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ARTICLE

Purification and Characterization of Parvalbumins, the Major Allergens in Red Stingray (*Dasyatis akajei*)

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Fish has received increasing attention because it induces IgE-meidated food allergy. Parvalbumin (PV) represents the major allergen of fish, and IgE cross-reactivity to PV in various teleost fish species has been shown, while little information is available about allergens in elesmobranch fish. In this study, two PV isoforms (named as PV-I and PV-II) from red stingray (*Dasyatis akajei*) were purified to homogeneity by a series of procedures including ammonium sulfate precipitation and column chromatographies of DEAE-Sepharose and Sephacryl S-200. Purified PVs revealed a single band on tricine−sodium dodecyl sulfate−polyacryalmide gel electrophoresis. The molecular masses of PV-I and PV-II were 12.29 and 11.95 kDa, respectively, as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Western blot using antifrog PV monoclonal antibody (PARV-19) showed positive reactions to the two proteins, confirming that they were PVs, although their immunological reactivities were weaker than those of PV from silver carp. The N-terminal amino acid sequence of PV-I was determined, and comparison with PVs from other fish species showed low homology between teleost and elasmobranch fish. The isoelectric points of PV-I and PV-II were 5.4 and 5.0, respectively, as determined by two-dimensional electrophoresis (2-DE), suggesting that both isoforms belong to the α-group. IgE immunoblotting analysis showed that sera from fish-allergic patients reacted to both PV-I and PV-II from red stingray. Thermal stability revealed that PV-I easily formed oligomers than PV-II, which might contribute to the maintenance of its allerginicity during heat processing.

KEYWORDS: *Dasyatis akajei*; parvalbumin; purification; characterization

INTRODUCTION

Fish plays an important role in human nutrition and health, especially in coastal areas, because of its high content of proteins and polyunsaturated fatty acids (*1*). However, increased production and consumption of fish have resulted in more hypersensitive reactions. In 2007, Vierk et al. showed that the self-reported prevalence of fish allergy in the United States was 0.7%, which was higher than that of eggs, tree nuts, wheat, and peanuts (*2*). A survey of 216 bronchial asthmatic children in China suggested that fish was the most important food allergen, with 20.3% (44 cases) of subjects allergic to it (*3*). Most fish allergies are IgE-mediated reactions that are the result of ingestion or contact with fish or fish products or even just inhalation of fish cooking vapors (*4*).

Parvalbumin (PV), which was first demonstrated as a major allergen in cod (*Gadus callarias*) (*5*), has been extensively studied in many different species of fish such as silver carp (*Hypophthalmichthy molitrix*) (*6*), commoin carp (*Cyprinus carpio*) (*7*,*8*), tilapia (*Oreochromis mossambicus*) (*9*), Pacific mackerel (*Scomber japonicus*) (*10*), Atlantic cod (*Gadus morhua*) (*11*, *12*), bigeye tuna (*Thunnus obesus*) (*13*), and Atlantic salmon (*Salmo salar*) (*14*, *15*). PV is a Ca^{2+}-binding protein with a molecular mass of about 12 kDa and an acidic isoelectric point. As a well-known major allergen in fish and fish products, different degrees of allergenic cross-reactivity among PVs from various species of fish have been established by immunoblotting and molecular studies (*16*−*18*), which might due to the various degrees of amino acid homologies (*19*). However, fish allergen research has been performed mostly on bony fish, and rare studies of allergens in cartilaginous fish have been systematically carried out.

Red stingray (*Dasyatis akajei*), which is mainly found in the northwestern Pacific Ocean off China, Japan, and Korea, is a member of the order Rajiformes of the class Elasmobranchii and is locally valued as a delicious food in China. However, the allergen profile of red stingray is still unknown. Therefore, the aim of this study is to purify PVs from red stingray and to investigate their characteristics.

MATERIALS AND METHODS

Fish. Red stingray (*D. akajei*) with a body weight of about 1300 g was purchased alive from a fish market in Xiamen, China. Fish were sacrificed

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instantly, and the muscle was collected and immediately used for the experiment.

Chemicals. DEAE-Sepharose and Sephacryl S-200 were purchased from Amersham Biosciences (Uppsala, Sweden). Protein standards for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were from Fermentas (Lithuania) or New England BioLabs (Beverly, MA). Mouse antifrog PV monoclonal antibody (PARV-19) and α-cyano-4-hydroxycinnamic acid (CHCA) were from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG antibody was purchased from DAKO (Glostrup, Denmark). Peroxidase-conjugated goat antihuman IgE antibody was from Kirkegaard & Perry Laboratories (MD). Enhanced chemiluminescent (ECL) substrate and 3,3′-diaminobenzindin tetrahydrochloride (DAB) for Western blot or dot blot were from Pierce (Rockford, IL). Dithiothreitol (DTT) was from Bio-Rad (Hercules, CA). Other reagents were all of analytical grade.

PV Purification. All procedures were performed at 4 °C. The muscle from red stingray was homogenized in 4-fold of 20 mM Tris-HCl (pH 7.5) buffer using a homogenizer (Kinematica, PT-2100, Switzerland). After centrifugation at 8000*g* for 30 min, the supernatant was collected and fractionated with 60−100% ammonium sulfate. After centrifugation at 15000*g* for 30 min, the resulting precipitate was dissolved in a minimum volume of 20 mM Tris-HCl (pH 7.5) and dialyzed against the same buffer extensively. The dialyzed solution was subsequently applied to DEAE-Sepharose (2.5 cm × 11 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.5). After the column was washed extensively with equilibrated buffer, binding proteins were eluted with a 0−0.5 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 20 mM Tris-HCl containing 0.15 M NaCl (pH 7.5). The column was eluted at a flow rate of 0.6 mL/min, and 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 all of the purification procedures.

Protein Concentration Determination. The protein concentration was determined by the method of Bradford using the Bio-Rad protein assay kit. Briefly, samples of bovine serum albumin (BSA) standard or PV samples were incubated with diluted dye reagent at room temperature for 5 min, and the absorbance at 595 nm was measured. Protein solutions were assayed in triplicate.

Tricine-SDS-PAGE and Western Blot. 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 (*20*). Purified samples were treated with 5% β-mercaptoethanol (β-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, France).

Western blot was performed as described by Towbin et al. (*21*). Briefly, proteins on polyacrylamide gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) followed by blocking with 5% skimmed milk in TBST (20 mM Tris-HCl, pH 8.0, containing 0.145 M NaCl and 0.05% Tween-20) at room temperature for 1 h. After it was washed with TBST, the membrane was immunoblotted with antifrog 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. Antibody binding was detected by enhanced chemiluminescence (ECL).

Two-Dimensional Electrophoresis (2-DE). Purified PVs were mixed and analyzed by 2-DE as described previously (*22*) for their isoelectric points comparison. Briefly, a mixed sample was applied to a 7 cm gel strip with immobilized pH gradient pH 4−7, 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 on 15% gel, followed by CBB staining.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Analysis. The molecular masses of purified PVs were further analyzed by MALDI-TOF MS (Applied Biosystems). Purified proteins were mixed with equal volumes of CHCA matrix and dried on a stainless steel MALDI target plate. MS spectra were acquired on a MALDI-TOF analyzer.

N-Terminal Amino Acid Sequence Determination. After electrophoresis, purified PV was electrophoretically transferred to a PVDF membrane. The membrane was briefly stained by CBB, and the protein band was excised. The N-terminal amino acid sequence of PV was determined using a protein sequencer (Shimadzu, PPSQ-33A).

Thermal Stability of PVs. To determine the thermal stability of PVs, purified proteins in microtubes were heated at 100 °C for different time intervals (5, 10, and 30 min and 1, 2, and 4 h, respectively). After incubation, samples were shortly centrifuged, and the supernatant was then analyzed by Tricine-SDS-PAGE.

Dot Blot Immunoassay. Sera from fish allergic patients were obtained from the hospital of Jimei University. The patients were all proposed to have fish allergy on the basis of their clinical history of immediate hypersensitivity reactions after ingestion of fish. Written, informed consent was obtained from each patient. All sera were stored at −80 °C until used. Purified PVs (1 μg) were blotted to a PVDF membrane, which was presoaked in methanol and PBS containing 10% methanol. The membrane was washed with TBST and blocked with 5% skimmed milk in TBST for 1 h at room temperature. After it was washed with TBST, the membrane was cut into strips and incubated with human sera (1:7 dilution) from 15 fish allergic subjects and two nonallergic individuals as the control at 4 °C overnight. HRP-conjugated goat antihuman IgE antibody was then allowed to react with the strips, followed by extensive washing and detection by DAB.

RESULTS AND DISCUSSION

For major allergen PV study in fish, many researchers focused on teleost fish. Although the amino acid sequence of PV from thornback ray (*Raja clavata*) and the crystal structure of PV from leopard shark (*Triakis semifasciata*) have already been described (*23*, *24*), rare research on PVs in elesmobranch fish concerning their immunological profiles was performed. More recently, Heffron et al. reported their work in determining the effects of urea, betaine, and TMAO on PVs' calcium-binding ability in Atlantic stingray (*Dasyatis sabina*) (*25*). Therefore, purification and characterization of PVs from edible elasmobranch red stingray (*D. akajei*) were carried out in the present study to elucidate their roles in food allergy. As a result, two isoforms of PV were purified to homogeneity from the muscle of red stingray by three steps including ammonium sulfate fractionation and sequential column chromatographies on DEAE-Sepharose and Sephacryl S-200. Because of the low content of aromatic amino acids of PV isoforms, measurement of the absorbance at 280 nm for most protein content was inappropriate to reveal the existence of PVs in column fractions. Therefore, in the present study, PVs were monitored at 220 nm during all purification procedures. The purity of PV was checked by Tricine-SDS-PAGE, which was better for stacking and separating low molecular mass proteins. As shown in **Figure 1A**, PVs were adsorbed to DEAE-Sepharose, and this ion exchange column was effective to separate PV isoforms. Fractions of different isoforms were pooled respectively for further purification by Sephacryl S-200 gel filtration (**Figure 1B**,**C**). The two isoforms were named as PV-I and PV-II according to their order of elution from DEAE-Sepharose column, and the purity was analyzed by Tricine-SDS-PAGE. The result showed that both PV isoforms migrated as a single band, indicating their high purity (**Figure 2A**). It was reported that 1−5 isoforms of PV are present in different species of teleost fish (*26*). Our present experiment revealed the existence of two PV isoforms in red stingray, which is similar to the case of Atlantic stingray (*D. sabina*) (*25*). However, only one PV isoform was identified in thornback ray (*23*) and leopard shark (*24*). Therefore, it is quite possible that the number of PV isoform was much less in elasmobranch than that in teleost. The yields of PV-I and PV-II were about 4.0 and 3.4 mg from 20 g of muscle, which were sufficient for following biochemical characterization.

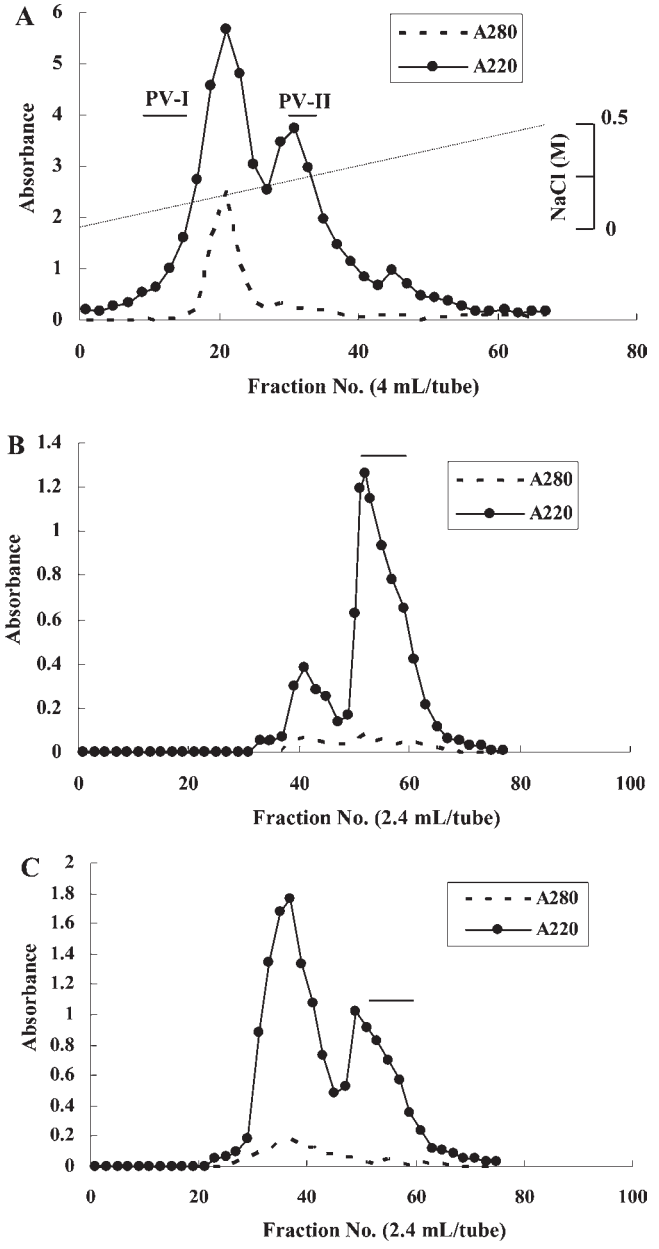

Figure 1. Chromatographic purification of PVs from red stingray. (**A**) DEAE-Sepharose chromatography, (**B**) Sephacryl S-200 gel filtration purification of PV-I, and (**C**) Sephacryl S-200 gel filtration purification of PV-II. Fractions under the bars were pooled.

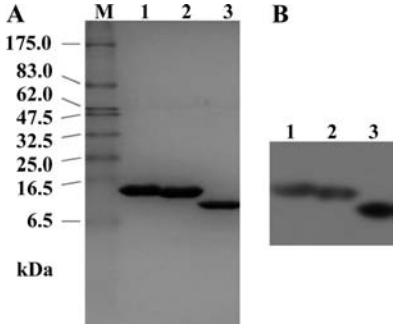

Figure 2. (**A**) Tricine-SDS-PAGE and (**B**) Western blot of purified PVs. M, protein marker; 1, PV-I; 2, PV-II; and 3, PV from silver carp as control. Approximately 5 ng of PV-I and PV-II and 1 ng of silver carp PV were loaded in each lane for Western blot. Pierce ECL substrate was used for development, and the membrane was exposed to film for 30 s.

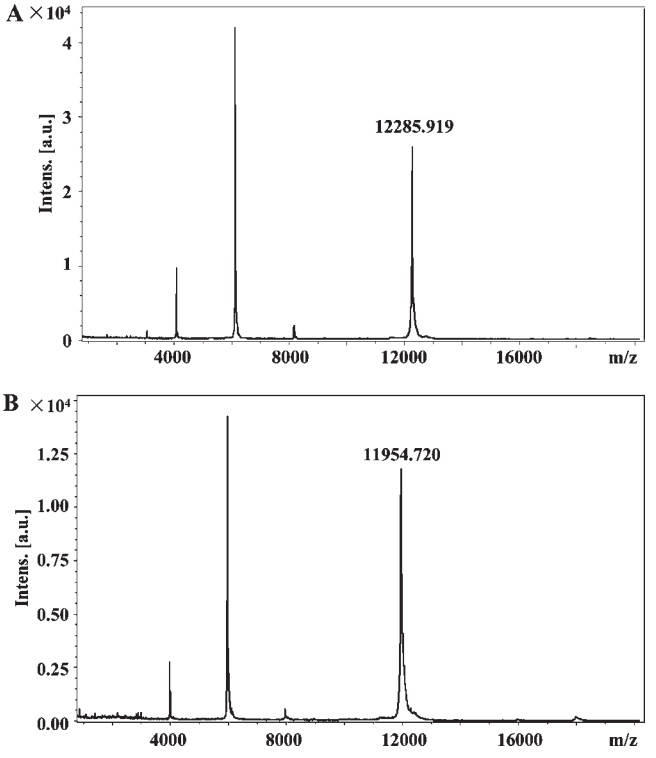

Figure 3. MALDI-TOF mass spectroscopic analysis of purified PV-I (**A**) and PV-II (**B**). The *x*-axis shows the mass/charge ratio; the signal intensity is displayed on the *y*-axis.

The molecular masses of PV-I and PV-II were estimated by Tricine-SDS-PAGE and MALDI-TOF mass spectrometry. Comparing the electrophoretic mobility of target proteins with protein molecular weight markers on Tricine-SDS-PAGE using Alphaview software, the estimated molecular masses of PV-I and PV-II were 13.05 and 12.68 kDa, respectively. MALDI-TOF mass spectrometry showed that the molecular masses of them were 12.29 and 11.96 kDa (**Figure 3**), which were slightly smaller than those obtained from Tricine-SDS-PAGE. These molecular mass results agreed well with PVs from other species of fish (10−14 kDa), such as silver carp (*H. molitrix*) (*6*), common carp (*C. carpio*) (*8*), and Atlantic stingray (*D. sabina*) (*25*).

Western blot analysis using antifrog PV monoclonal antibody (PARV-19) showed slight positive reactions with both PV isoforms from red stingray and positive reaction with PV from silver carp (**Figure 2B**), supporting that the purified proteins are PVs. In fact, wide cross-reactivity of PV among different species of teleost fish was proved (*10*, *16*), and reactivity of serum IgE from fish-allergic patients to bullfrog PV was also reported (*13*). This commercial monoclonal antifrog PV IgG (PARV-19) has been proved to cross-react with many species of teleost fish, and its binding site was considered to associate with the calcium-binding motifs, which were the most conserved regions of PVs (*26*−*28*). However, here, we observed that the immunological reaction of PARV-19 to PVs from red stingray was much weaker than that to PV from fresh water fish silver carp as the loading amount of purified PV from silver carp was just one-fifth of that from red stingray. These differences in IgG-binding ability might ascribe to the variances in their primary structures between elasmobranch and teleost fish.

The N-terminal amino acid sequences of purified PVs were then analyzed to compare their homologies. Purified PVs were

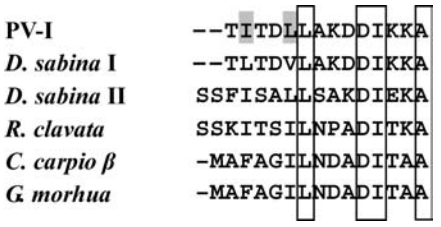

Figure 4. N-terminal amino acid sequence alignment of PVs. PV-I from red stingray was compared to Atlantic stingray [*D. sabina* (*25*)] and thornback ray (*R. clavata*, GenBank accession no. P02630) as well as teleost common carp (*C. carpio*, GenBank accession no. CAC83658) and Atlantic cod (*G. morhua*, GenBank accession no. AAK63087). Residues of PV-I differing from *D. sabina* I are indicated in shade. Residues identical among these species are boxed.

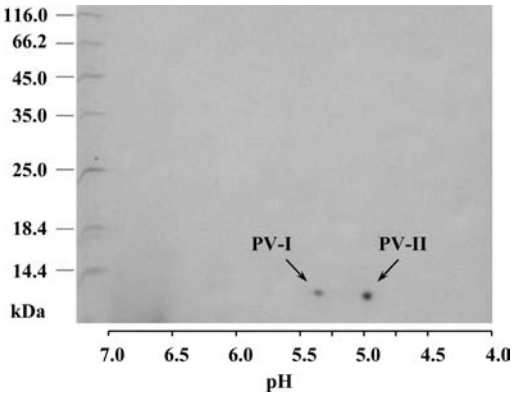

Figure 5. 2-DE map of purified PVs from red stingray. The gel was stained with CBB.

subjected to N-terminal amino acid microsequencing. The first 14 amino acid residues of PV-I were identified. Sequence alignment of PVs from different fish species is shown in **Figure 4**. The result indicated that PV-I from red stingray shared highest identity (85.7%) with PV-I from *D. Sabina* (*25*). Approximately 50% identities to PV-II from *D. Sabina* (*25*) and PV from *R. clavata* (*23*) were also identified. The low homology of about 28.6% to PVs from *C. carpio* β (*8*) and *G. morbua* (*12*) together with the result of Western blot revealed an evolutionary distance of PVs between teleost and elasmobranch fish. In the present study, PV-II was also applied to N-terminal sequencing, while it did not afford any amino acid residue, indicating the blocking of its N terminus. Such sequence blocking cases were also recognized previously in PVs from Atlantic salmon (*14*), Pacific mackerel (*29*), and bullfrog (*13*). Therefore, further research by molecular cloning to determine the full-length amino acid sequences of PV-I and PV-II is necessary to elucidate such variations.

To better characterize the purified PVs, 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) (*30*). The PV protein family could be subdivided into two distinct lineages, α and β, based on their isoelectric points and amino acid sequences. It is regarded that α-PV consists of less acidic amino acid residues with isoelectric points at pI 5.0 or higher, while β-PV consists of more acidic amino acid residues with isoelectric points at pI 4.5 or lower (*31*). **Figure 5** shows the 2-DE profile of mixed samples of PV-I and PV-II from red stingray, and just two spots were detectable, supporting the homogeneity of purified PVs. The molecular masses of these two spots were consistent with the result of Tricine-SDS-PAGE. The pI values of PV-I and PV-II were approximately 5.4 and 5.0, respectively, suggesting that both isoforms quite possibly belong to the α-group. However, full amino acid sequencing is necessary to verify their belonging. Thatcher et al. reported that thornback rays (*R. clavata*), which is included in the order of Rajiformes and thus evolutionarily close to red stingray, possess a major PV with the sequence more closely related to the ancestral sequence than any other extant PV, as its N-terminal region is similar to that of α-lineage, while possessing a distinct feature in position 66 of the β-lineage (*23*). The pI values of PVs from our 2-DE map were higher than values (4.7 and 4.8) of PVs from Atlantic stingray (*D. sabina*) (*25*), suggesting their difference. It has also been reported that monoclonal antibody PARV-19 reacts much stronger with acidic, smaller molecular mass PV isoform in tilapia (*26*). Thus, together with the pI values (5.4 and 5.0) and molecular masses, weak reaction of PVs from red stingray to PARV-19 antibody in Western blot might be explained.

In general, β-PVs are considered as the main allergens of fish allergy (*32*). A positive IgE reaction of sera from fish-allergic patients with α-PV from bullfrog (*Rana catesbeiana*) was also reported (*13*). To verify the IgE-binding activity of PVs from red stingray, purified PV-I and PV-II were applied as antigens and investigated by dot blot. Sera samples from 15 fish allergic patients and two nonallergic individuals as controls were used. In comparison with the results of control sera, all patient sera immunologically reacted with PV-I and PV-II (**Figure 6A**), suggesting that PVs contain the IgE-binding epitopes. However, the reaction potencies varied largely among the 15 sera samples, and the reactivities were complicated. To make a better comparison, the dots' density was analyzed using Alphaview 3.0 software. As shown in **Figure 6B**, all sera, except sera from patient 10 and 14, revealed relatively stronger immunoreactions with PV-II than PV-I. Sera from patient 14 reacted almost equally to both isoforms, while sera from patient 10 reacted strongly to PV-I than to PV-II. These results indicate that PV-II from red stingray may be more allergenic than PV-I in most cases.

Resistance to boiling is one of the characteristics of PV to induce allergy in sensitive patients. Therefore, heating treatment of PVs from red stingray was performed, and the proteins were checked by Tricine-SDS-PAGE. As can be seen from **Figure 7A**, protein dimer appeared in PV-I even before heating, suggesting that monomer and dimer coexist in its native state. Such a proposal was confirmed in **Figure 2A**, as merely a single band was observed under reducing conditions. As the heating time increased, the dimer and tetramer bands of PV-I progressively intensified, and a decrease in the band density of PV-I monomer was also observed. On the other hand, no dimer band could be found in PV-II even after heating for 30 min (**Figure 7B**), and only a slight dimer band appeared after 1 h. However, this band did not intensify even after heating for 4 h. It is of interest to notice that a band with the molecular mass of about 13 kDa appeared as the heating time was prolonged, which might be formed due to conformational change of PV-II after heat treatment. Our results suggested that PVs from red stingray are highly thermal stable. This thermal stability mainly depends on the presence of binding calcium ions, and the steric structures of PVs are variable during thermal processing (*33*). It was reported that the dimer of PV in snook was the product of the intermolecular reduction of Cys-18 (*34*). Therefore, in the present study, PV-I might possess more readily reducible cysteine residues that facilitate the formation of oligomers in PV-I than PV-II. Our result using patients' sera further indicated that the formation of oligomers maintained the allergenicity of PV-I, while the IgE-binding ability of PV-II was decreased as heating time was prolonged (**Figure 7C**). This result was consistent with the reports about the contribution of disulfide bonds to the allergenicity in allergens (*35*).

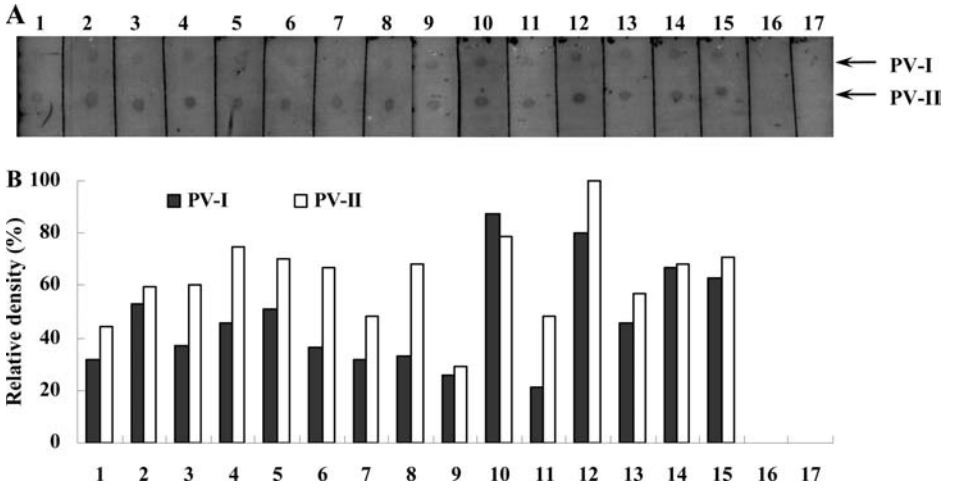

Figure 6. IgE immunoblotting of purified PVs from red stingray. (**A**) Result of dot blot and (**B**) semiquantitative analysis on dot blot image using Alphaview 3.0 software. Lanes: 1−15, fish-allergic patient sera; and 16 and 17, sera from nonallergic individuals as control.

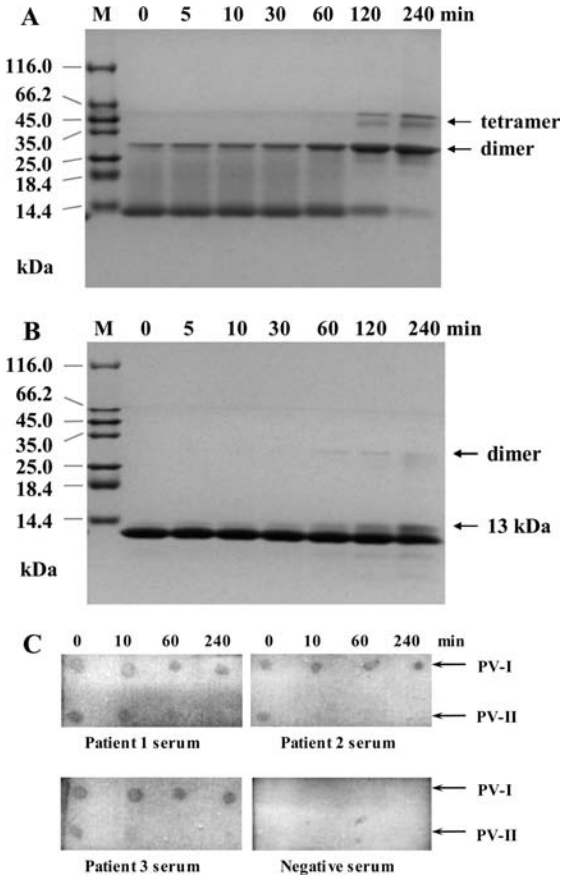

Figure 7. Thermal stability of purified PVs from red stingray. PV-I (**A**) and PV-II (**B**) were heated for different time intervals and then applied to Tricine-SDS-PAGE. M, protein marker. The incubation time was indicated above each lane. (**C**) IgE-binding ability of heated PVs from red stingray.

In conclusion, two PV isoforms were purified to homogeneity from red stingray (*D. akajei*). These two isoforms were different in their immunological reactions and biochemical characteristics including molecular masses, N-terminal amino acid sequence information, isoelectric points, IgE-binding ability, and thermal stability. Previous serological cross-reactions have indicated that β-PVs are major allergens in various species of fish. Here, we showed evidence that PVs from red stingray, which might belong to the α-group, are another candidate of IgE-mediated anaphylaxis. Therefore, individuals allergic to teleost fish had better avoid consuming cartilagenous fish, such as red stingray.

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