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Purification of Parvalbumin from Carp: A Protocol That Avoids Heat Treatment

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Abstract
Parvalbumin from carp, a major allergen,was purified to homogeneity using ion exchange chromatography and size exclusion chromatography (estimated purity > 95% to 98% based on SDS-PAGE and native PAGE) with a yield of 318 mg, and a number of basic biochemical characteristics were determined. The identity was confirmed by peptide-mass fingerprinting, and IgE-binding was demonstrated. The UV/Vis absorbance spectra were explained using the previously published amino acid sequences. Far UV-CD spectroscopy was used to confirm the folding character of parvalbumin. We conclude that parvalbumin from carp can be purified on a comparatively large (hundreds of milligrams) scale using a purification protocol that does not include denaturing steps. The purified protein resembles biochemical characteristics as were earlier published for carp parvalbumin, that is, a molecular weight of approximately 12 kDa, amino acid sequence identity and a secondary structure containing α-helices and β-structures. The described method provides a yield sufficient to produce and characterize antibodies to construct immunochemical methods to detect parvalbumin in food as well as for use as a standard calibrator for such assays.
Practical Application: Parvalbumin is a major allergen from fish. Here,we have purified a comparatively large quantity from carp that can be used to develop antisera for use in an assay method to detect fish allergens.

Keywords: allergen, carp, fish, parvalbumin

Introduction

Parvalbumin from fish is considered to be a pan-allergen for fish-allergic patients, and parvalbumins from many different fish that are commonly consumed in Western countries share biochemical and immunochemical characteristics (Taylor et al. 2004; van Do et al. 2005a). Evidence also indicates that parvalbumin is the major cross-reacting allergen in tropical fish commonly consumed in Asian-Pacific countries (Lim et al. 2008). Parvalbumins are proteins conserved in lower vertebrates (Lehky et al. 1974) and are proposed to be involved in the muscle contraction/relaxation cycle, calcium buffering, and signal transduction (Kretsinger 1980). Parvalbumins are typically 10 to 12 kDa in size, acidic (pH = 4 to 5.2), and binders of divalent cations (Elsayed and Aas 1971; Kretsinger and Nockold 1973). Up to now, the purification of parvalbumin from fish has been described only on a small scale. With increasing attention to the labeling of allergenic foods, immunoassays to detect allergens or residues of allergenic foods are needed. Purified allergens are required to raise antibodies for such immunoassays and to calibrate these assays.

Cod parvalbumin was the first reported fish allergen (Aas 1966; Aas and Jebsen 1967; Elsayed and Aas 1970, 1971). In those studies, fish fillets were extracted with water and the resulting extracts dialyzed against water as the first step of purification. Similar extraction and purification procedures were subsequently used by many other researchers to investigate parvalbumin as an allergen in other fish species (Hamada et al. 2003; van Do et al. 2005b). Bushana-Rao et al. (1969) used buffers containing 2 mM EDTA for the extraction of parvalbumin. The aim of that study was to investigate the metal ion-binding properties of parvalbumin by means of spectroscopy. The inclusion of EDTA during extraction and purification removed intrinsically bound calcium ion. On the other hand, the majority of reports pertaining to parvalbumin extraction/purification related to the characterization of the allergenic properties of parvalbumin use aqueous extraction methods without EDTA because the presence of the calcium ion in the calcium-binding sites of parvalbumin is relevant for protein structure, structural stability, and IgE-binding (Bugajska-Schretter et al. 1998; Bugajska-Schretter et al. 2000).

The original purification of carp parvalbumin applied a heat-treatment step (30 min at boiling temperature) to recover the relatively heat-stable parvalbumin from fish extracts (Bugajska-Schretter et al. 2000). Although the denaturation of parvalbumin upon boiling is only limited, the secondary structure is affected to some extent as was observed in minor differences between the far UV CD spectra of native and heat-treated parvalbumin (Bugajska-Schretter et al. 2000). Therefore, heat treatment should be avoided when aiming to purify native parvalbumin.

The aim of the current study is to develop a purification protocol for carp parvalbumin in its native form in yields sufficient for biochemical characterization and to immunize laboratory animals to raise anti-sera for the development of immunochemical assays as was recently accomplished for parvalbumin from cod (Faeste and Plassen 2008). Because we anticipate using purified parvalbumin as a calibration standard for such an immunochemical assay, our target yield is at least 100 mg. The key steps to purify parvalbumin (without applying a potentially denaturing step such as heat treatment or adding EDTA) are extraction, dialysis, size exclusion chromatography, and ion-exchange chromatography.

These steps allow for scale-up of the purification, provided that appropriate chromatography media are used. Dialysis may form a limiting step for large-scale purification and should be avoided as much as possible. Reverse-phase HPLC approaches may result in high purity (Hamada et al. 2003) but are impractical for large-scale purifications. We describe a method to purify parvalbumin from carp without the use of heat treatment and without EDTA, on a scale of at least 100 mg, with the possibility to further improve the yield.

Materials and Methods

Origin and extraction of carp fillets

For all experiments except the dot blotting, carp extracts were made as follows: carp fillets (1.2 kg) were obtained from a fresh fish market in Omaha, Nebraska, USA. Two live carp were selected based on size to result in at least a total of 1 kg of skinless fillets. Fillets were removed from both fish and immediately placed on ice and then frozen in a –80°C freezer within 1.25 h. Frozen fillets were shipped on dry ice to the Netherlands laboratory for extraction. The frozen fillets were partially thawed and combined into 1 sample. A portion of this sample (400 g) was homogenized in 400 mL of deionized water followed by addition of 800 mL of deionized water, 32 mL 1 M Tris-HCl (pH 8), and 12 mL of 1 M NaOH to adjust the pH to 8. Thereafter the pH was maintained around pH 8 using 1 M NaOH. The slurry was stirred for 10 min on ice and then was centrifuged for 30 min at 4000 rpm (1800 g) at 4°C. The supernatant was collected, filtered through medium speed filter paper (Whatman/Schleicher and Schuell, Keene, New Hampshire, USA), and frozen at –32°C. For dot-blotting experiments, carp extracts were prepared by mixing homogenized carp fillet with 0.01 M sodium phosphate buffered saline (0.85% NaCl), pH 7.5 (PBS), in a ratio of 1:10 (w/v), overnight at 4°C. Extracts were clarified by centrifugation at 3000 rpm (1000 g) for 30 min at 4°C, and the soluble protein concentration was determined. Throughout the purification procedure, the 12 kDa protein bands as visualized on SDS-PAGE were used as a marker for the presence of parvalbumin.

Diafiltration

Diafiltration was performed using the tangential flow filtration technique. A Quickstand diafiltration system (GE Healthcare, Uppsala, Sweden) employing the 3 and 30 kDa cutoff hollow fiber cartridges (Xampler, GE Healthcare) was used for diafiltration and concentration. A 550 mL extract (prepared with Tris-HCl as described previously) was thawed at 37°C in a water bath. Nondissolved material was removed by centrifugation (15 min, 5500 g at room temperature). The supernatant was filtered over a 30 kDa hollow fiber cartridge, and the parvalbumin-containing filtrate was collected. The retentate was diafiltered with 1.5 L 20 mM Tris, pH 8.1, over a 30 kDa hollow fiber cartridge. The parvalbumin-containing filtrates were pooled and concentrated over a 3 kDa hollow fiber cartridge. The filtrate did not contain parvalbumin (not shown). The 3 kDa concentrate (260 mL) was used for further purifications (referred to as 3 kDa concentrate). The 30 kDa concentrate was produced from 250 mL of the extract prepared with Tris-HCl buffer as described previously by concentrating on the 30 kDa hollow fiber cartridge.

Concentrating of protein solutions

A centrifugational filter (3 kDa cut-off, Vivaspin (VS2091) Sartorius, Nieuwegein, the Netherlands) was washed with 10 mL of buffer in which the protein was solubilized (depending on purification step described subsequently) and centrifuged at 4800 g (5min, 4°C). The eluant and remaining buffer were removed, and the protein sample was loaded onto the filter. Samples were centrifuged at 4800 g (4°C) until the volume was reduced sufficiently. For samples that were applied on the Superdex75 column, the volume was reduced to 1.6 mL. The concentrated protein sample was transferred to an appropriate tube, and the filter was washed at least 2 times with 150 μL of the appropriate buffer to recover residual protein.

SDS-PAGE and native PAGE

For SDS-PAGE, sample buffer was prepared by mixing one-third of Invitrogen NP0009 with two-thirds of Invitrogen NP0008 (v/v). A 24 μL volume of each concentrated protein solution was mixed with 12 μL of sample buffer. Samples were incubated for 10 min at 70°C. Next, samples were briefly centrifuged, and 14 μL was applied to the gel (12% gradient gel, Invitrogen NP0232box). Total of 6 μL of 5-fold diluted (in sample buffer) dual color marker was loaded as a reference. Gels were run for 35 min at 200 V, were then transferred to a washing tray and approximately 200 mL demineralized water (obtained by reverse osmosis) were added. Gels were incubated in a microwave oven (max power = 900 W for 1 min). The gels were washed twice more with fresh demineralized water. Next, the gel was incubated in 20 mL PageBlue stain (Fermentas R0571, St. Leon-Rot, Germany), first for 30 s in a microwave oven at maximum power (900 W), followed by a 15-min incubation at room temperature. The stained gels were destained in demineralized water (overnight at room temperature).

Native PAGE was run in a mini Protean® 2 system (Bio-Rad Laboratories Inc., Hercules, California, USA) using 10% to 20% acrylamide Tris-HCl precast gradient gels from the manufacturer. Parvalbumin was loaded at 10, 5, and 2.5 μg per lane. Sample buffer consisting of 62.5 mM Tris-HCl, pH 6.8 with 40% glycerol, and 0.01% bromophenol blue was mixed 1:2 (v/v) with the carp parvalbumin solution. Molecular weight standards for non-denaturing PAGE (Sigma-Aldrich Inc., St. Louis, Missouri, USA) were used. The electrophoresis was run with Tris/glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 180 volts for 30 to 45 min depending on the progress of the tracking dye. Resulting gels were fixed with 12% (w/v) trichloroacetic acid and 3.5% (w/v) sulfosalicyclic acid and stained with Brilliant Blue G-Colloidal solution (Sigma-Aldrich Inc.) according to the manufacturer's instructions.

Protein concentration determination

A standard curve of bovine serum albumin (BSA) was prepared as follows: 2, 1.5, 1, 0.75, 0.50, 0.25, 0.125, 0.025, 0.00 mg BSA per mL in 0.9% saline, 0.05% sodium azide. Total of 3 different dilutions for each parvalbumin sample were made. The BSA standard curve and parvalbumin samples were measured in duplicate in the following way: samples of BSA standard (10 μL) were pipetted into a 96-well microtiter plate (Costar 2595, Cambridge, Massachusetts, USA) and 100 μL bicinchoninic acid (BCA) working reagent was added (BCA Kit 23225, Pierce Protein Research Products, Rockford, Illinois, USA). The plate was

incubated for 30 min at 37°C in a humidified tray. Next, the tray was cooled to room temperature for 10 min. The OD at 550 nm was measured within 30 min in a microplate reader (Biorad model 680, Bio-Rad Laboratories Inc.).

UV-VIS Spectroscopy

Samples were measured at 280 nm in an 80 μL, 1 cm quartz micro cuvette (Amersham Biosciences) using an Ultrospec2100pro spectrophotometer (Amersham Biosciences Benelux, Roosendaal, the Netherlands).

Far UV spectroscopy

The far-UV circular dichroism (CD) spectra of 0.20 mg/mL parvalbumin in 10 mM phosphate (pH 6.8) in the presence of 2 mM EDTA were recorded as averages of 20 spectra of a singleton preparation on a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan). Quartz cells with an optical path length of 0.1 cm were used. The scan range was 190 to 260 nm, the scan speed 50 nm/min, the spectral resolution 0.2 nm, bandwidth 1 nm, and the response time 0.125 s. Spectra were corrected for a protein-free spectrum obtained under identical conditions, and subsequent noise reduction was applied according to the Jasco software.

Peptide mass fingerprinting

SDS-PAGE bands were cut from the gel, and each band was processed for in-gel digestion according to the method of Shevchenko et al. (2000). Briefly, bands were washed/dehydrated 3 times in 50 mM ammonium bicarbonate pH 7.9/50 mM ammonium bicarbonate + 50% acetonitrile. Subsequently, cysteine bonds were reduced with 10 mM DTT (dithiotreitol) for 1 h at 56°C and alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. After 2 subsequent wash/dehydration cycles, the bands were dried 10 min in a vacuum centrifuge and incubated overnight with 0.06 μg/μL trypsin (TPCK-modified porcine trypsin sequence grade (V5111) (Promega Benelux BV, Leiden, the Netherlands) at 25°C.

Peptides were extracted once in 100 μL 1% (v/v) formic acid and subsequently 2 times in 100 μL 50% (v/v) acetonitrile in 5% (v/v) formic acid. After concentrating the peptides in a vacuum centrifuge to 50 μL, equal volumes of peptide mixture and α-cyano hydroxycinnamic acid (HCCA) matrix (0.5 μL) were mixed and dried on a stainless-steel MALDI target (Applied Biosystems Opti-TOF 384-well plate). MS and MS/MS spectra were acquired on a 4800 MALDI ToF/ToF analyzer (Applied Biosystems, Foster City, California, USA) in positive reflector mode. MS and MS/MS spectra were searched by Mascot (Matrix Science, London, UK) against the Swissprot protein sequence database (www.expasy.ch/sprot) with 52 ppm mass accuracy. This mass accuracy allows a maximum deviation of 0.052 Da for a peptide with a mass of 1000 Da. The search was restricted to the taxon Actinopterygii because proteins only from fish were expected in the samples.

IgE dot blotting

Carp extracts and purified carp parvalbumin were used for IgE dot blot experiments. The samples and buffer controls were applied to nitrocellulose membranes (0.45 μm pore size)

according to the dot blot apparatus manufacturer's instructions (Bio-Rad Bio-Dot® Micro-filtration Apparatus, Hercules, California, USA). Briefly, nitrocellulose was prewetted with Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.4). Equal volumes of test materials were allowed to filter by gravity through the membrane and then the membrane washed with PBS. The membrane was removed from the apparatus and blocked for 2 h using radioallergosorbent test (RAST) buffer (0.05M sodium phosphate, 2.5% NaCl, 0.2% BSA, 0.5% Tween 20, and 0.05% sodium azide). The blocked membrane was cut into strips and incubated overnight with serum from fish-allergic subjects diluted 1:10 in RAST-buffer. Strips were washed with RAST buffer and incubated overnight with Iodine-125 labeled antihuman IgE (Siemens Medical Solutions, Los Angeles, California, USA) diluted 1:10 in RAST buffer. Bound antihuman IgE was visualized by autoradiography. Sera were obtained by FARRP from various clinical collaborators under the conditions of approved IRB protocols of the University of Nebraska–Lincoln and the respective institutes of the clinical collaborators. Sera from 9 fish-allergic subjects, 1 nonallergic negative control subject, and 1 subject with seasonal pollen allergy but no food allergy were used. Fish-allergic subjects were selected based on a convincing history of allergic symptoms experienced after the consumption of fish, positive skin prick tests (SPT) to fish extracts, and specific IgE levels to cod and/or other fish species. Symptoms included throat, mouth, and lip itching and swelling; dyspnea; wheezing; urticaria; conjunctivitis; and anaphylactic shock (Table 1).

All fish-allergic subjects had a positive SPT to either cod or a mixture of cod, flounder, and haddock, or positive to both a fresh water fish extract mixture and a salt water fish extract mixture. In addition, serum from each fish-allergic subject had specific IgE to more than one fish species at levels > 0.35 kU_A/L. Total 7 of the 9 fish-allergic sera had specific IgE ranging from 1.1 to 14.9 to various fish species. Specific IgE was determined using the Phadia ImmunoCAP® method (Phadia AB, Uppsala, Sweden).

Table 1. Subject characteristics

ID	Symptoms[a]	Cod IgE[b]	Other specific IgE scores	Cod SPT[c]	SPT[d]
1	C, D, LE	1.23	Tuna 4.35, salmon 3.52, pollack 1.5, haddock 3.08, sardine 1.47, herring 1.51		4 + to salt water mix[e], 4 + to fresh water mix[f]
2	LE, LS, OAS	2.54	Pollock 3.33, herring 3.96, haddock 3.51, salmon 3.32, sole 2.76, sardine 3.20		4 + to salt water mix, 4 + to fresh water mix
3	A, LE, LS, ES, D, W, C	3.98	Flounder 3.27, walleye pike 8.49, ocean perch 3.39, tuna 1.83, salmon 3.21, trout 4.16		3 + to salt water mix, 4 + to fresh water mix, and perch
4	U, V, OAS, DZ, BV	0.91	Flounder 0.52, salmon 0.48, halibut 0.54	5 × 10	
5	A, U, LE, EZ	14.5	Flounder 12.1, tuna 11.0, salmon 14.9, halibut 14.8	13 × 60	
6	LE, MB, CT, U, LS, CJ	0.64	Tuna 0.60		Tuna 6 × 30 mm, salmon 5 × 20 mm
7	LE, U	0.44		12 × 20	
8	U, W	1.43		21 × 45	
9	P, U, OAS	3.48		13 × 43	
NA		< 0.35			
A-NFA		< 0.35	Seasonal pollen allergy		

[a]A = anaphylaxis, BV = blurred vision, C = choking, CJ = conjunctivitis, CT = chest tightness, D = dyspnea, DZ = dizziness, ES = eye swelling, EZ = eczema, LE = laryngeal edema, LS = lip swelling, MB = mouth blistering, OAS = oral allergy symptoms, P = pruritis, U = urticaria, W = wheezing
[b]Expressed in kilo units specific allergen/L (kU_A/L)
[c]Diameter in millimeters of skin prick test with extract of cod or containing cod in a mix—wheal × flare
[d]Grading system for skin prick test: 4+: wheal diameter > 15 mm and flare diameter > 40 mm; 3+: wheal diameter 10 to 15 mm, flare diameter 31 to 40 mm
[e]Salt water mix = haddock, blue fish, herring, sardine, salmon
[f]Fresh water mix = pike, trout, pickerel

Purification of carp parvalbumin

Chromatography columns were connected to an Äkta Explorer protein purification system (Amersham Biosciences,Uppsala, Sweden). Buffers were prepared using demineralized water. During the chromatographic procedures, the absorbance of eluents at 280 nm was recorded. The following columns (Amersham Biosciences) were used: 28mL Q-Sepharose FF (column YK16/20, 16 × 200 mm, equilibrated with 25mM Tris, pH 8.1, and eluted with a salt gradient to 1 M in the same buffer, flow rate 3 mL/min.); 320 mL Superdex 75 (YK 26/600, diameter 26 mm, length 600 mm, equilibrated and eluted with 20 mM Tris, 100 mM NaCl, pH 8.1, flow rate 3 mL/min, gradient volume: 280 mL); 120 mL Superdex 75 (YK16/600, diameter 16 mm, length 600 mm, equilibrated with and eluted with 20 mM Tris, 100 mM NaCl, pH 8.1, flow rate 0.5 mL/min). Tris buffers were adjusted to the desired pH using a 1 M solution of HCl.

Purification without the use of heat treatment was done as schematically shown in Figure 1. 260 mL of the 3 kDa concentrate were applied on a 28 mL Q-Sepharose FF column.

Two pools were prepared after elution: Pool 1 from 0 to 80 mM NaCl and Pool 2 from 80 to 145 mM NaCl. Both pools were concentrated on a 3 kDa centrifugation filter unit. 1.6 mL of the concentrated pool from 0 to 80 mM NaCl were applied on a 320 mL Superdex75 column. A 12 kDa protein band was detected by SDS-PAGE in several fractions. Fractions representing the main peak of the chromatogram were pooled, and the SDS-PAGE pattern is shown in Figure 2. This sample is referred to as Sample 1. Fractions at the high-MW shoulder of the main peak were pooled. The pool eluting from the Q-Sepharose FF column at 80 to 145 mM NaCl was concentrated and applied to a 75 mL Superdex75 column. The main peak containing among others a 12 kDa protein band as analyzed by SDS-PAGE was stored frozen for further purification.

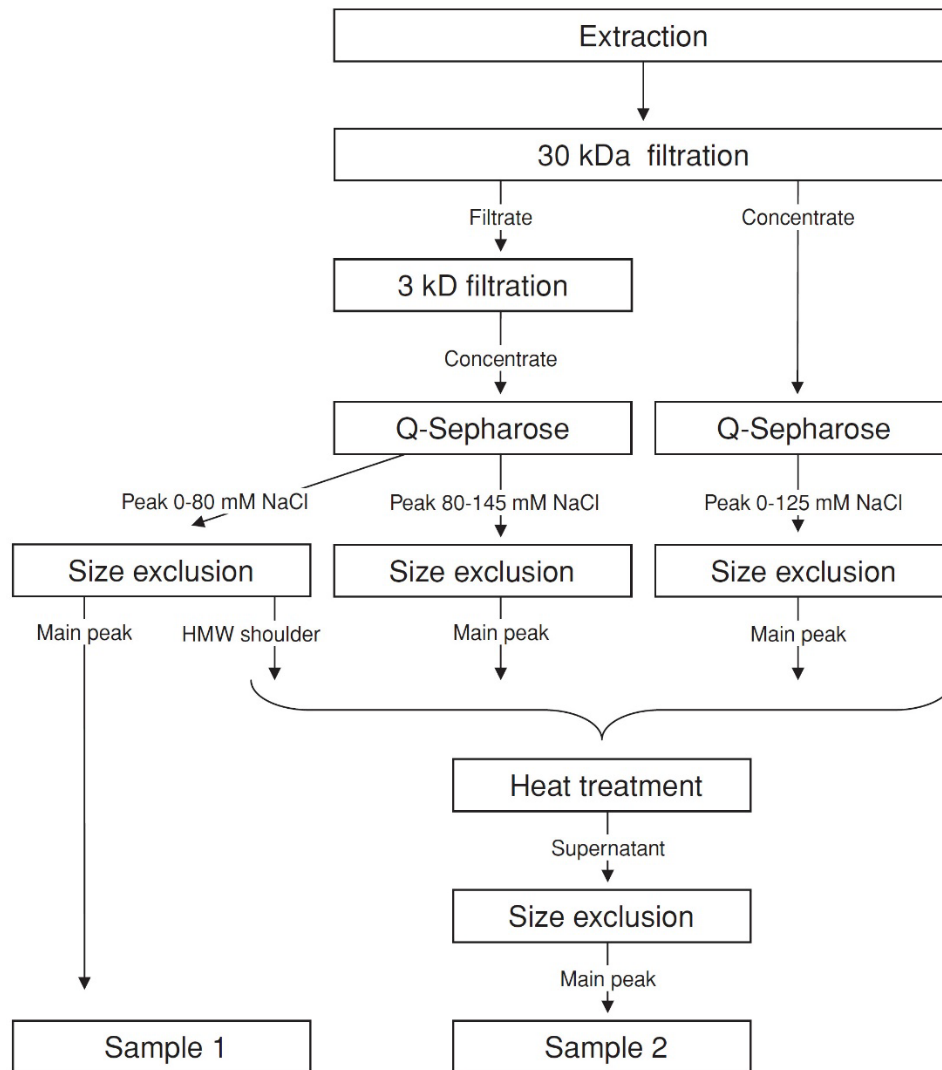


Figure 1. Purification steps.

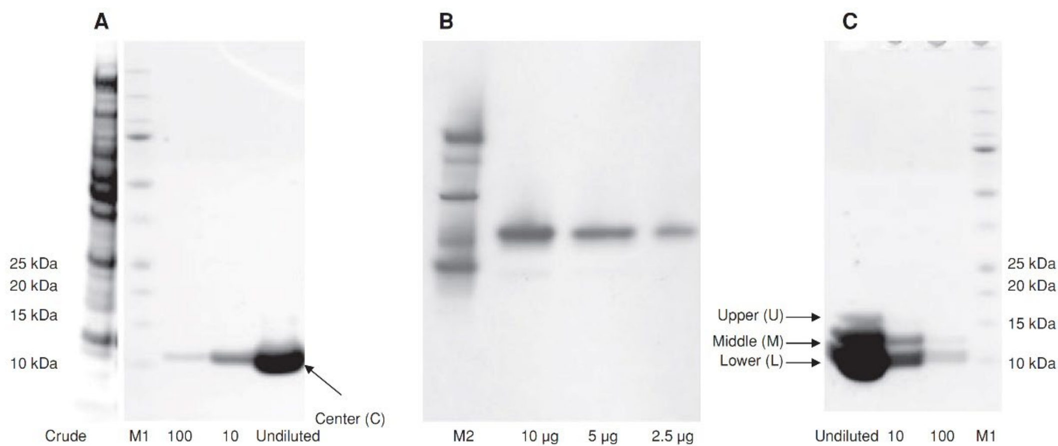


Figure 2. Native and SDS-PAGE analysis of Sample 1 and Sample 2. *Panel A:* SDS-PAGE of Sample 1; *Panel B:* Native PAGE of Sample 1; *Panel C:* SDS-PAGE of Sample 2 (see Figure 1 for explanation of sample codes). Arrows indicate excised bands for mass spectroscopy. Crude: Crude extract; M1: marker proteins for SDS-PAGE (assigned in left and right margin); M2: marker proteins for native PAGE (not assigned); 100: 100-fold diluted sample; 10: 10-fold diluted sample; Undiluted: undiluted sample.

The purification of carp parvalbumin with the use of heat treatment was done using a side fraction of the procedure described previously, as shown in Figure 1. Dory et al. (1998) and Das Dores et al. (2002) described the tendency of parvalbumin to form dimers and tetramers with molecular weights up to approximately 130 kDa. Therefore the 30 kDa concentrate was also used as a source of parvalbumin, even though it was expected that only a part of the parvalbumin would remain on a diafiltration membrane with a 30 kDa cut-off. A 250 mL sample of the 30 kDa concentrate was applied to a 28 mL Q-Sepharose FF column. The column was eluted using a salt gradient, and fractions from 0 to 125 mM NaCl were pooled. SDS-PAGE analysis (not shown) indicated abundant protein bands at higher molecular weight (25 to 50 kDa) in addition to a 12 kDa protein band. The pool was concentrated on a 3 kDa filter, and 2.1 mL of this concentrated pool were applied to a 120 mL Superdex 75 column. Although some separation occurred between the higher molecular weight proteins and a 12 kDa protein band, this separation technique is not sufficient to purify the 12 kDa band to homogeneity. SDS-PAGE analysis revealed a protein band at approximately 10 kDa that co-elutes with the 12 kDa band, as is expected based on the small difference in molecular weight. This 10 kD band may represent another isoform of parvalbumin. To determine if there are indeed more isoforms of parvalbumin present, it was decided to heat treat partially purified carp extracts. Thereto, fractions from 3 different size exclusion chromatography runs (see scheme in Figure 1) were pooled and heat treated by keeping 1 mL portions of the pool for 10 min in a water bath of 100°C. The supernatant was concentrated on a 3 kDa filter. Concentrated sample (0.75 mL) was applied to a 120 mL Superdex 75 column. The main peak contained a homogenous protein band at approximately 12 kDa. Peak fractions were pooled and the SDS-PAGE analysis of this pool is shown in Figure 2. Three bands are seen: an intense doublet at 10 to 12 kDa, and a band at

a higher molecular weight (approximately 15 kDa). This sample is referred to as Sample 2. Purity of protein samples was estimated by analyzing such sample in serial dilution on SDS-PAGE and comparing the intensities of the protein bands, assuming a similar sensitivity for staining.

Results and Discussion

Anion exchange and size exclusion chromatography are key steps in the purification of parvalbumin from fish as already described by other investigators (Aas 1966; Bushana-Rao et al. 1969; Hamada et al. 2003). The present report is the first to describe a method to purify carp parvalbumin without the use of heat treatment (Sample 1, see Materials andMethods). The SDS-PAGE pattern of the purified protein is shown in Figure 2. The yield is 318 mg of parvalbumin obtained from 1.2 kg carp fillets, and the estimated relative purity of the 12 kDa protein band is > 98%, based on serial dilutions of the purified sample analyzed by SDS-PAGE. The vast majority of the proteins in the carp extract are proteins other than parvalbumin (Figure 2, panel A, left lane) indicating the effectiveness of this purification. Native PAGE was performed for Sample 1 to investigate homogeneity further, as this technique is sensitive to differences in charge and shape of proteins with the same molecular weight. Figure 2B shows that Sample 1 at 2.5 and 5 μg protein migrates as a single band. At 10 μg, a very faint band with higher migration is observed. It is estimated, based on this gel that the amount of this faint band relative to the main band is well below 5%. Subsequently, we showed that Sample 1 is a single isoform, even though more isoforms of parvalbumin are reported to exist in carp (Ma et al. 2008). To confirm that other isoforms are indeed present in our source material, we also applied a heat treatment step on the side-fractions obtained during the purification of Sample 1 (see Figure 1). The resulting protein is shown in Figure 2 and is referred to as Sample 2. The yield of Sample 2 is 58.5 mg, and the estimated relative purity of the 10 and 12 kDa protein bands is approximately 80% to 90%, an estimate based on the comparison of the serial dilutions in Figure 2C. Impurities were also detected by means of size exclusion chromatography. Because the isoforms of carp parvalbumin have a low and variable number of aromatic amino acids among the different isoforms, their absorbances at 280 nm as measured during the size exclusion chromatography are not proportional to the protein concentration. Therefore, quantitative statements about the purity of Sample 2 cannot be made based on size exclusion chromatography. Various techniques were applied to document that the purified protein was parvalbumin and to investigate some of its relevant biochemical characteristics.

The target protein is an IgE-binding protein

Parvalbumin is the pan-allergen in fish-allergic patients (Bernhisel-Broadbent et al. 1992; Sten et al. 2004; Taylor et al. 2004) and the IgE-reactivity of carp parvalbumin was demonstrated for fish-allergic patients by other investigators (Ma et al. 2008). To test if the carp protein purified in these experiments (Sample 1) is indeed IgE-reactive, a dot-blot experiment was performed. Figure 3 shows that the crude carp extract is IgE-reactive for sera from all of the fish-allergic patients except patient nr 7. For serumsample nr 9, the reactivity is only moderate. Serum IgE from the patients who reacted to the carp extract, except

serum sample nr 6, reacted with purified carp protein resulting in clear positive dots. The reason that serum sample nr 6 does not react with purified carp protein while the crude extract is reactive may be that this serum sample recognizes another allergen in carp. Other fish allergens have been described previously (Mata et al. 1994; Kondo et al. 2006). It should also be noted that serum samples nr 6 and nr 7 had specific IgE levels to fish that were classed as "weakly positive," even though these subjects had compelling clinical histories of allergic reactions when fish was ingested. Although there are differences between serum samples, it is clear that the purified carp protein is recognized by IgE in sera of fish-allergic individuals.

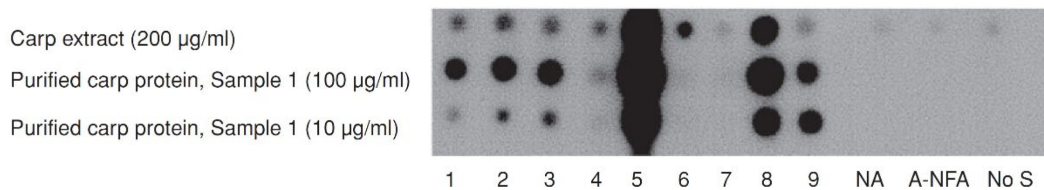
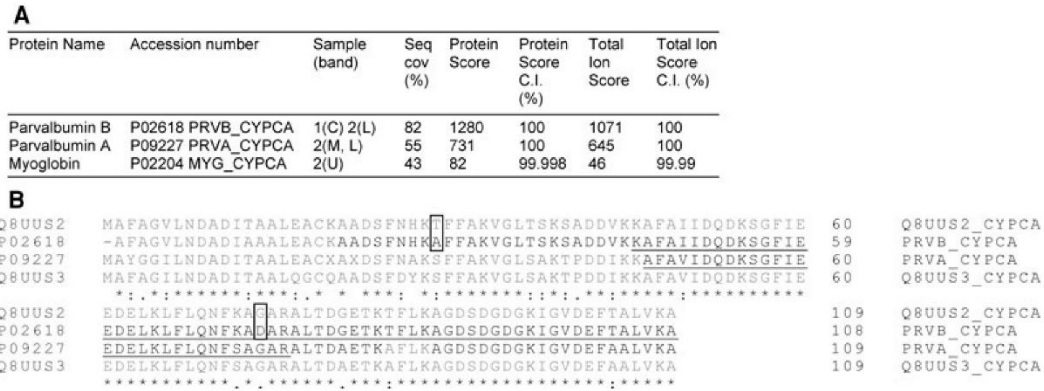


Figure 3. IgE dot-blot of carp preparations. Rows contain different preparations as indicated in left margin 1 to 8: patient serum codes; NA = serum from nonallergic individual; A-NFA = serum from allergic but non-fish allergic individual; No S = no serum applied.

Identification of Sample 1 and Sample 2 as carp parvalbumin

The 12 kDa protein band in Sample 1 and the 12 and 10 kDa bands in Sample 2 (Figure 2) are assumed to be parvalbumins based on their molecular weight, acidity as observed during anion exchange chromatography, and IgE-binding (not tested for Sample 2). For Sample 2, the thermostability of the 10 and 12 kDa band is another indication that these bands may indeed be parvalbumin. Peptide mapping by mass spectrometry (Figure 4A) identified the protein in band C of Sample 1 (Figure 2) unambiguously as carp parvalbumin isoform PRVB˙CYPCA with SwissProt database accession code P02618. The protein in Sample 1 is identified with 82% sequence coverage (percentage of amino acids found in peptides identified with peptide mass fingerprinting relative to total amount of amino acids in the target protein), with identified peptides localizing to the C-terminus of the protein (Figure 4, panel B). The protein was distinguished from the highly homologous isoform Q8UUS2 because of the differing amino acids at residues 28 (alanine) and 73 (aspartic acid) (boxed in Figure 4). The fact that a single isoform is found in Sample 1 is in line with the results of the native PAGE, where a single band was found for Sample 1, even though the methodology used is sensitive for (small) differences in charge as often observed between isoforms. In Sample 2, parvalbumin is identified in both bands L and M (Figure 4 , panel A) as the top scoring protein with up to 55% sequence coverage. The lower sequence coverage as compared to Sample 1 may be due to a somewhat lower protein concentration in Sample 2, and to the possible ionization suppression caused by peptides originating from the other isoforms/proteins present in the excised gel pieces as contaminants from higher/lower migrating bands. It was not possible to distinguish between the isoforms with accession numbers Q8UUS3 and P09227 in Sample 2. Both sequences were supported equally well because the differential amino acids are localized in the N-terminal

region of the sequence, a region where no peptides were detected by MALDI MS. Band L in Sample 2 furthermore contains the isoform with accession number P02618 that was found in Sample 1 too, as the 2nd highest scoring protein. The highest band in Sample 2, band U, is identified as a contaminating carp myoglobin with 43% sequence coverage (Figure 4, panel A).



A

Protein Name	Accession number	Sample (band)	Seq cov (%)	Protein Score	Protein Score C.I. (%)	Total Ion Score	Total Ion Score C.I. (%)
Parvalbumin B	P02618 PRVB_CYPCA	1(C) 2(L)	82	1280	100	1071	100
Parvalbumin A	P09227 PRVA_CYPCA	2(M, L)	55	731	100	645	100
Myoglobin	P02204 MYG_CYPCA	2(U)	43	82	99.998	46	99.99

Figure 4. Peptide mapping by mass spectrometry. *Panel A:* Mascot Search results summary. *Panel B:* Aligned sequences of the 4 reported parvalbumin α and β variants (gray: amino acid sequence, black: MS identified peptides, underlined: supporting MS/MS spectra, box: important amino acid variations).

UV/Vis spectroscopy

Absorbance in the far-UV regime is dictated by the aromatic amino acids. Typically tryptophan residues have absorption maxima around 280 to 285 nm; tyrosine at 270 to 275 nm and phenylalanine around 250 to 260 nm. The molar extinction coefficient for each of these amino acids decreases from tryptophan > tyrosine >> phenylalanine. None of the carp parvalbumin isoforms contains tryptophan. A single tyrosine is found in isoforms Q8UUS3 and P09227 (both present in our Sample 2), but none are found in isoform P02618 (our Sample 1). Isoforms P09227, Q8UUS3, and P02618 contain phenylalanine residues (9 for P09227 and Q8UUS3, 10 for P02618). The UV/Vis spectra of the 2 samples of carp parvalbumin we have purified are remarkably different (Figure 5). Sample 1 has a maximum absorbance at 260 nm, and no absorbance at 275 to 290 nm in line with the absence of both tryptophan and tyrosine residues in the isoform P02618 found in Sample 1. Sample 2 has a maximum absorbance at 260 nm, and a distinct absorbance at 270 to 280 nm as well, in line with the presence of a single tyrosine in isoform P09227. The (minor) contaminant, myoglobin, in Sample 2 may add to the UV absorbance, although its contribution is considered to be low based on the estimated level of purity in Sample 2. Assuming myoglobin is present at 5% of the total protein content, and taking into account the A280 at 1 mg/mL (0.716, SwissProt accession code Q2LC33), the contribution of myoglobin to the absorbance at 280 nm as presented in Figure 5 would be approximately 0.03 to 0.04 absorbance units.

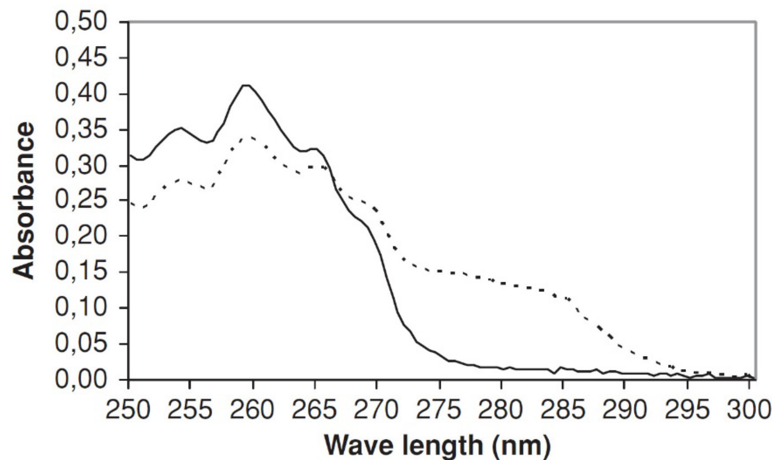


Figure 5. UV spectrum of carp parvalbumin. Solid line: Sample 1; dotted line: Sample 2 (see Figure 1 for explanation).

Far UV CD spectroscopy

Far UV CD spectra of Sample 1 and Sample 2 recorded in the presence of EDTA are shown in Figure 6. In this case, calcium was removed to assure that all the parvalbumin present was in the same state, that is, the apo-form without calcium. The spectra for both Sample 1 and Sample 2 are very similar and show minima at 208 and 222 nm, combined with a zero-crossing at approximately 200 nm. This indicates the presence of α-helices and a minor contribution of β-structures. Spectral analysis using a nonlinear regression method with reference spectra for the 4 different secondary structure types as input (de Jongh et al. 1993) provides estimates for the secondary structure content of 49% alpha-helix, 9% beta-structures (β-sheet + β-turn), and 42% nonordered structure. Identical estimates were obtained for both Sample 1 and Sample 2. The small difference in intensity is related to slight differences in protein concentration. The spectra are in line with those of Bugajska-Schretter et al. (2000), who also measured far UV CD spectra of the apo-form of carp parvalbumin at neutral pH. Recently recombinant carp parvalbumin has been expressed and purified, and its far UV CD spectrum has been published (Swoboda et al. 2002; Ma et al. 2008). The apo-form of this recombinant carp parvalbumin resembles the secondary structure of the natural protein as described earlier (Bugajska-Schretter et al. 2000) and as we show here.

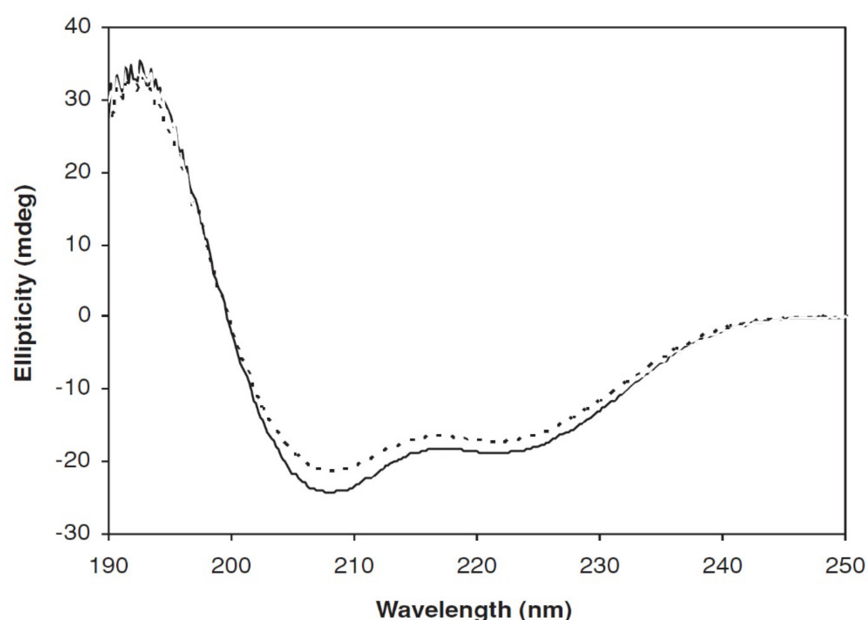


Figure 6. Far UV CD spectrum of carp parvalbumin. Solid line: Sample 1; dotted line: Sample 2.

Description of the purified carp parvalbumin

Using a protocol that avoids potential denaturing steps such as heat treatment and addition of chelating compounds like EDTA, parvalbumin can be purified from carp fillets. The protocol was based on extraction with aqueous buffer, ultrafiltration through a 30 kDa cut-off membrane, anion exchange, and size exclusion chromatography. The purified parvalbumin, identified by means of peptide mapping using mass spectrometry, has earlier been cloned by Swoboda et al. (2002) and is known as PRVB˙CYPCA with SwissProt accession number P02618. Our purified parvalbumin as obtained here has the ability to bind IgE from fish-allergic individuals, and its secondary structure is similar to that of carp parvalbumin purified by others, cod parvalbumin and recombinant carp parvalbumin (Ma et al. 2008). Other isoforms of parvalbumin, although present in the carp fillets, were not purified using the current protocol. At least one other isoform was shown to be present in the concentrate of a 30 kDa ultrafiltration experiment but not in the 30 kDa filtrate. This could possibly be explained if this parvalbumin isoform has a greater tendency to form dimers/multimers as observed by others (Dory et al. 1998; Das Dores et al. 2002). Although the occurrence of fish parvalbumin as dimers or oligomers is not well established, any dimers/oligomers that may be present under native conditions will not be observed in IgE-immunoblotting studies because of the denaturing conditions enforced by SDS. In many previous experiments, the IgE-binding properties of various fish parvalbumins has been evaluated after SDS-PAGE (van Do et al. 2005b), conditions where any dimers/oligomers would be missed. Furthermore, in previous studies describing the purification of fish parvalbumin, heat treatment and addition of chelating compounds were often applied, conditions that may induce dissociation of (weakly) associated multimers. However, no experimental evidence

is presented to further support the hypothesis that certain isoforms of carp parvalbumin form dimers or multimers. To purify the other isoforms, a heat treatment step was applied as no other common purification method (except for ultrafiltration) was suitable to remove the 25 to 50 kDa proteins from the preparation. High-performance liquid chromatography (HPLC) and reverse phase–HPLC (RP-HPLC) were not considered because of the low scale of such purification steps. It has been shown that heat treatment does not affect the secondary structure of carp parvalbumin (Bugajska-Schretter et al. 2000) to a great extent. In our study, we did not make a direct comparison between native and heat-treated carp parvalbumin. We did analyze Sample 1 (native) with Sample 2 (heat-treated), which seemed to contain different isoforms of carp parvalbumin. Therefore, it still may be that heat treatment leads to denaturation which results in precipitation of aggregated material or adherence of denatured protein to walls of vials and tubing. Such denatured protein will not be detected with far UV CD spectroscopy, resulting in overestimation of the heat stability as described by others earlier (Bugajska-Schretter et al. 2000; Ma et al. 2008). Therefore, for studies investigating the structural stability of allergens, heat treatment and other potentially denaturing steps should be avoided during purification as far as possible.

Up to now, 4 isoforms of carp parvalbumin have been submitted to the SwissProt database. It is, however, not known which of these isoforms is the most relevant in carp allergenicity. Considering the very high degree of homology between the 4 isoforms of carp parvalbumins (Figure 4, panel B), and taking into account the fact that parvalbumins from different fish species are highly cross-reactive on the level of IgE-binding, it is probably fair to say that the isoform we have purified in this study is well suited as a model protein for investigating carp parvalbumin allergenicity. The purified carp parvalbumin (Sample 1) was subjected to IgE-binding studies using sera from fish-allergic individuals and indeed proved to be IgE-reactive.

Conclusions

The protocol provided in this report allows easy purification of relatively large amounts of carp parvalbumin, that is, hundreds of milligrams. The applied steps are all suitable for further scale-up. The scale of hundreds of milligrams is sufficient not only for biochemical analysis but also for raising polyclonal antibodies that are needed for detection and quantification of fish allergens in food products. The use of nondenaturing conditions in the current purification protocol will allow characterization of the resultant native carp parvalbumin on the level of structural stability, a topic of current studies in our laboratories. We conclude that this protocol is well suited to prepare native carp parvalbumin on a scale that will allow for various biochemical and immunochemical experiments.

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