

Purification and characterization of parvalbumins from silver carp (*Hypophthalmichthys molitrix*)

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Abstract

BACKGROUND: As the largest producer and consumer of freshwater fish in the world, many people suffer from allergy for consuming freshwater fish in China. However, the allergen profiles of freshwater fish are rarely known.

RESULTS: Parvalbumins (PVs) from the white muscle of silver carp (*Hypophthalmichthys molitrix*) were purified by ammonium sulfate fractionation and column chromatography including DEAE-Sepharose and Superdex 75. Three PV isoforms – PV-I, PV-II, and PV-III – were obtained and their molecular masses as estimated by tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis were 12, 11, and 14 kDa, respectively. All the PVs could be detected by anti-frog PV monoclonal antibody. PV-I and PV-II were quite possibly glycoproteins, while PV-III was not glycosylated, as analyzed by periodic acid–Schiff (PAS) staining. Thermal stability revealed that PV-I and PV-II easily formed polymers, while these proteins were stable in a pH range of 4.0–10.0. A PV gene encoding 110 amino acid residues was cloned and it revealed high identity with PVs from other species of fish.

CONCLUSION: Three isotypes of PV were purified to homogeneity and one distinct PV gene was cloned in silver carp white muscle.

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Keywords: silver carp; parvalbumin; purification; cDNA cloning; characterization; tricine SDS-PAGE

INTRODUCTION

Parvalbumin (PV) represents the major allergen involved in IgE-mediated food hypersensitivity to fish and fish products.^{1–3} A variety of clinical symptoms, such as urticaria, angioedema, respiratory symptoms (asthma and rhinoconjunctivitis), gastrointestinal symptoms (diarrhea and vomiting) and, in severe cases, fatal anaphylaxis, can be caused after ingestion of fish.^{1,2}

PVs with molecular masses of about 12 kDa belong to the family of Ca²⁺-binding proteins, which not only play important roles in muscle relaxation but are also regarded as major allergens in fish.^{3,4} They are resistant to heat, denaturing chemicals and proteinase treatment, and thus represent the major allergens for 95% of fish-allergic patients suffering from IgE-mediated hypersensitivity to fish.^{2–4} The PV content in fish white muscle is much higher than that in red muscle.⁵ PVs have been identified as the major allergen in different species of fish, including tuna (*Thunnus tonggol*, *T. albacares*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), Atlantic mackerel (*Scomber scombrus*), herring (*Clupea harengus*), wolf fish (*Anarhichas* sp.), halibut (*Hippoglossus hippoglossus*) and flounder (*Platichthys flesus*).^{2–6} PVs have also been purified from Atlantic cod (*Gadus morhua*),⁷ snook (*Centropomus undecimalis*),⁸ Alaska pollack (*Theragra chalcogramma*),⁹ mackerel (*Scomber japonicus*, *S. australasicus*, *S. scombrus*)¹⁰ and Pacific pilchard (*Sardinops sagax*).¹¹ Furthermore, cDNA cloning and expression of PVs from Atlantic salmon¹²

and Pacific mackerel (*Scomber japonicus*)^{13,14} were performed. PVs also have been identified or purified in vertebrates such as frog,¹⁴ chicken,¹⁵ rabbit¹⁶ and even human.¹⁷

It is noteworthy that much research concerning fish allergy has been performed on marine fish. So far, concerning food allergy caused by freshwater fish, only PVs from carp (*Cyprinus carpio*),^{18,19} tilapia²⁰ and kokanee (*Oncorhynchus nerka*)²¹ have been studied. China is the largest producer and consumer of freshwater fish in the world, and the total production of freshwater fish exceeded 25 million tons in 2007. Many people suffer from allergy from consuming freshwater fish.^{22,23} A study by Peng *et al.* showed that some cod-specific IgE-positive children were also allergic to freshwater fish by skin tests.²² A survey of 216 bronchial asthmatic children in China using specific allergen IgE test kits produced by EU-ROIMMUN Co. (Lübeck, Germany) suggested that freshwater fish was the third most important food allergen, with 8.3% (18 cases) of subjects allergic to it.²³ Silver carp (*Hypophthalmichthys molitrix*) is

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one of the most widely cultured and consumed freshwater fish in China, and thus is economically important. However, the allergen profiles of silver carp are still unknown. In the present study, three isotypes of PV from the skeletal muscle of silver carp were purified and their characteristics were investigated. Furthermore, the primary structure of the protein was determined by cDNA cloning.

MATERIALS AND METHODS

Fish

Cultured silver carp (*Hypophthalmichthys molitrix*) with a body weight of about 900 g was purchased live from a fish market of Jimei, Xiamen. The fish was sacrificed instantly. After decapitation and evisceration, white muscle was collected and immediately used for experiment.

Chemicals

DEAE-Sepharose and Superdex 75 10/300 GL were products of GE Healthcare (Waukesha, WI, USA); the protein marker for tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (tricine-SDS-PAGE) was from Fermentas (Vilnius, Lithuania); periodic acid–Schiff (PAS) staining kit was from Genmed (Grand Rapids, MI, USA); anti-frog PV monoclonal antibody (PARV-19) was from Sigma (St Louis, MO, USA); horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was from Dako (Glostrup, Denmark); 3,3'-diaminobenzidine tetrahydrochloride (DAB) was from Pierce (Rockford, IL, USA). All other reagents were of analytical grade.

Purification of PVs

All procedures were conducted at 4 °C. Silver carp white muscle was homogenized in 1 volume of muscle with 4 volumes of 20 mmol L⁻¹ Tris-HCl (pH 7.5) containing 5 mmol L⁻¹ EDTA (buffer A) using a homogenizer (PT-2100, Kinematica, Littau/Lucerne, Switzerland). After centrifugation at 8000 × g for 30 min, the supernatant was collected and fractionated with ammonium sulfate from 60% to 100% saturation. After centrifugation at 15 000 × g for 30 min, the resulting precipitate was dissolved in buffer A and dialyzed extensively against the same buffer. The dialyzate was subsequently applied to a DEAE-Sepharose ion-exchange column (2.5 × 11 cm) previously equilibrated with buffer A. After extensively washing the column with buffer A, binding proteins were eluted with a linear gradient of NaCl from 0 to 0.5 mol L⁻¹ in a total volume of 300 mL at a flow rate of 1 mL min⁻¹. Subsequently, fractions (PV-I, PV-II, PV-III) were respectively collected and concentrated by ultrafiltration using a YM-3 membrane (Millipore, Billerica, MA, USA), then applied to a gel filtration column of Superdex 75 10/300 GL, which was equilibrated with 50 mmol L⁻¹ phosphate buffer containing 0.15 mol L⁻¹ NaCl (pH 7.0). The column was eluted at a flow rate of 0.5 mL min⁻¹ and fractions were collected and used for electrophoretic analysis.

Tricine-SDS-PAGE, western blot and native PAGE analysis

Tricine-SDS-PAGE was based on a glycine-Tris and tricine-Tris buffer system according to the method of Schägger.²⁴ Approximately 2–10 µg protein per lane was applied to the gel. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (CBB). Western blot was performed as described by Towbin *et al.*²⁵ Briefly, proteins on polyacrylamide gel were electroblotted onto nitrocellulose membranes followed by blocking with 5% skimmed milk powder in TBST (20 mmol L⁻¹

Tris-HCl buffer, pH 8.0, containing 0.145 mol L⁻¹ NaCl and 0.05% Tween 20) at room temperature for 1 h. After washing with TBST, the membrane was reacted with monoclonal antibody PARV-19 (1 : 2000 dilution) overnight at 4 °C. HRP-conjugated rabbit anti-mouse IgG antibody (1 : 3000 dilution) was then allowed to react with the membrane for 1 h, followed by extensive washing with TBST. Immunodetection was carried out using DAB as substrate.

Native PAGE was performed in a similar manner to the SDS-PAGE procedure, without heating of samples and addition of SDS both to samples and gel. After electrophoresis, the gel was stained with CBB.

PAS Staining for PVs

To detect whether PVs were glycosylated proteins, PAS staining was performed according to the manufacturer's instructions. Briefly, after electrophoresis the tricine-SDS-PAGE gel was immersed in reagent A for 30 min. The gel was then washed with distilled water, transferred to reagent B, and incubated for 1 h. After washing thoroughly with distilled water, the gel was incubated with reagent C in a dark room at 4 °C for 1 h. The gel was then incubated with reagent D until a magenta band appeared on a clear background. Finally, the gel was washed with distilled water, stored in reagent E and the gel image was recorded by an imaging system (Syngene, Cambridge, UK).

Protein concentration assay

Protein concentration was estimated by measuring the absorbance at 220 nm of the sample solution or by the method of Lowry *et al.*²⁶ using bovine serum albumin (BSA) as standard.

pH and thermal stability of PVs

To determine thermal stability of PVs, purified proteins were heated at 100 °C for different time intervals (5 min, 30 min, 1, 2, 4, 6 and 8 h, respectively). Samples were then electrophoresed on tricine-SDS-PAGE gels, followed by CBB staining. To determine the pH stability of PVs, samples were incubated in buffers with different pH values (pH 4.0–10.0) for 6 h at 4 °C, followed by tricine-SDS-PAGE analysis. The buffers used were HAc-NaAc (pH 4.0–5.0), PBS (pH 6.0–7.0), Tris-HCl (pH 8.0) and Na₂CO₃–NaHCO₃ (pH 9.0–10.0).

Isolation of RNA, cDNA cloning and sequencing

White muscle of silver carp was obtained from live fish and immediately frozen in liquid nitrogen. Total RNA was isolated from muscle using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with reverse transcriptase (Invitrogen) and an oligo(dT)₁₈ primer according to the manufacturer's instructions. Both sense (CO-F: 5'-ATGGCATTGCTGGAATTCTG-3') and antisense (CO-R: 5'-TGCCATTATGCCTTGACCAG-3') primers based on the sequences of common carp (*Cyprinus carpio*) parvalbumin mRNA (GenBank nos. AJ292211 and AJ292212) were designed to amplify the PVs. The PCR program was performed as following: 4 min at 94 °C followed by 35 cycles of 40 s at 94 °C, 45 s at 55 °C, 60 s at 72 °C, and a final extension of 7 min at 72 °C. Anchor primer (5'-GACCACGCGTATCGATGTCGAC-3') and oligo(dT)₁₈ primer were used in 3'-RACE. The PCR product was purified from agarose gel and was ligated to pBS-T vector (Tiangen, Beijing, China) for nucleotide sequencing. DNA sequencings were analyzed by Invitrogen Biotechnological Co. Ltd (Shanghai, China) with the DNA sequencer ABI PRISM 3730 (CA). According to the

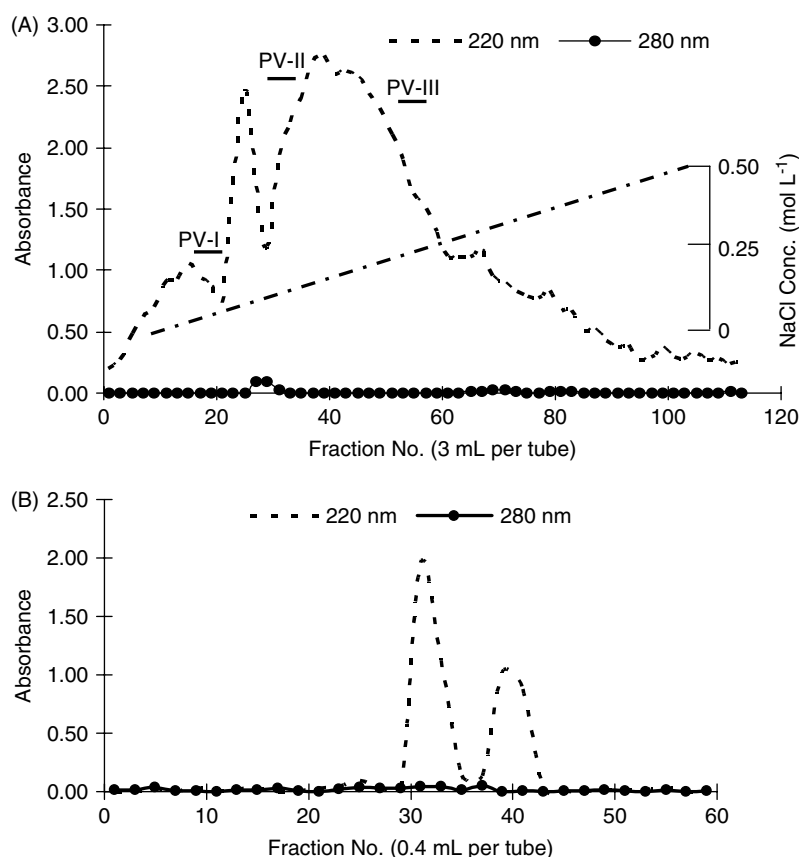


Figure 1. Column chromatography purification of silver carp PVs. (A) DEAE-Sepharose chromatography. (B) Superdex 75 gel filtration of PV-II. The bars indicate each pooled fraction.

sequence of cDNA fragment, additional specific antisense primers were designed (5'-AGTTTGAGCTCATCCTCTCAA-3') and used in 5'-RACE, which was carried out according to the instructions of the manufacturer of the 5'-RACE kit (Clontech, Mountain View, CA, USA).

RESULTS AND DISCUSSION

Purification of PVs

In the present study, PVs were purified from the white muscle of silver carp by ammonium sulfate fractionation and sequential column chromatography with DEAE-Sepharose and Superdex 75. Three isotypes of PVs were separated by DEAE-Sepharose column chromatography (Fig. 1(A)). They were pooled respectively and applied to a Superdex 75 gel filtration column for further purification (Fig. 1(B)). After Superdex 75 chromatography, all these fractions migrated as single bands with a molecular mass of 12, 11, and 14 kDa, respectively, on tricine-SDS-PAGE (Fig. 2(A)). Western blot analysis using anti-frog PV monoclonal antibody showed that it reacted positively with all these PVs (Fig. 2(B)). These results strongly suggest that all the purified proteins were PVs and were thus named PV-I, PV-II, and PV-III. However, there is a minor band under the main band of PV-III which is quite possibly the degradation product of PV-III and which cannot be separated by the methods used. Molecular masses of PVs of silver carp are similar to those from various other fish species such as tuna, Atlantic cod, snook, Alaska pollack and mackerel,^{5–9} and are also comparable to those from other vertebrates, including frog, chicken and rabbit.^{9,15,16}

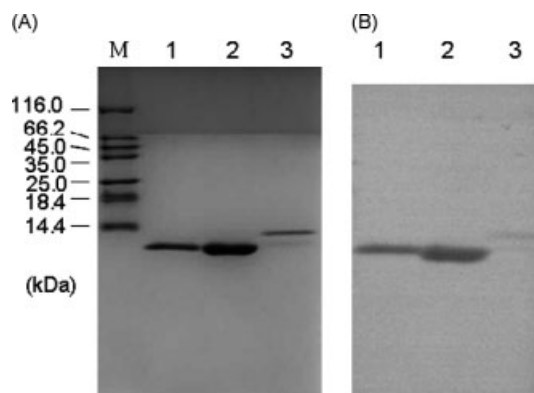


Figure 2. (A) Tricine-SDS-PAGE and (B) western blot of purified silver carp PVs. Western blot was developed with anti-frog PV monoclonal antibody. M, protein marker; 1, PV-I; 2, PV-II; 3, PV-III.

Native PAGE analysis further revealed that silver carp PVs are of high purity. It is of interest to note that their motilities on native PAGE are quite different (Fig. 3(A)). PV-I is the slowest one, suggesting it is less negatively charged in native form. PV-III moved to the foremost position, indicating that it is most acidic and thus most negatively charged under the same conditions.

PAS staining

Some food allergens were identified as glycoproteins with molecular masses in the range of 10–40 kDa.^{27,28} Therefore, PAS

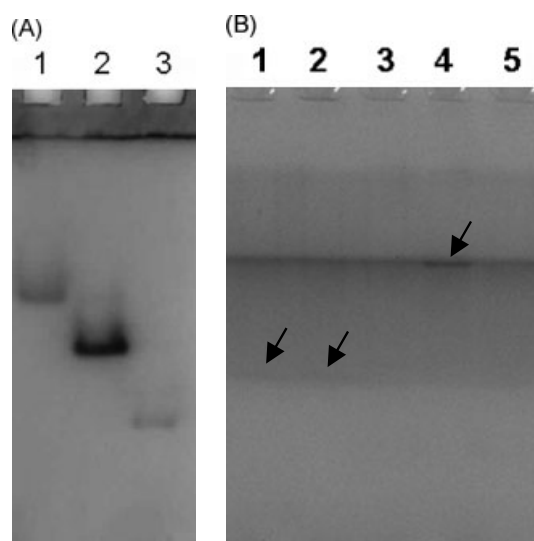


Figure 3. Native PAGE and PAS staining of purified silver carp PVs. (A) Native PAGE. 1, PV-I; 2, PV-II; 3, PV-III. (B) PAS staining of PVs. 1, PV-I; 2, PV-II; 3, PV-III; 4, IgG heavy chain as positive control; 5, BSA as negative control.

staining was used to determine whether PVs from silver carp are glycosylated or not. As a positive control, a magenta band was observed on IgG heavy chain (Fig. 3(B), lane 4) while as a negative control no such band could be observed in BSA (Fig. 3(B), lane 5). Bands of PV-I and PV-II revealed slight magenta staining (Fig. 3(B), lane 1 and 2), suggesting PV-I and PV-II are possibly glycoprotein, while the glycosylation degree was very low. On the other hand, no magenta band could be observed in PV-III, suggesting it is not a glycosylated protein or its glycosylation extent was extremely low, so that it cannot be detected by the PAS staining kit in the recommended detection range of 30–100 μ g. Glycosylation was assumed to be associated with the IgE-binding capacity of parvalbumin from Atlantic cod, while no difference was observed between recombinant carp parvalbumin and its

natural counterpart.^{13,19} Therefore, further research to investigate the relationship between glycosylation and allergenicity of PVs from silver carp is needed.

Thermal stability and pH stability of PVs

The thermal stability of PVs at 100 °C was detected by tricine-SDS-PAGE. After heating, protein aggregates appeared to form in PV-I (Fig. 4(A)) and PV-II (Fig. 4(B)). The formation of PV-I and PV-II aggregates can occur even after heating for only 5 min. On the other hand, the protein band of PV-III disappeared after heating for 2 h (Fig. 4(C)). The results indicated that the stability of PV-III is not so high compared with PV-I and PV-II, suggesting that the protein structures of PVs are variable during thermal processing. It has been reported that in food allergenic protein loss of tertiary structure is typically followed by unfolding, loss of secondary structure (55–70 °C), cleavage of disulfide bonds (70–80 °C), formation of new intra/intermolecular interactions, rearrangements of disulfide bonds (80–90 °C) and formation of aggregates (90–100 °C).²⁹ It was also reported that there is a dimer of size 22 kDa in snook PV which is the product of the intermolecular reduction of Cys-18.⁸ Therefore, quite possibly, PV-I and PV-II from silver carp may contain readily reducible cysteine residues that facilitate the formation of PV polymers. PVs from four fish species of the genus *Channa* showed species- and isotype-specific electrophoresis profiles on non-denaturing gels after heating at 90 °C for 3 h,³⁰ indicating that the thermal stability capacity of PVs is different among isoforms, which was consistent with our results.

Stability of PVs at various pH values were also examined by tricine-SDS-PAGE. As shown in Fig. 5(A), PV-I revealed high stability in the range pH 4.0–10.0, as no protein band change could be observed. PV-II (Fig. 5(B)) and PV-III (Fig. 5(C)) showed similar results to PV-I. These results indicated that all of these isoforms of PVs are stable at pH 4.0–10.0. However, whether their conformation and other optical properties changed or not should be further confirmed.

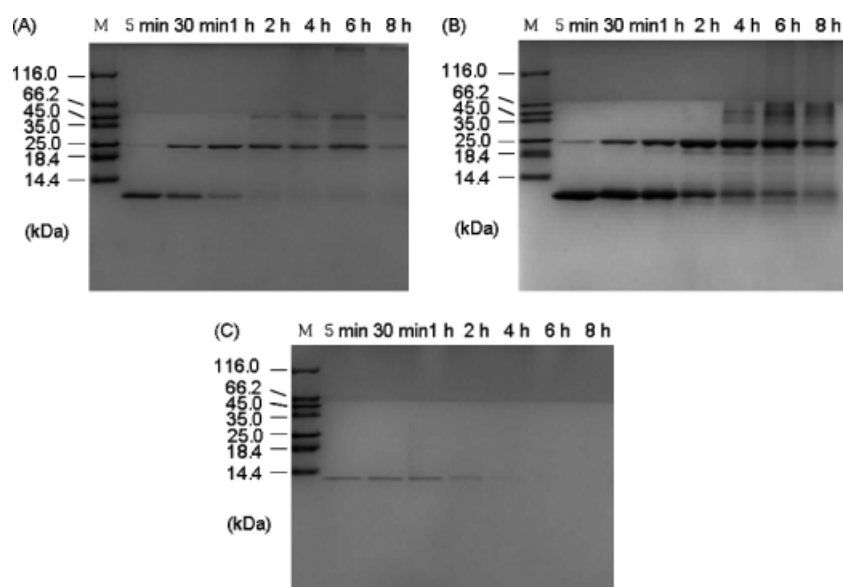


Figure 4. Detection of the effect of thermal stability. Three isoforms of PV were allowed to incubate at 100 °C for different time intervals. After incubation, samples were applied to tricine-SDS-PAGE. (A), PV-I; (B), PV-II; (C), PV-III. M, protein marker. Incubation time was indicated above each lane.

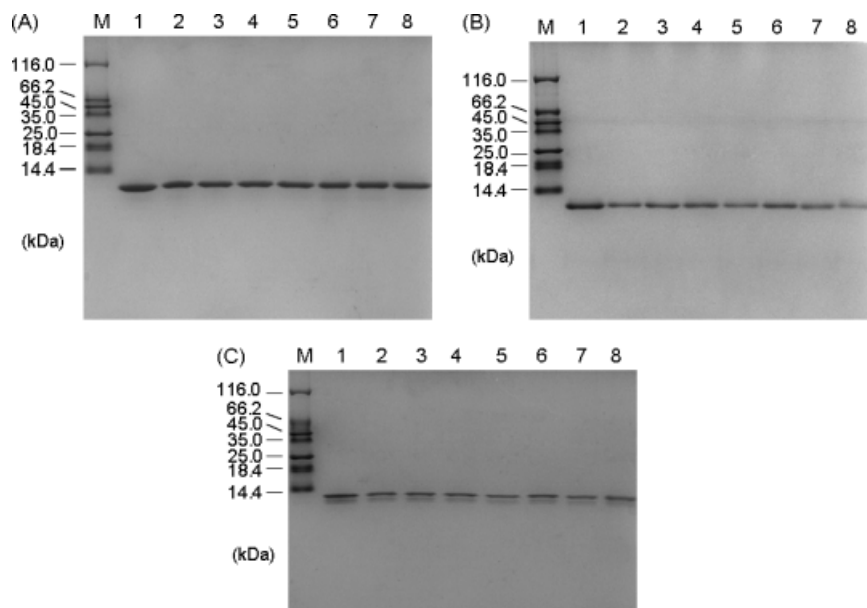


Figure 5. Detection of the effect of pH stability. PVs were allowed to incubate at 4 °C for 6 h in different buffers with pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, respectively. After incubation, samples were applied to tricine-SDS-PAGE. (A) PV-I; (B) PV-II; (C) PV-III. M, protein marker; lane 1, control (non-heated); lanes 2–8 correspond to pH 4.0–10.0.

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1      CCTAAAGACAACCAGAGGCTAATCATGGCATTGCTGGAATTCTGAATGAGGCTGACGTC
1      M A F A G I L N E A D V

61     ACTGCAGCCCTGCAGGCTGCCAAGCTGCTGACTCCTTCAAGTACAAGGACTTCTTCGCC
21     T A A L Q A C Q A A D S F K Y K D F F A

121    AAGGTTGGCCTTTCCGCCAAGAGTCCTGATGACATCAAGAAGGCCTTTGCTGTCATTGAC
41     K V G L S A K S P D D I K K A F A V I D

181    CAGGACAAGAGCGGCTTCATTGAGGAGGATGAGCTCAAACTGTTCTGTCAGGACTTCTCT
61     Q D K S G F I E E D E L K L F L Q D F S

241    GCTGGTGCCAGGGCACTCACTGATGCAGAGACAAAGGCCTTCTTGAAGCTGGAGACTCT
81     A G A R A L T D A E T K A F L K A G D S

301    GATGGTGATGGCAAGATTGGAGTTGATGAGTTCGCCGTCCTGGTCAAGGCATAAATGGCA
101    D G D G K I G V D E F A V L V K A *

361    CCTGACTAACAACTTTTGTGACAAACAGTATCGTAGCCTGAGATCTCCACTTTTTTATC

421    TCGGTGGCTGATTTTATACACAATTCTGACTGTCCCATGCTGCCTTCGTTTACAATGA

481    CTGTCAATGCCACACGGTCTTTTCGATTGTAGATATGAATGTTGGTTTCTGTAAATCTT

541    TGATCCCGTTTTTTGGGAAAGGACAATGAGAAAATAAATACTATTTTCAAAAAAAAAAAA
      AAAAAAAA

601    AAAAAAA
  
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Figure 6. The nucleotide and deduced amino acid sequence of silver carp PV. The stop codon is indicated by an asterisk and the polyadenylation signal is underlined.

Cloning of silver carp PV

A cDNA sequence of PV was amplified by reverse transcriptase (RT)-PCR silver carp white muscle, which was designated as Hyp1. The deduced amino acid sequence of silver carp PV gene is shown in Fig. 6. It was 607 bp in length including the start codon ATG at positions 25–27, the stop codon TAA at positions of 352–354, and a poly-A tail. The open reading frame of silver

carp PV is 330 bp and the predicted protein consists of 110 amino acid residues with a theoretical molecular weight of 12 kDa and isoelectric point of 4.6. Only one cysteine residue exists in this PV. Protein sequence alignment showed that PV from silver carp has high homology to PVs from other fish (Fig. 7). Silver carp PV gene revealed 73.4%, 73.4%, 92.7%, 82.6%, 82.6%, 77.1%, 74.0% and 74.0% identities, respectively, with PVs from *Scomber*



Figure 7. Sequence alignment of deduced amino acid sequence of silver carp PV with other fish PVs. Mackerel (*Scomber japonicus*) (GenBank accession nos. AB091470 and AB211366); carp (*Cyprinus carpio*) (GenBank accession no. AJ292211 and AJ292212); Atlantic cod (*Gadus morhua*) (GenBank accession no. AM497927 and AM497928); Alaska pollack (*Theragra chalcogramma*) (GenBank accession no. AY035586 and AY035587). Two calcium binding sites are boxed.

japonicus (AB091470), *Scomber japonicus* (AB211366), *Cyprinus carpio* (AJ292211), *Cyprinus carpio* (AJ292212), *Gadus morhua* (AM497927), *Gadus morhua* (AM497928), *Theragra chalcogramma* (AY035586) and *Theragra chalcogramma* (AY035587). Such high identity in protein sequence established the molecular basis for the immunological cross-reactivity among fish PVs. Based on amino acid sequence data, the PV family can be subdivided into two evolutionarily distinct lineages: the α group and the β group. The former is less acidic, with isoelectric points at or above 5.0, while the latter is more acidic, with an isoelectric point at or below 4.5.¹⁹ From the cloned silver carp PV sequence, it is clear that no potential glycosylation site exists and the deduced pI is 4.6, which is quite acidic and suggests inclusion in the β group. However, it is necessary to confirm its pI value by experiments such as isoelectric focusing. Combined with the result of PAS staining in accordance with its non-glycosylation characteristic in cDNA sequence, native PAGE, and its predicated acidic pI value that reflects its delayed elution position in DEAE-Sepharose ion exchange chromatography (Fig. 1), it is quite possible that the cloned Hyp1 is the gene of PV-III.

CONCLUSIONS

In the present study, three PV isoforms – PV-I, PV-II, and PV-III – were obtained with molecular masses of 12, 11, and 14 kDa from freshwater fish silver carp white muscle. All these PVs immunologically cross-reacted with anti-frog PV monoclonal antibody. PV-I and PV-II were quite possibly glycoproteins, while PV-III was not glycosylated. PV-I and PV-II were easily formed polymers during heating, while all these proteins were stable in a pH range of 4.0–10.0. A 607 bp gene was cloned and predicted to encode 110 amino acid residues and it has high identity to PVs from other species of fish. Further characterization

of PVs from freshwater fish, such as determination of the protein conformation and the establishment of a molecular strategy for specific immunological diagnosis and therapy of fish allergy, are necessary.

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