

Purification and Characterization of Parvalbumin Isotypes from Grass Carp (*Ctenopharyngodon idella*)

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ABSTRACT: The prevalence of fish allergy is rapidly increasing because of a growing fish consumption driven mainly by a positive image of the fish and health relationship. The purpose of this study was to characterize parvalbumin isotypes from grass carp (*Ctenopharyngodon idella*), one of the most frequently consumed freshwater fish in China. Three parvalbumin isotypes were purified using consecutive gel filtration and reverse-phase chromatography and denoted as PVI, PVII, and PVIII. The molecular weights of the isotypes were determined to be 11.968, 11.430, and 11.512 kDa, respectively. PVI showed 74% matched amino acids sequence with PV isotype 4a from *Danio rerio*, while PVII and PVIII showed 46% matched amino acids sequence with PV isotypes from *Hypophthalmichthys molitrix*. PVII is the dominant allergen, but it was liable to gastrointestinal enzymes as PVIII; however, PVI was resistant to pepsin digestion. A further study is to characterize the epitopes of PVII, the dominant allergen.

KEYWORDS: parvalbumin isotypes, purification, characterization, IgG binding, IgE binding

INTRODUCTION

Fish consumption has grown tremendously over the past several decades, driven mainly by a positive image of the fish and health relationship and as an alternative animal protein source over pork and beef.^{1,2} Fish (including shellfish) allergies are common in both the western world and Asian countries, affecting 0.2–2.29% of the general population;³ unlike other allergies, most of the children cannot outgrow fish allergy. Although fish allergies occur mainly via the gastrointestinal tract (eating fish), handling and inhalation of cooking vapors can also cause fish allergic reactions.^{4,5} Fish allergies cause urticaria, rhino conjunctivitis, asthma, oral allergy syndrome, diarrhea, and even life-threatening anaphylaxis.⁶

Parvalbumin (PV), accounting for 90% of fish allergic reaction, was first identified as the major allergen in cod⁷ and then from many different species.^{8–10} As the major clinical cross-reactive fish allergen, PV is a subfamily of closely related three EF-hand calcium-binding proteins, with a molecular weight of 10–12 kDa and isoelectric points of 3.9–5.5.¹¹ PV is often subdivided into two distinct isoform lineages, α -PV and β -PV, with β -PV as the major allergen.³ α -PV and β -PV consist of 109 and 108 amino acid residues, respectively. PV consisting of 110 amino acid residues was also reported in herring recently.¹² The pI value of α -PV is $\sim$ 5.0, while that of β -PV is more acidic, with a pI value $\sim$ 4.5.^{13,14} The presence of isotypes with different levels of immunoglobulin G (IgG)- and immunoglobulin E (IgE)-binding capacities was reported in several kinds of fish species.^{15–17} Cai et al. indicated that PVII (11.95 kDa) may be more allergenic than PVI (12.29 kDa) in red stingray.¹⁵ However, two isotypes of PV with the same molecular weight (11.5 kDa) and similar IgG- and IgE-binding capacities but different isoelectric points (4.39 and 4.6, respectively) were reported from Alaska pollack.¹⁶

Grass carp (*Ctenopharyngodon idella*) is one of the most frequently consumed freshwater fish in China, with an annual

harvest of 4.78 million metric tons in 2012.¹⁸ There were reports about the main allergen in carp⁹ and silver carp,¹⁹ but PV from grass carp has not been characterized. Carp, grass carp, silver carp, and zebrafish are from the same family (Cyprinidae) but different genus. Liu et al. revealed that silver carp PV had 92.7 and 82.6% similarities with PVs from carp.¹⁹ Therefore, it is important to investigate and characterize the main allergen in grass carp. The objectives of this study were to purify and characterize PV isotypes from grass carp, determine their IgG- and IgE-binding capacities, and compare their digestibility using a simulated gastrointestinal condition.

MATERIALS AND METHODS

Materials. Live grass carps were purchased from a local fish market (Beijing, China). After sacrifice, white muscle was collected and used for PV extraction. Human sera were purchased from Plasma Lab International (Everett, WA). According to the supplier, these patients were selected on the basis of their allergic symptoms, such as itchy mouth, hives, wheezing, swelling throat, and IgE levels, against codfish. The IgE levels were measured by the Thermo Fisher Scientific ImmunoCAP at levels of 68.4, 77.5, 84.4, and 44.7 kU/L for 17716 (female, age: 28), 21290 (male, age: 22), 21473 (female, age: 56), and 17485 (male, age: 38), respectively. Anti-grass carp PV sera from rabbit was prepared in the lab (China Agricultural University, China). Mouse anti-frog PV monoclonal antibody (PARV-19), *para*-nitrophenylphosphate (*p*-NPP) substrate solution, tetramethylbenzidine (TMB) substrate solution, anti-mouse IgG peroxidase-conjugated antibody produced in goat, anti-rabbit IgG peroxidase-conjugated antibody produced in goat, and anti-human IgE peroxidase-conjugated antibody produced in goat were from Sigma (St. Louis, MO). Molecular weight protein standard and 4–20% gel were purchased from Bio-Rad Laboratories,

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Inc. (Hercules, CA). Trypsin used in gel digestion was purchased from Promega (Madison, WI). High-performance liquid chromatography (HPLC)-grade acetonitrile was from Acros Organics (Fair Lawn, NJ). Pepsin (from porcine gastric mucosa) and trypsin (from porcine pancreas) used in simulated gastrointestinal digestion were purchased from Sigma (St. Louis, MO).

Extraction and Purification of PV. For PV extraction, the white muscle was homogenized with three volumes of 20 mM Tris-HCl (pH 7.5). After centrifugation at 10000g for 20 min, the supernatant was incubated in a water bath at 95 °C for 30 min. The heated extract was subjected to gel filtration chromatography on a Superdex 75 column coupled with an ÄKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden) and eluted with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). Fractions were collected every 7 min, and absorbance was measured at 220 and 280 nm. Cytochrome *c* from horse heart (molecular mass of 12.4 kDa) was run under the identical conditions as a standard. Fractions containing PV from gel filtration chromatography were further applied to a Waters XBridge C₁₈ column (5 μ m, 3.0 $\times$ 250 mm, Waters Corporation, Milford, MA) coupled with a guard column (40 $\times$ 10 mm, Waters Corporation, Milford, MA) attached with a Waters 600 HPLC system. Instrumental control and data collection were carried out by Empower, version 2. Samples were automatically injected by Waters 2707 autosampler at a volume of 25 μ L. The column was eluted by two solvents, solvent A [HPLC-grade water containing 0.1% trifluoroacetic acid (TFA)] and solvent B (70% acetonitrile containing 0.1% TFA), at a flow rate of 0.5 mL/min using a gradient from 0 to 50% B within 5 min, to 50% B within 30 min, to 0% B over 5 min, and maintained for 10 min for the next injection. Fractions were collected every 0.5 min from 19 to 24 min, and elution was monitored by a Waters 2998 photodiode array at 220 nm. The fractions were concentrated by a vacuum rotary evaporator at 35 °C and then freeze-dried for further analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed with 4–20% gel (Bio-Rad) according to the method by Laemmli.²⁰ The load volume was 15 μ L at a protein concentration of 2 mg mL⁻¹. SDS-PAGE was performed in a Mini-PROTEAN tetra cell at a constant voltage of 200 V. Protein bands were stained using Brilliant Blue R-250. After destaining, the gel was scanned in an Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, CA) with FluorChem SP software.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) Analysis of PV Isoforms. Molecular weight of the purified PV isoforms was analyzed by MALDI-TOF-MS according to the procedure of two-layer sample preparation.^{21,22} For the first thin matrix layer, 0.7 μ L of 10 mg mL⁻¹ 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 80% acetone/20% methanol (HPLC grade) (v/v) was loaded onto a clean MALDI target, and then the matrix layer was spread and dried immediately. A total of 2 μ L of diluted PV isotype sample was mixed with 2 μ L of saturated sinapinic acid in 50% acetonitrile/50% water (v/v). A total of 1 μ L of treated sample was applied to the first layer of the matrix. After drying, 5 μ L of water was added on top of the dry spot to desalt the sample, and after 10 s, the liquid was blown off by an air pulse; this was repeated 5 times. Each sample was applied to six spots. At least, three spots of each sample were analyzed. MALDI analysis was carried out on an Applied Biosystems Voyager Elite MALDI (Foster City, CA) time-of-flight mass spectrometer in a positive linear ion mode.

In-Gel Digestion of PV Isoforms and Liquid Chromatography (LC)-Tandem Mass Spectrometry (MS/MS) Analysis. Excised gel pieces containing PV isoforms were digested by trypsin, as Offengenden et al. described.²³ First, the protein in gel pieces were reduced with 10 mM dithiothreitol (DTT) and then alkylated with 50 mM iodoacetamide. After washing with 100 mM ammonium bicarbonate and acetonitrile, the gel pieces were dried in a SpeedVac. The dried gel pieces were digested by 0.8 μ g of trypsin (20 ng μ g⁻¹ in 50 mM ammonium) at 37 °C. After overnight digestion, the peptides were extracted with 30 μ L of 100 mM ammonium bicarbonate, followed by two steps of extraction with 30 μ L of solution containing 5% formic acid and 50% acetonitrile in water. At last, the extracts were dried to about 15 μ L in a SpeedVac. The sample after in-gel trypsin digestion was

analyzed by a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, QToF Premier (Waters, Milford, MA), online connected to a Waters nanoAcquity ultrahigh-performance liquid chromatography (UPLC) system. A total of 5 μ L of sample was loaded onto a nanoAcquity UPLC system with a peptide trap (180 μ m $\times$ 20 mm, Symmetry C18 nanoAcquity column, Waters Corporation, Milford, MA) and a nano analytical column (75 μ m $\times$ 100 mm, Atlantis dC18 nanoAcquity column, Waters Corporation, Milford, MA). A solution of 1% acetonitrile and 0.1% formic acid in water (solvent A) was used to flush the trap column at a flow rate of 10 μ L min⁻¹ for 3 min to desalt the trapped sample. Peptides were separated with a gradient of 1–65% solvent B (acetonitrile and 0.1% formic acid) over 35 min at a flow rate of 300 nL min⁻¹. The column was connected to a QToF Premier (Waters Corporation) for electrospray ionization-mass spectrometry (ESI-MS) and MS/MS analyses of the effluent. Then, analysis using the online search algorithm Mascot (Matrix Science) was carried out for peptide identification.

Preparation of Anti-grass Carp PV Antibodies in Rabbit. Anti-PV antibody was prepared from three female New Zealand rabbits by injection of purified PV from gel filtration chromatography, as described everywhere.²⁴ The first injection was 0.5 mL of PV solution (1 mg mL⁻¹) emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO) to the hind leg muscle of a rabbit. After 2 weeks, 0.5 mL of PV solution (1 mg mL⁻¹) emulsified with an equal volume of incomplete Freund's complete adjuvant (Sigma, St. Louis, MO) was injected to the back muscle of a rabbit. After 2 weeks, the rabbits were injected with another 1 mL solution containing 2 mg of PV via the ear vein of a rabbit. After 1 week, the rabbit sera were collected and kept at -80 °C until use.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA). IgG- and IgE-binding capacities of PVs from grass carp were analyzed by indirect ELISA using PARV-19, anti-grass carp PV produced in the sera of rabbits and fish allergic patients. Briefly, samples were coated on a 96-well high binding polystyrene plate and incubated at 4 °C overnight, and bovine serum albumin (BSA) was coated as the blank. After four steps of washing, 100 μ L of phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 1% BSA was added to each well to block the unoccupied space of the wells and incubated at 37 °C for 1 h. Then, 100 μ L of antibodies was added to wells after four steps of washing. Four human serum diluted 1:10 with PBS contained 0.1% Tween-20 and 1% BSA. Mouse serum and rabbit serum were diluted 1:400 000 and 1:160 000 with the same dilution, respectively. After incubation at 37 °C for 1 h, 100 μ L of the corresponding peroxidase-conjugated antibody produced in goat (anti-mouse IgG, anti-rabbit IgG, or anti-human IgE) was added to the plate after diluted 1:10 000 with PBS contained 0.1% Tween-20 and 1% BSA and incubated at 37 °C for 1 h. For anti-human IgE, the enzyme reactions was performed using *p*-NPP substrate solution at room temperature for 30 min and stopped by 3 mol L⁻¹ sodium hydroxide, and the color that developed was measured by absorbance at 405 nm. For the anti-mouse IgG and anti-rabbit IgG, the enzyme reaction was performed using TMB substrate solution at 37 °C for 10 min, stopped by 2 mol L⁻¹ sulfuric acid, and measured by absorbance at 450 nm.

Simulated Gastrointestinal Digestion. Simulated gastrointestinal digestion of purified PVs was performed according to a modified method by Majumder et al.²⁵ PVs were dispersed in 0.15 M KCl-HCl (pH 2.0) buffer at a concentration of 2 mg mL⁻¹ and first digested with pepsin (E/S, 2%, w/w) at 37 °C for 1.5 h, and then the pH of the digest was raised to 7.4. Half of the pepsin digest was removed and heated in a 95 °C water bath for 15 min. The rest of the sample was further digested by trypsin (E/S, 2%, w/w) at 37 °C for 3 h and then was inactivated by heating the sample at 95 °C for 15 min. The digested samples were stored at -20 °C for further analysis.

Separation of Digested PV Isoforms by HPLC. The digested PV isoforms were applied to a Waters XBridge C₁₈ column (5 μ m, 3.0 $\times$ 250 mm, Waters Corporation, Milford, MA) coupled with a guard column (40 $\times$ 10 mm, Waters Corporation, Milford, MA) attached with a Waters 600 HPLC system for further separation. Instrumental control and data collection were carried out by Empower, version 2. Samples were automatically injected by a Waters 2707 autosampler at a volume of

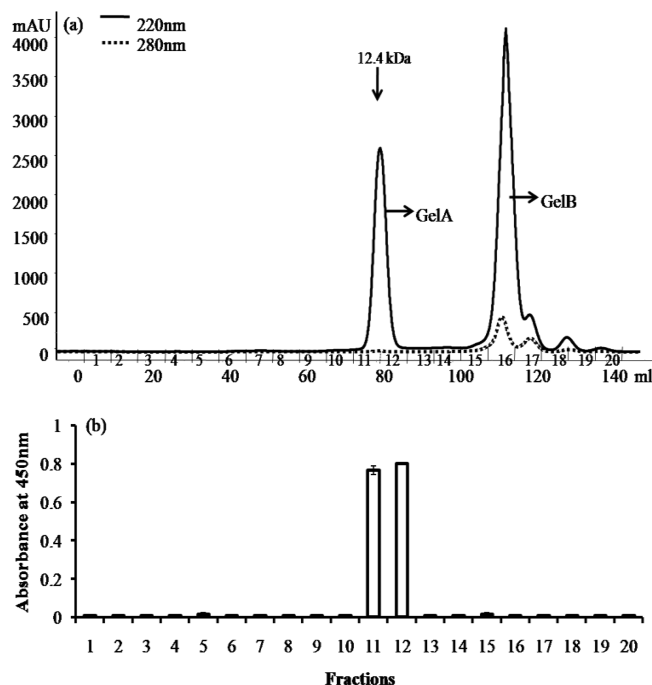


Figure 1. (a) Fractionation of PV, extracted from grass carp white muscle, by gel filtration chromatography on a Superdex 75 (1.6×60 cm) column. (b) IgG-binding capacity of fractions with mouse anti-frog PV monoclonal antibody (PARV-19). Data were expressed as the mean $\pm$ SD ($n = 3$).

25 μ L. The column was eluted at a flow rate of 0.5 mL/min using a gradient consisting of solvent A (HPLC-grade water containing 0.1% TFA) and solvent B (70% acetonitrile containing 0.1% TFA) from 0 to 50% B within 5 min, to 50% B within 30 min, to 0% B over 5 min, and maintained for 10 min for the next injection. Fractions were collected every 1 min from 21 to 41 min, and elution was monitored by a Waters 2998 photodiode array at 220 nm. The fractions were concentrated by a vacuum rotary evaporator at 35 $^{\circ}$ C and then freeze-dried for further analysis.

Data Analysis. All sample determinations were performed in triplicate, and the results were expressed as the mean value $\pm$ standard deviation (SD). Analysis of variance (ANOVA) with Tukey's post-hoc test was used to determine statistical differences, and differences were considered significant with a p value of <0.05 .

RESULTS AND DISCUSSION

Purification of PV Isotypes from Grass Carp White Muscle

PV extracted from grass carp was first purified by gel filtration chromatography, as shown in Figure 1a, where two major fractions, GelA and GelB, were collected. The GelA fraction displayed high binding capacity to PARV-19, whereas no binding activity was found in fraction GelB, indicating fraction GelA containing PV (Figure 1b). Furthermore, fraction GelA did not have absorbance at 280 nm because of the lack of Trp and Tyr residues in PV.^{15,17} The retention time of fraction GelA was close to the protein standard, indicating that the molecular weight of fraction GelA was close to the molecular weight of the standard protein (12.4 kDa).

Fraction GelA was further purified by reverse-phase (RP)-HPLC. Three distinct peaks were collected and denoted as PVI, PVII, and PVIII (Figure 2a). PV samples were subjected to SDS-PAGE analysis (Figure 2b). GelA fraction revealed two bands, similar to the band of PVI, whereas PVII and III contain one band at a molecular weight of ~ 10 kDa. The presence of a lower molecular weight band in PVI was probably due to cross-contamination of PVII. Different isotypes of PV was previously reported in several fish species.^{16,26} Hamada et al. purified two isotypes by reverse-phase HPLC from Japanese flounder and Japanese eel but did not detect isotypes in red sea bream and skipjack.²⁷ Liu et al. purified three PV isotypes by DEAE-Sepharose chromatography and Superdex 75 gel filtration chromatography from silver carp with the molecular weights of 12, 11, and 14, respectively.¹⁹

Characterization of PV Isotypes. As shown in Figure 3, the molecular weights of PVI, PVII, and PVIII were 11.968, 11.430, and 11.516 kDa, respectively, which were in the range of PV molecular weight (10–12 kDa) as previously reported.³ However, a 14 kDa isotype was recently reported in sea bream¹⁷ and red sea bream.²⁸ The molecular weight of PVI was close to that of a PV isotype (11.954 kDa) in red stingray.¹⁵ There are three small peaks that appeared in the MS spectrum of PVI as inserted, corresponding to the smaller band observed in Figure 2b of PVI, with the molecular weights of 11 430, 11 462, and 11 479 Da, respectively. The 11 430 kDa peak was the contamination of PV II, whereas the peak of 11 462 showed the same/similar molecular weight to isotypes of Atlantic cod, with the molecular weight of 11 462 Da¹³ or 11 459 Da.²⁹ The peak

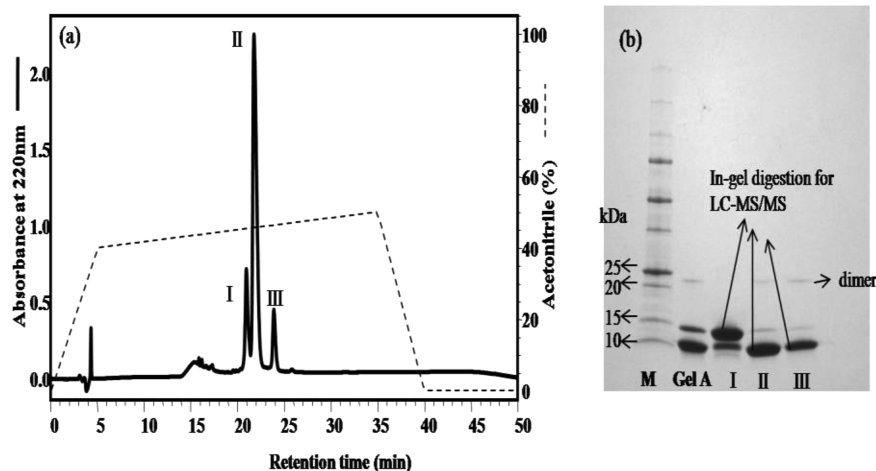


Figure 2. (a) Reverse-phase chromatogram of the PV GelA fraction obtained from gel filtration chromatography. (b) SDS-PAGE analysis of fractions. M, protein markers; GelA, a fraction prepared from Figure 1; PVI, PVII, and PVIII, fractions prepared from panel a.

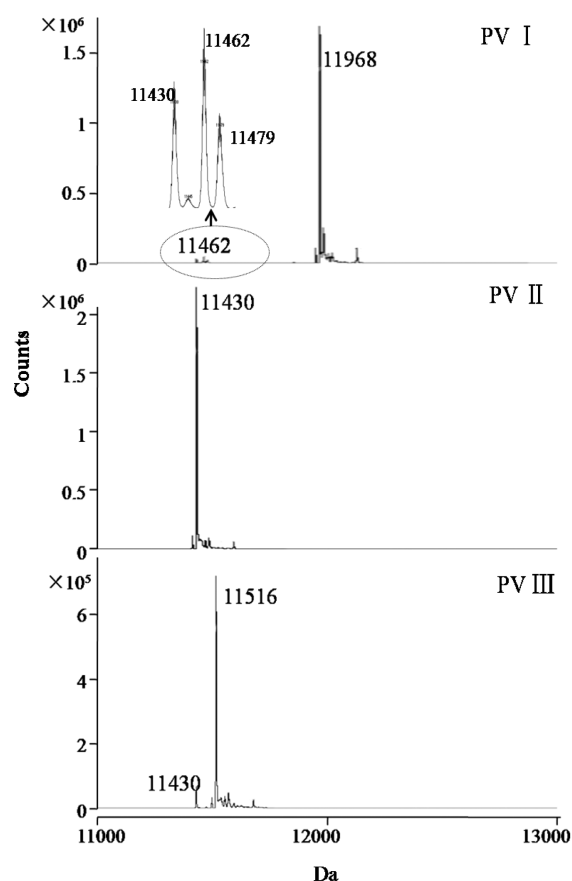


Figure 3. MALDI-TOF-MS analysis of molecular weights of PV isotypes.

of 11 479 Da showed a similar molecular weight to PV (11 487 Da) of carp.⁹

To characterize the sequences, three bands of PVI, PVII, and PVIII in Figure 2b were digested by trypsin and subjected to LC–MS/MS. As shown in Table 1, PVI showed 74% sequence coverage with the PV isoform 4a from *Danio rerio* (gil28194094), supporting that PVI was an isotype of PV. Perez-Gordo et al.³⁰ reported a 15 amino acid residue (DGKIGVDEFGAMIKA) of PV from Atlantic cod, which was considered as a major epitope of the protein. A 15 amino acid residue (DGKIGIDEFEALVHE) of PVI displayed 60% similarity with the major epitope of PV from Atlantic cod; it is to be determined if this epitope in PVI is the major epitope. PVII and PVIII showed 46% sequence coverage with PV isotypes from *Hypophthalmichthys molitrix*

(gil209902357 and gil209902355). In addition, PVII and PVIII shared two epitopes, 51–65 (IDQDKSGFIEEDELK) and 89–103 (AG DSDGDGKIGVDEF). The 51–65 amino acid epitope was also reported in the PV isotype of crimson sea bream, Japanese eel, carp, Atlantic salmon, cod, and horse mackerel.¹⁷ The 89–103 amino acid epitope was identified in the PV isotype of cod, horse mackerel, and skipjack.¹⁷ Therefore, these two epitopes are likely to be antigenic epitopes of PV.

IgG- and IgE-Binding Capacities of PV Isotypes.

Absorbance of the immunosorbent assay was used to reflect the binding capacity of IgG or IgE. IgG-binding capacities with anti-frog and anti-grass carp PV antibodies increased dose-dependently at increasing concentrations of PVI, PVII, and PVIII, further supporting that the purified proteins are PV isotypes. PVI showed the highest IgG-binding capacity with anti-frog antibody (Figure 4a), whereas PVII displayed the highest IgG-binding capacity with anti-grass carp antibody (Figure 4b). The difference in IgG-binding capacities of fish PV with anti-frog PV antibody and anti-grass carp antibody was due to the difference in PV amino acid sequences between frog and grass carp PVs. Lee et al. also demonstrated that antibodies raised against fish and frog PVs displayed varying specificity for different fish species.³¹

The IgE-binding capacity of PV samples with sera from four allergic patients also increased dose-dependently with an increasing concentration of PVI, PVII, and PVIII, as shown in panels c–f of Figure 4. The PV isotypes were recognized as allergens by the sera of all four fish allergic patients. Three isotypes purified from pilchard PV and two isotypes from anchovy, yellowtail, and bake purified PVs were also reported to have an IgE-binding capacity with different sera of fish allergic patients.³² When the coated protein concentration was 0.1 or 10 $\mu\text{g mL}^{-1}$, the IgE-binding capacity of the three isotypes showed no significant difference. At the coated protein concentration of 1 $\mu\text{g mL}^{-1}$, PVII showed the highest IgE-binding capacity, whereas PVIII showed the lowest IgE-binding capacity. Kuehn et al.³³ pointed out that, besides the difference in PV IgE epitopes in different fish species, variability in the PV content was likely to contribute to the variation in clinical reactivity to different fish species. In this study, PVII showed the highest content in grass carp PV (Figure 2a); therefore, PVII is the dominant allergen in PV isotypes. Cai et al. also reported that PVII from red stingray is more allergenic than PVI.¹⁵ Guo et al. reported that the 14 kDa PV isotype showed lower antibody binding capacity than the 12 kDa PV isotype from crimson sea bream.¹⁷

Table 1. LC–MS/MS Analysis of the Amino Acid Identify of PV Isotypes

protein name	number	possible amino acid sequence ^a	sequence coverage (%)
PVI	1	MAMKNILKDD DIKKALDQFK AADSFHKKF FDVVGKALS ADNVKLVFKA	74
	51	LDVDASGFIE EEELKFVLKG FSADGRDLTD KETKAFLAAA DKDGDGKIGI	
	101	DEFEALVHE	
PVII	1	MAFAGILNDA DIAALEACKAADSFNHKAF FAKVGLSAKS GDDVKKAFI	46
	51	IDQDKSGFIE EDELKFLQN FKAGARALTD AETKIFLKAG DSDGDGKIGV	
	101	DEFAALVKA	
PVIII	1	MAFAGILNEA DVTAALQACQ AADSFYKDF FAKVGLSAKS PDDIKKAFV	46
	51	IDQDKSGFIE EDELKFLQD FSAGARALTD AETKAFLKAG DSDGDGKIGV	
	101	DEFAVLVKA	

^aPVI was matched to PV isoform 4a from *D. rerio* (gil28194094), and PVII and PVIII were matched to PV isotypes from *H. molitrix* (gil209902357 and gil209902355).

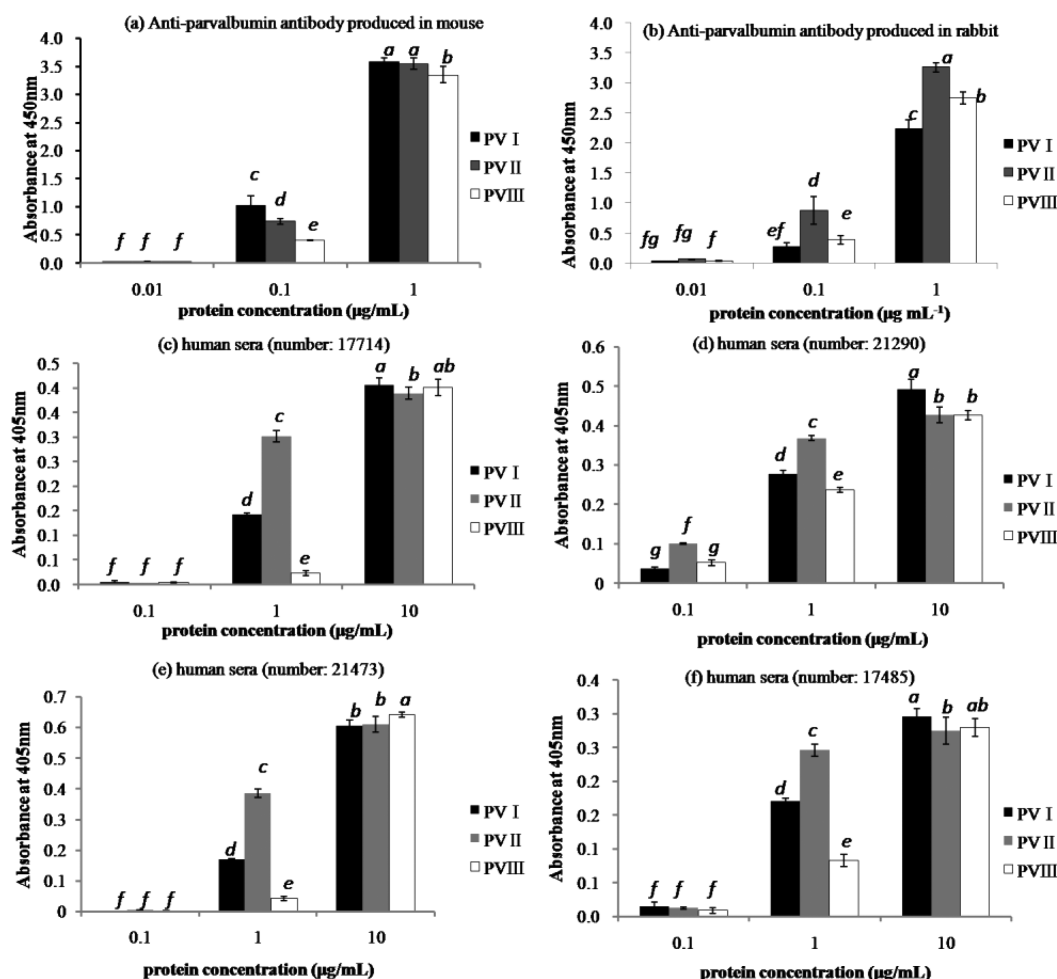


Figure 4. Analysis of IgG- and IgE-binding capacities of fractions PVI, PVII, and PVIII by ELISA. Data are the mean $\pm$ SD ($n = 3$). Means with different letters in the same panel are significantly different ($p < 0.05$): (a) IgG-binding capacity with anti-frog PV antibody produced in mouse, (b) IgG-binding capacity with anti-grass carp PV antibody produced in rabbit, and (c–f) IgE-binding capacity with four codfish allergic human sera.

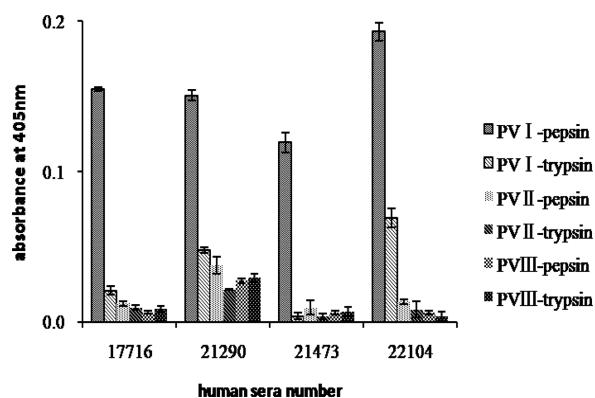


Figure 5. IgE-binding capacity of digested PVs by indirect ELISA. The concentration of samples coated on the plate was $10 \mu\text{g mL}^{-1}$. Data are the mean $\pm$ SD ($n = 3$).

Simulated Digestibility of the Three PV Isotypes.

Normally, primary food allergens present strong resistance to gastrointestinal degradation and are therefore believed to sensitize via the gut,³⁴ and an incomplete digestion of dietary protein may cause an inappropriate immune response in the gut.³⁵ Therefore, digestibility of allergens is one major factor that might affect the allergenic potential of an allergen. The effect of pepsin and trypsin digestion on IgE-binding capacity is shown

in Figure 5. In comparison to the IgE-binding capacity of PV isotypes in panels c–f of Figure 4, it was apparent that digestion significantly decreased the IgE-binding capacity of the digested PV isotypes. Untersmayr et al. also reported that peptic digestion of codfish PV could significantly reduce allergenic potency probably because of the generation of small fragments.³⁶ The absorbance of pepsin-digested PVI was higher than 0.1, while the absorbance of pepsin-digested PVII and PVIII was less than 0.05; although the IgE-binding capacity of PVI was further decreased by trypsin digestion, the IgE-binding capacity of trypsin-digested PVI was still higher than those of pepsin-digested PVII and PVIII. Our results implied that PVI was resistant to digestion or there are IgE-binding sites after pepsin and trypsin digestions. A number of food allergens were also claimed to be resistant to simulating gastrointestinal digestion.^{37–39}

To determine if there was residual PV or there were new peptides generated from digestion that could contribute to IgE-binding capacity of peptic and tryptic digests, these two digests were further fractionated by PR-HPLC (Figure 6a). Peptic digestion showed residual PVI in the digest, but most of the residual PVI can be further digested by trypsin. For the peptic digestion, 20 fractions were collected from 21 to 41 min, and each fraction was subjected to an IgE-binding capacity assay. As shown in Figure 6b, the two fractions collected from 39 to 41 min showed the highest IgE-binding capacity, suggesting that the undigested

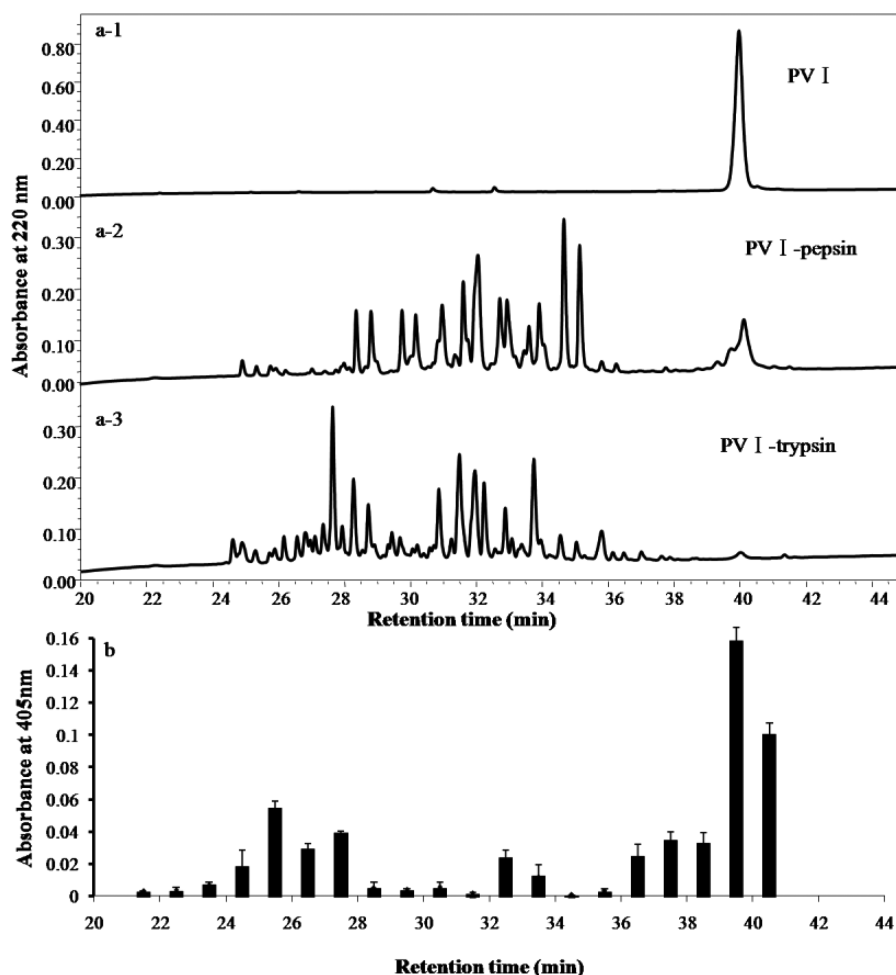


Figure 6. (a) Reverse-phase chromatogram of PVI and its pepsin and trypsin digests. (b) IgE-binding capacity of each fractions (collected every 1 min) from 21 to 41 min. Data were expressed as mean $\pm$ SD ($n = 3$).

PVI in the peptic digest was responsible for its IgE-binding capacity. RP-HPLC analysis was also performed on digested PVII and PVIII (data not shown), and almost all of the PVII and PVIII were digested by pepsin after 1.5 h. In summary, three PV isotypes were first purified from grass carp and identified by LC-MS/MS. These isotypes were different in molecular weight, amino acid sequence, IgG-binding capacity, IgE-binding capacity, and digestibility. PVII may be the main allergen but was liable to gastrointestinal enzymes, as well as PVIII; however, PVI was resistant to pepsin digestion.

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Notes

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