500 g was purchased from the eighth seafood market in Xiamen, China. The muscle tissue was collected and immediately used for the experiment.

Chemicals. DEAE-Sepharose and Sephacryl S-200 were purchased from GE Healthcare. Mouse antiserum carp PV monoclonal antibody was prepared by our laboratory as previously described.²⁷ Horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG antibody was purchased from DAKO (Glostrup, Denmark). HRP-conjugated goat antihuman IgE antibody was from Kirkegaard & Perry Laboratories (MD). Other reagents were all of analytical grade.

Patient Sera. Sera of 15 fish-allergic patients and 2 nonallergic individuals were collected from the Xiamen First Hospital (Xiamen, China).

Fish-specific IgE levels in sera were measured using the ImmunoCAP system (Phadia AB, Uppsala, Sweden). Levels lower than 0.35 kU_A/L were considered negative. All subjects provided their sera with informed consent voluntarily. All of the experiments using sera were performed in accordance with a protocol approved by the internal Ethical Committee of the hospital (XSH2012-EAN019).

Purification of PVs. All procedures of PV purification were performed at 4 °C. The muscle from *Mustelus griseus* was homogenized in 4-fold of buffer A (20 mM Tris-HCl pH 7.5) using a homogenizer (Kinematica, PT-2100, Switzerland). After centrifugation at 10 000g for 20 min, the supernatant was collected and fractionated with 60–100% ammonium sulfate. After centrifugation at 15 000g for 30 min, the resulting precipitate was dissolved in a minimum volume of buffer A and dialyzed against the same buffer extensively. The dialyzed solution was subsequently applied to DEAE-Sepharose (2.5 cm × 14 cm) previously equilibrated with buffer A. After the column was washed extensively with equilibrated buffer, binding proteins were eluted with a 0–0.3 M NaCl linear gradient at a flow rate of 1 mL/min. Fractions of interest were pooled respectively and concentrated by ultrafiltration using a YM-3 membrane (Millipore), followed by application to a gel filtration column of Sephacryl S-200, which was equilibrated with buffer A. The column was eluted at a flow rate of 0.6 mL/min. The fractions containing purified PVs were pooled and used for further characterization. Tricine-SDS-PAGE together with measurement of the absorbance at 220 nm was used to show the presence of PV throughout the purification procedures. Purified PVs were analyzed by Tricine-SDS-PAGE followed by Western blot assay.

Tricine-SDS-PAGE. Tricine-SDS-PAGE, which is commonly used to separate proteins in the molecular mass range of 1–100 kDa, was employed to analyze the molecular mass and purity of isolated PVs according to Schägger's protocol.²⁸ Purified samples were treated in the absence and presence of 1% 2-mercaptoethanol (2-ME). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). Molecular mass analysis was performed with Alphaview 3.0 software (Alpha Innotech, San Leandro, CA, USA).

IgG Immunoblot Assay. Proteins on Tricine-SDS-PAGE were transferred to a nitrocellulose (NC) membrane followed by blocking

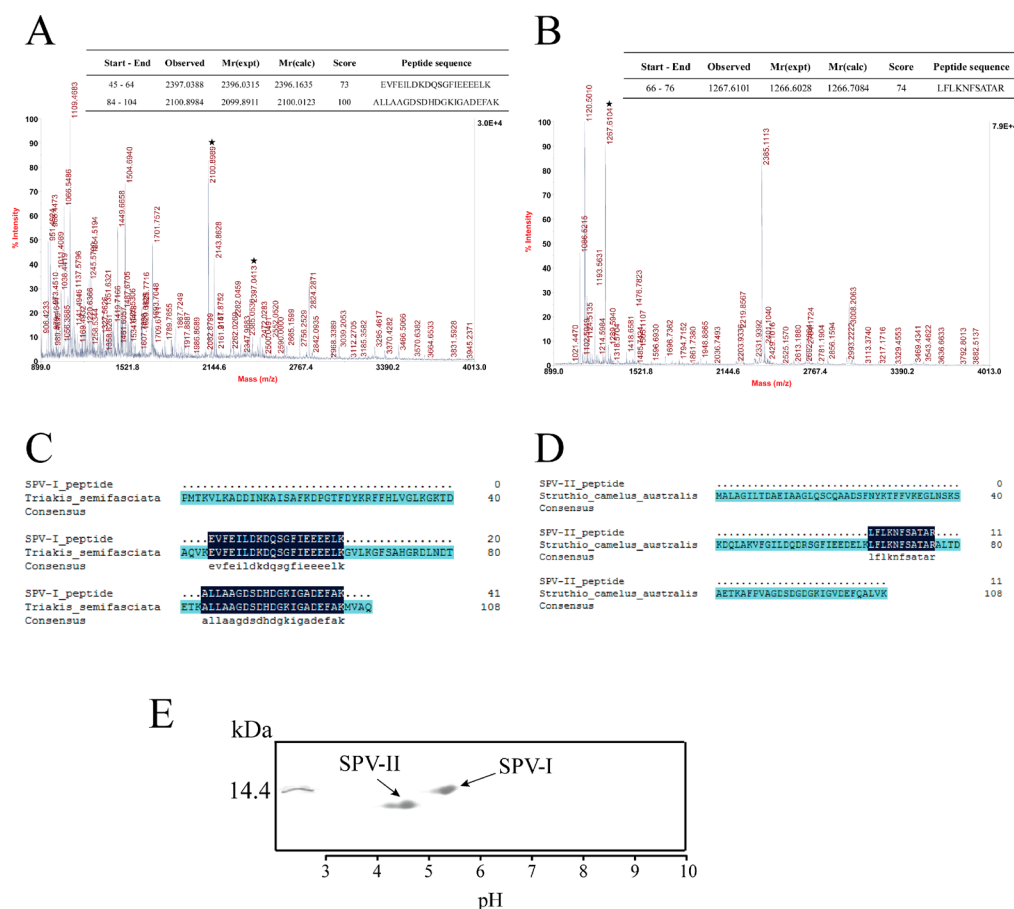


Figure 2. Identification of SPVs. (A) SPV-I and (B) SPV-II peptide segments obtained by MALDI-TOF-MS. * indicates the matched peptides. (C) SPV-I and (D) SPV-II sequence alignment. (E) Two-dimensional electrophoresis (2-DE) of SPV-I and SPV-II.

with 5% skim milk in TBST (0.145 M NaCl, 0.05% Tween-20, 20 mM Tris-HCl pH 8.0) at room temperature for 1 h. After it was washed with TBST, the membrane was immunoblotted with antisliver carp PV monoclonal antibody overnight at 4 °C. The membrane was then rinsed with TBST and incubated with HRP-conjugated rabbit antimouse IgG antibody for 1 h. Immunodetection was carried out using enhanced chemiluminescence (ECL) as substrate and the results were recorded using a fluorescence, chemiluminescence and visible light detection gel imaging system (Alpha Innotech).

Two-Dimensional Electrophoresis (2-DE). A mixed sample was applied to a 7 cm gel strip with immobilized pH gradient (pH 3–10), and isoelectric focusing was performed in an Ettan IPGphor 3 unit (GE Healthcare). After isoelectric focusing, the strip was equilibrated and subjected to SDS-PAGE.

Identification of PVs by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). The potential target protein bands were excised from the Coomassie Brilliant Blue stained SDS-PAGE gel and subjected to MALDI-TOF-MS in Shenzhen Wininnovate Biotechnology Company (Shenzhen, China). Gel digestion was performed according to the method of Katayama et al.²⁹ Sample mixture was spotted on a stainless sample plate. Peptide MS and MS/MS were performed on an ABI 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA). Both the MS and MS/MS data were integrated and processed by using the GPS Explorer V3.6 software (Applied Biosystems) with default parameters.

Dot Blot Immunoassay. Purified PVs (1 µg) were blotted to a NC membrane, followed by incubating at room temperature for 30 min. The membrane was blocked with 5% skim milk in TBST. After washing with TBST, the membrane was cut into strips and incubated with human sera (1:4 dilution in TBST buffer containing 1% skim milk) from 15 fish allergic subjects and two nonallergic individuals at 4 °C

overnight with gentle shaking. HRP-conjugated goat antihuman IgE antibody (diluted at 1:20 000) was used as the secondary antibody and the results were visualized by enhanced chemiluminescence (ECL) using the ECL substrate (Pierce, Rockford, Illinois, USA).

Thermal Denaturation of SPVs by Circular Dichroism (CD). The heating curve of the sample was recorded from 25 to 95 °C and the cooling curves were recorded from 95 to 25 °C at a rate of 2 °C/min from the wavelength of 190 to 260 nm using a Circular Dichroism instrument (Applied Photophysics, England). The data (Θ_{220}) was fitted by Origin 7.5 in order to calculate the ΔH and T_m values.³⁰

Thermal and pH Stabilities of SPVs and Their Effects on IgE-Binding Capabilities. To determine the thermal stability, purified PVs were adjusted to a final concentration of 1 mg/mL in microtubes and heated at 100 °C for a designated time (5, 10, and 30 min and 1, 2, and 4 h, respectively). To determine the pH stability, SPVs were adjusted to desired pH (pH 3.0–11.0) and incubated for 4 h at room temperature. After incubation, all samples were briefly centrifuged and the supernatant was subjected to Tricine-SDS-PAGE and dot blot immunoassay.

Crystallization, X-ray Diffraction Data Collection and Structure Determination. The purified PV proteins were concentrated to over 20 mg/mL and crystallization conditions were screened with available commercial as well as in-house designed crystallization kits. Several hits were readily identified within 2 weeks after the setup of hanging drops. High quality SPV-I crystals were obtained with a condition containing 2.5 M ammonium citrate, 0.1 M HEPES pH 7.0. Additional 10% (v/v) ethylene glycol and 10% (v/v) glycerol was added to the crystallization condition, and was used as the cryoprotective solution to flash-cool the crystals in liquid nitrogen. Thin plate crystals were obtained for the SPV-II protein at a condition composed of 2.3 M ammonium sulfate, 5% ethylene glycol, 0.1 mM HEPES pH 7.0. Additional 15% ethylene glycol was added to the crystallization condition and used as its cryoprotectant solution. See Table 2.

X-ray diffraction data were collected at beamline BL17U, BL18U, and BL-19U1 at Shanghai Synchrotron Radiation Facility (SSRF). Data were processed with HKL2000 program suite³¹ and XDS.³² The SPV-I structure was determined by molecular replacement calculation with Phaser³³ using the leopard shark SPV-I structure (PDB: 5PAL) as a searching template. The structure of SPV-II was determined by molecular replacement using the C-alpha model of SPV-I as a search model. The structural models were improved by rounds of manual model fitting in coot and structural optimization in phenix refine in Phenix GUI.³⁴ The final structural models were validated by the Molprobit server³⁵ and RCSB ADIT validation server.³⁶ Molecular graphics were displayed with Pymol (Delano Scientific LLC, San Carlos, CA). Protein sequence alignment was prepared with MEGA7,³⁷ ClustalX 2.1³⁸ and ESPript 3³⁹ together. Structure superposition and rmsd of structures was calculated in Pymol.

RESULTS

Purification and IgG-Immunoblot Analysis of SPVs.

Two lineages of PVs were purified from the muscle of *Mustelus griseus* by ammonium sulfate fractionation and sequential column chromatography with DEAE-Sepharose and Sephacryl S-200 columns. As shown by Tricine-SDS-PAGE (Figure 1A), which is better for stacking and separating low molecular weight proteins.^{28,40} PVs were adsorbed to DEAE-Sepharose and this ion exchange column effectively separated the two PV isoforms. Fractions of different isoforms were pooled respectively for further purification by Sephacryl S-200 gel filtration (Figure 1B and 1C). The two shark PV isoforms were named as SPV-I and SPV-II according to their elution order from DEAE-Sepharose column, and the purity was analyzed by Tricine-SDS-PAGE. We analyzed the intersubunit disulfide bonds by the absence and presence of 1% (v/v) 2-ME. In the absence of 2-ME, SPV-I migrated predominantly as a single band while SPV-II had a minor dimer band (Figure 1D, panel I). Interestingly, the band corresponding to SPV-II dimer disappeared after adding 2-ME (Figure 1D, panel II), suggesting that the dimerization of SPV-II molecules was mediated by disulfide bond (s). Furthermore, both SPVs showed IgG-binding capabilities with antiserum carp PV antibody (Figure 1D, panel III). PV has at least 50% cross-reactivity among different species.² Hence, these results established the existence of two PV isoforms in *Mustelus griseus*, and both PV isoforms have distinct disulfide bond forming capabilities.

Identification of SPVs. The purified SPVs were subjected to MALDI-TOF-MS analysis. Peptide mass fingerprinting of the purified protein showed multiple peaks ranging from 800 to 4000 Da, and the peaks with signal/noise ratio >50 were analyzed by MS/MS (Figure 2A and 2B). As shown in Figure 2A, two peptides of SPV-I were identified (EVFEILDKDQSGFIEE-EELK and ALLAAGDSHDGKIGADEFAK), which were mapped to the α -PV of *Triakis semifasciata* (GenBank: P30563.1, Figure 2C), indicating that SPV-I belonged to α lineage. Blast search showed that a peptide (LFLKNFSATAR, Figure 2B) of SPV-II displayed high sequence similarity with that of β -PV from *Struthio camelus australis* (GenBank: XP_009681637.1, Figure 2D), indicating that SPV-II belonged to β lineage. Therefore, these results showed that SPV-I and SPV-II were isotypes of PV and were α -PV and β -PV respectively.

To better characterize the purified SPVs, samples were subjected to 2-DE for separation by isoelectric point in one dimension (the horizontal direction) and by molecular mass in the second (the vertical direction).²¹ Figure 2E showed the 2-DE profile of mixed samples of SPV-I and SPV-II, and two spots were detectable. The molecular masses of these two spots were consistent with the result of Tricine-SDS-PAGE (Figure 1D).

Table 1. Clinical and Laboratory Characterizations of 15 Fish-Allergic Patients and 2 Nonallergic Individuals

sample no.	patient no.	age	sex	symptoms ^a	sIgE fish (f3) by ImmunoCAP (kUA/L)
1	7600	8	F	A, B	0.5
2	3809	3	M	N	0.59
3	7822	2	M	B, Dy	0.7
4	2775	4	M	A, Dy	1.5
5	3551	1	M	U	1.8
6	1784	3	M	A, B	2.5
7	0781	2	M	B	3.2
8	5286	76	M	H	3.5
9	5500	1	M	U, D	3.6
10	4323	25	F	U	5.6
11	5464	3	F	B, Dy	5.6
12	8475	1	F	A, B	13.5
13	0991	28	F	D	17.5
14	3580	2	M	A, Dy	43
15	1444	5	M	R	46
N1 ^b	8676	2	F	ND	0.14
N2 ^b	0297	4	M	ND	0.08

^aAbbreviation of clinical symptoms: U, urticaria; A, asthma; B, bronchitis; Dy, dyspnea; R, rhinitis; D, dermatitis; N, nephropathy; H, hectic; ND, not determined/unknown. ^bN1 and N2 are two nonallergic individuals.

Table 2. X-ray Data Collection and Structure Refinement Statistics

	SPV-I	SPV-II
Data collection	TJ15–4	TJ15–15
Space group	P1	P12 ₁
Unit cell (<i>a</i> , <i>b</i> , <i>c</i>) (Å)	26.21, 43.29, 52.57	44.87, 41.80, 60.34
(α , β , γ) (deg)	65.97, 88.93, 88.61	90, 110.40, 90
Resolution (Å) (Last shell)	50–1.40 (1.42–1.40) ^a	50–1.54 (1.58–1.54) ^a
No. of reflections (total/unique)	133405/37631	166757/30489
Redundancy (last shell)	3.6 (3.2) ^a	5.5 (4.0) ^a
Completeness (%)	90.9 (90.9) ^a	97.5 (77.4) ^a
<i>I</i> / σ (<i>I</i>) (last shell)	25.15 (3.34) ^a	16.22 (6.33) ^a
<i>R</i> _{meas} (last shell) (%) ^b	11.7 (84.8) ^a	8.6 (20.1) ^a
Refinement		
Resolution (Å)	50–1.40	50–1.54
Number of protein atoms	1686	1614
No. of solvent/heteroatoms	272/22	228/24
rmsd bond lengths (Å)	0.005	0.014
rmsd bond angles (deg)	0.713	1.228
<i>R</i> _{work} (%) ^c	16.19	19.43
<i>R</i> _{free} (%) ^d	18.56	21.25
Ramachandran plot favored/disallowed ^e	100/0	100/0
PDB code	SZGM	SZH6

^aNumbers correspond to the last resolution shell. ^b $R_{meas} = \sum_i (n/n - 1)^{1/2} \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h) \rangle$ are the *i*th and mean measurement of the intensity of reflection *h*. ^c $R_{work} = \sum_i |F_{obs}(h) - F_{calc}(h)| / \sum_i F_{obs}(h)$, where $F_{obs}(h)$ and $F_{calc}(h)$ are the observed and calculated structure factors, respectively. No *I*/ σ cutoff was applied. ^d*R*_{free} is the *R* value obtained for a test set of reflections consisting of a randomly selected 5% subset of the data set excluded from refinement. ^eValues from Molprobit server (<http://molprobit.biochem.duke.edu/>).

The pI values of SPV-I and SPV-II were approximately 5.3 and 4.7, suggesting that the SPV-I isoform possibly belonged to the α -group and the SPV-II isoform belonged to β -group.

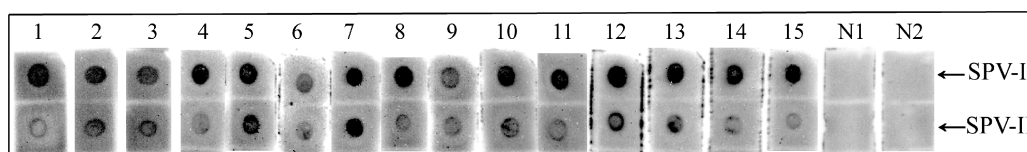


Figure 3. IgE immunoblot of purified SPVs by dot blot analysis. 1–15, dot blot profiles of IgE-binding activity of purified SPVs to patients' sera. N1 and N2, pooled nonallergic sera.

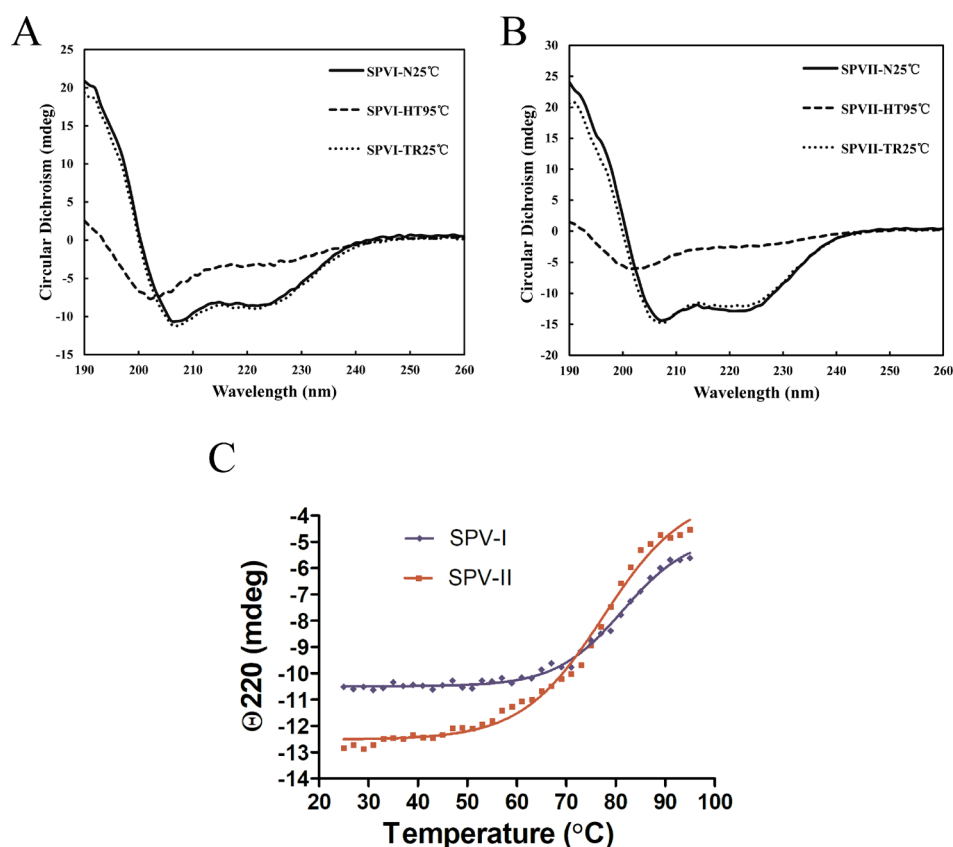


Figure 4. Thermal denaturation of SPVs by CD spectrum. Reversible conformational change of SPVs were induced by heat. (A) The CD spectra of SPV-I with at 25 °C (N25 °C), heating to 95 °C (HT 95 °C) and temperature reduced to 25 °C (TR25 °C). (B) The CD spectra of SPV-II at 25 °C (N25 °C), heating to 95 °C (HT 95 °C) and temperature reduced to 25 °C (TR25 °C). (C) Structural changes of SPVs during thermal denaturation as monitored by CD spectrum at 220 nm.

IgE-Binding Activities of SPVs. To verify the IgE-binding activities of SPVs, purified SPV-I and SPV-II were subjected to dot blot analysis with sera from 15 fish allergic patients and two nonallergic individuals as controls were used (Table 1). The results showed that the SPV-I and SPV-II had good specificity with fish-allergic patients' sera when compared with controls. Among them, the sera from six fish allergic patients (sample number: 2, 3, 5, 6, 7, 9) reacted almost equally to both isoforms, while the other sera reacted more intensely to SPV-I than SPV-II (Figure 3). These results indicated that SPV-I was more allergenic than SPV-II in most cases.

Thermal Denaturation Curves of Purified SPVs. High thermal as well as chemical stability are major characteristics of common food allergens.⁴¹ Therefore, we studied the thermal denaturation curves of SPVs by CD spectrum. Figure 4A and 4B, native SPVs show typical characteristic α -helical configuration of proteins (Figures 4A and 4B) at 25 °C with negative maximum peaks at 208 and 220 nm, and a positive minimum peak at 190 nm in the CD spectrum. When SPVs were heated to 95 °C (HT95 °C), the peak at 220 nm disappeared, with a negative

maximum peak emerging at 204 nm. As the temperature reduced to 25 °C (TR25 °C), the SPVs secondary structure recovered (Figure 4A and 4B), suggesting the thermal denaturation of SPVs were reversible. By monitoring the conformational changes of SPVs with CD spectrum at 220 nm, the results showed that SPV-I structure changed minimally in the range 25–65 °C, indicating that the α -helices were not destroyed. A faster change of SPV-I structure was observed in the range 65–85 °C with a $T_m = 82.6 \pm 2.3$ °C. At temperatures above 85 °C, SPV-I structure remained unchanged, implying the completion of SPV-I denaturation. When compared with SPV-I, SPV-II is less thermal stable. As the temperature rose, SPV-II structure slowly changed (Figure 4C), with a fitted $T_m = 79.2 \pm 3.4$ °C.

Effects of Thermal Stability on Dimerization and IgE-Binding Capabilities of SPVs. To study the effects of thermal stability on dimerization of SPVs, samples in buffer A were heated at 100 °C for 240 min. Samples were taken at different time points and analyzed by nonreducing Tricine-SDS-PAGE. As shown in Figure 5A, SPV-I stayed as a monomer up to 120 min, and only a minor dimer band appeared after heating for

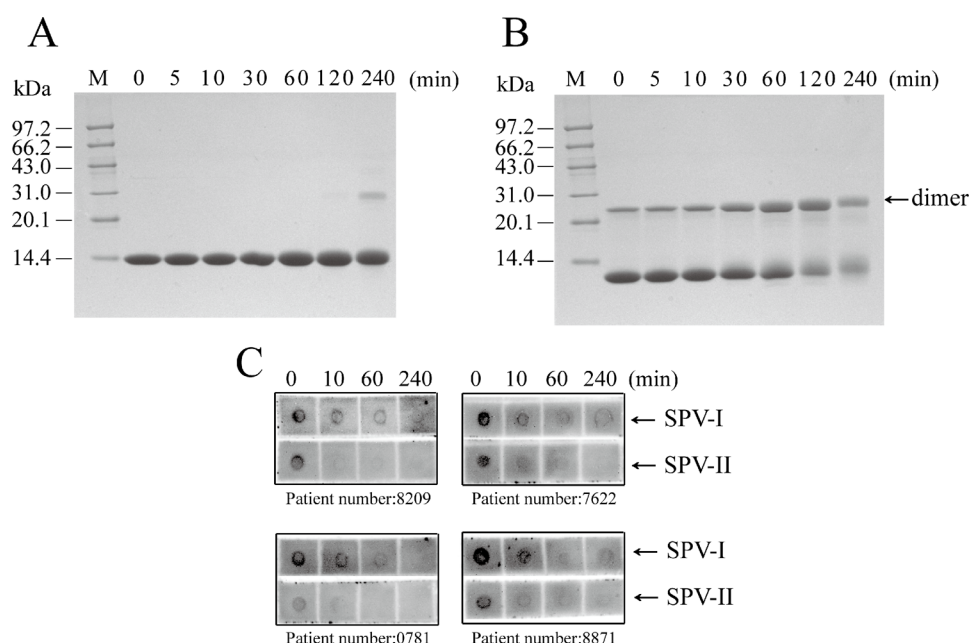


Figure 5. Thermal stability of purified SPVs. SPV-I (A) and SPV-II (B) were heated for different times and then applied to nonreducing Tricine-SDS-PAGE. M, protein marker. The incubation time was indicated above each lane. (C) IgE-binding capabilities of heated SPVs.

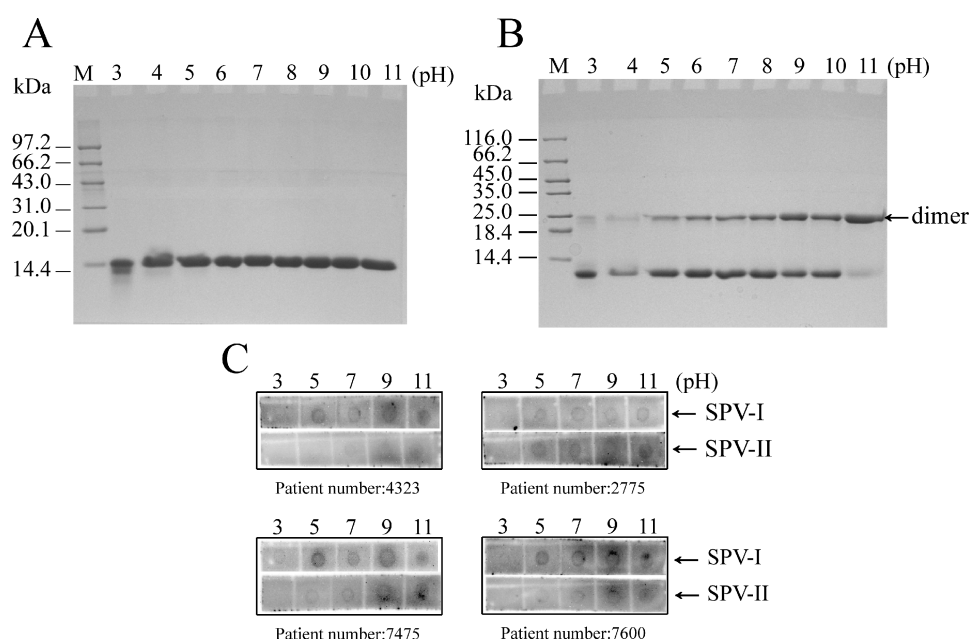


Figure 6. Effects of pH on the allergenicity of SPVs. SPV-I (A) and SPV-II (B) proteins were adjusted to desired pH by buffers and then applied to nonreducing Tricine-SDS-PAGE. M, protein marker. The pH was indicated above each lane. (C) SPV allergens were adjusted to desired pH by buffers and blotted on nitrocellulose membrane. Patients' sera were blotted and bound IgE were visualized.

240 min. On the other hand, a band corresponding to dimer appeared in SPV-II even before heating, suggesting that monomer and dimer coexisted in its native state (Figure 5B). As the heating time increased, the dimer population of SPV-II increased while its monomer population decreased gradually (Figure 5B). In order to find out whether thermal denaturation alters the IgE-binding of both SPV allergens, dot blot analysis of heat-treated SPVs was conducted with four fish-allergic patients' sera. Our data showed that both of the SPV allergens lost their reaction capability with allergic patients' sera after heat treatment in a time-dependent manner. SPV-I lost its IgE-binding capability much slower when compared with SPV-II.

The IgE-binding ability of SPV-II started to decrease after thermal treatment for 10 min, and almost disappeared at 60 min (Figure 5C).

Effects of pH on IgE-Binding Capabilities of SPVs. Protein structure is sensitive to solvent conditions. pH conditions may affect the structure of allergens, and therefore affect their dimerization and IgE-binding. In this study, we investigated the effects of pH on the IgE-binding capabilities of SPVs using patients' sera. As shown in Figure 6A, SPV-I as merely a single band was observed. As the pH increased, the SPV-I bands were almost unchanged. On the other hand, SPV-II tended to be cross-linked into dimers (Figure 6B) when pH increased.

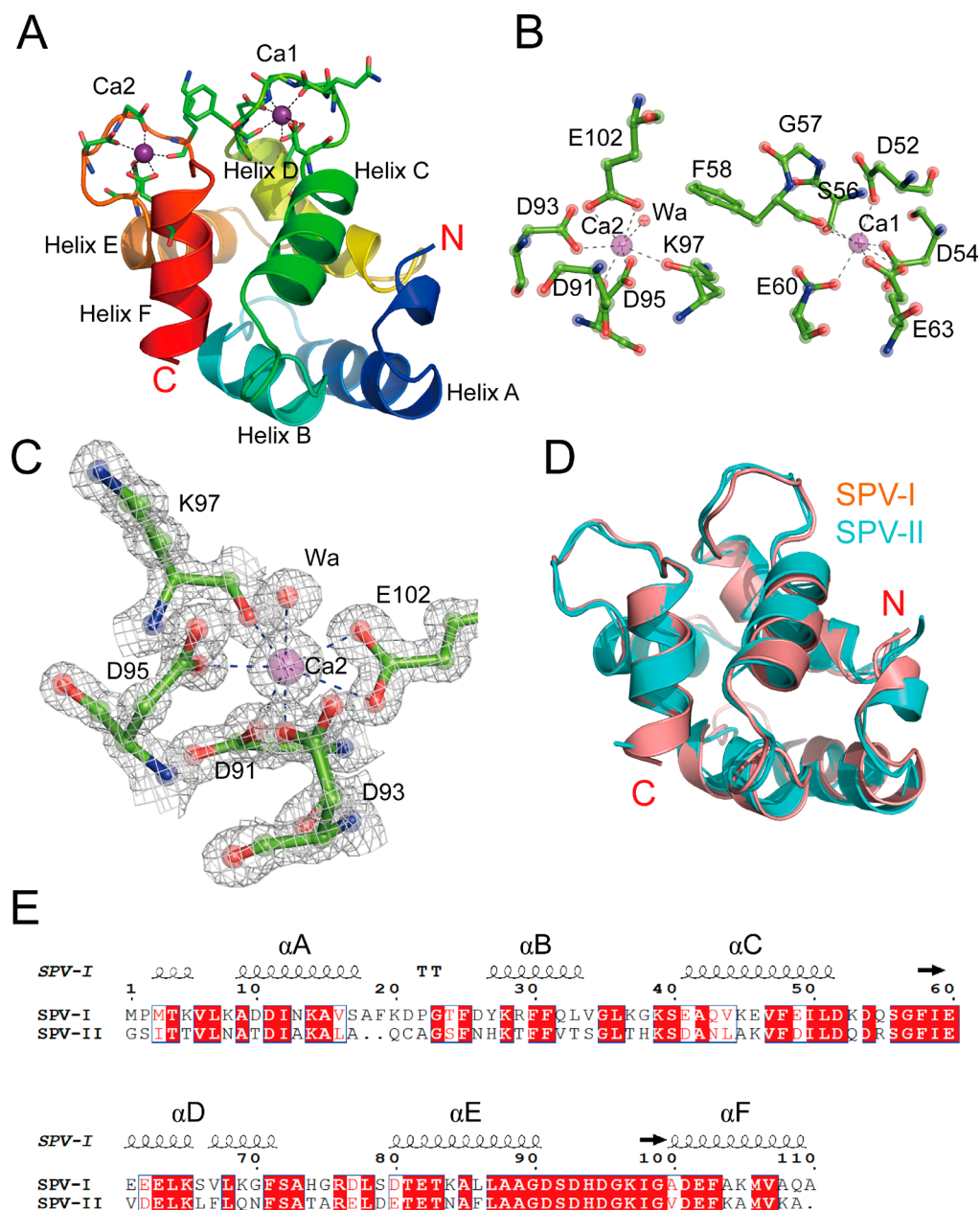


Figure 7. X-ray crystal structure of SPVs. (A) Overall structure of SPV-I. SPV-I was shown in cartoon and colored by rainbow. Both N- and C-termini as well as its 6 helices were labeled. The two calcium-binding sites were shown in sticks and the calcium ions were colored in purple. (B) The two calcium-binding sites of SPV-I were highlighted. The seven coordinating bonds were shown in dash lines, which form a pentagonal bipyramid type geometry surrounding a calcium ion. (C) Electron density map of the second calcium center. Five residues and a water molecule participated the interaction with calcium ion. (D) Structural superposition of SPV-I and SPV-II. Both proteins had very similar overall structure despite of ~57% sequence identity. (E) Structure based sequence alignment of SPV-I and SPV-II. The residues involved in calcium coordination are denoted by stars.

Otherwise, we found that SPV-I retained comparable reaction capability with all of the four patients' sera at all of the tested pH from 3 to 11 (Figure 6C). However, SPV-II showed higher reaction capability at higher pH and lost the IgE-binding capability at lower pH (Figure 6D).

Atomic Structures of SPVs. Structural characterization of allergens is important to understand the molecular nature of allergens and probably find potential correlation of three-dimensional structure with allergenicity.^{42,43} To this end, we crystallized both SPV allergens and determined their crystal structures. The structure of SPV-II was determined using the model derived from SPV-I. Both crystals diffracted to

very high resolution, SPV-I to 1.40 Å and SPV-II to 1.57 Å respectively.

Belonging to the EF-hand family of calcium-binding protein, both SPV-I and SPV-II displayed similar structural features despite of only 57% sequence identity (Figure 7A and 7E). Both of the SPV-I and SPV-II harbored two calcium-binding sites, and the calcium ions were coordinated by seven ligands forming a pentagonal bipyramid molecular geometry (Figure 7B). Close inspection of the calcium-binding sites identified minor differences. The Ca1 site of SPV-I was formed by D52, D54, S56, G57, F58 and E60, while the Ca2 site of SPV-II was formed by D91, D93, D95, K97, E102 and a water molecule (HOH9)

(Figure 7B and 7C). Structural superposition of SPV-I and SPV-II produced an rmsd of 0.70 Å of the aligned 707 atoms by Pymol, showing that parvalbumins of different lineages were highly conserved in their structure (Figure 7D).

DISCUSSION

The major pan-fish allergen, PV, is reported to account for up to 90% of cross-reactive fish allergy.¹⁷ PV has been extensively studied in bony fish.^{13,19,24,44–48} In comparison, studies on PV from cartilaginous fish are limited to red stingray,²¹ leopard shark,²² Atlantic stingray,⁴⁹ and thornback ray.⁵⁰ Two distinct phylogenetic lineages of PV, α and β , have been identified.² As a result, two isoforms of PV were purified from the muscle of *Mustelus griseus* by ammonium sulfate fractionation and sequential column chromatographies of DEAE-Sephacryl S-200. A search for homologous proteins in the NCBI protein database showed that the peptides of SPV-I had 100% sequence coverage with α -PV from *Triakis semifasciata*, while SPV-II showed high similarity with β -PV from *Struthio camelus australis*. The pI values of SPV-I and SPV-II were approximately 5.3 and 4.7 as determined by 2-DE analysis to further verify the SPV lineages.

Previous studies revealed that β -PV was more abundant in bony fish and appeared to be responsible for the majority of allergic reactions, while the α -isoform was predominant in cartilaginous fish.²⁵ Cai et al. revealed two α -PV isoforms (named as PV-I and PV-II) from red stingray, showing that PV-II displayed higher IgE-binding capacity than PV-I.²¹ To verify the IgE-binding activity of α and β isoforms of SPV, purified SPV-I and SPV-II were applied as antigens and investigated by dot blot with the sera of fish-allergic patients. The results showed that SPV-I possessed higher IgE-binding capacity, whereas SPV-II lower, indicating that SPV-I was likely more allergenic than SPV-II. However, elucidation of the IgE-binding epitopes was necessary to better understand allergen–antibody interactions.

The thermal denaturation processes of both SPVs were reversible, with unfolding of α -helix upon heating and refolding upon cooling. However, SPV-I was more resistant to heating than SPV-II as determined by CD spectrum at 220 nm.

Thermal stability is an important characteristic of SPVs as it conveys stability to heat processing and preparation of fish. Our results suggested that SPVs from *Mustelus griseus* were highly thermal stable. This thermal stability was mainly dependent on the presence of binding calcium ions, and the steric structures of PVs were variable during thermal processing.^{20,41} Therefore, in the present study, SPV-II might possess more readily reducible cysteine residues that facilitated the formation of oligomers. Further result revealed that SPV-I maintained the IgE-binding capability as heating time prolonged, while the IgE-binding ability of SPV-II was reduced significantly, indicating that the formation of SPV-II oligomers might hide the SPV-II IgE-binding epitopes.

The effect of pH on the IgE-binding characteristics is mainly due to the change of protein structure. In this study, the SPV-I monomer and IgE-binding were stable at different pH conditions. However, we found that at basic conditions, SPV-II tended to be cross-linked into dimers by disulfide bonds, probably due to the high oxidizing potential of oxygen at basic conditions. Chen et al. reported the tertiary structure of proteins might be partly unfolded in the circumstance of extreme pH conditions.⁵¹ Future studies are needed to investigate the relationship between dimer formations of SPV-II with its allergenicity.

ASSOCIATED CONTENT

Accession Codes

The coordinates and structural factors of SPV-I and SPV-II have been deposited in the Protein Data Bank with accession code SZGM and SZH6.

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Notes

The authors declare no competing financial interest.

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