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Research Article

Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp

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Allergic reaction following fish consumption can trigger life-threatening reactions in predisposed individuals. Parvalbumins from different species have been identified as the major fish allergens. There are two distinct phylogenetic lineages of parvalbumins, alpha and beta. Most allergic reactions are caused by β-parvalbumins. We cloned and expressed cDNAs encoding cod (*Gadus morhua*) and carp (*Cyprinus carpio*) β-parvalbumins and purified natural cod β-parvalbumin. CD spectra of the purified proteins showed that their overall secondary structure contents were very similar. No differences in thermal stability were monitored in the calcium-bound or calcium-depleted form of natural cod parvalbumin. IgE reactivity was assessed using 26 sera of fish allergic patients from Spain, The Netherlands, and Greece in immunoblot and ELISA experiments. Twenty-five of the 26 patients with IgE reactivity to native and recombinant cod parvalbumin also reacted to the recombinant carp parvalbumin. IgE inhibition assays were performed using cod and carp extracts and purified recombinant parvalbumin of cod and carp. High crossreactivity among cod and carp parvalbumins was observed in immunoblots as well as in fluid phase assays. Natural and recombinant parvalbumins gave comparable results when performing various *in vitro* diagnostic assays.

Keywords: Carp parvalbumin / Cod parvalbumin / Fish allergy / Food allergy / β-Parvalbumin

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1 Introduction

Fish are among the most common sources of food allergens. In many countries where seafood is an integrate part of the diet, fish represent a frequent cause of food allergy [1]. The parvalbumins of fish represent the second largest animal food allergen family, the largest being the tropomyosins of crustaceans and molluscs [2]. Parvalbumins which are abundant in the white muscle of many fish species constitute a subfamily of a large evolutionary related family of proteins with mixed type binding sites for Ca^{2+}/Mg^{2+}, the so-called EF-hand [3]. The family of EF-hand proteins is the third ranking protein family in terms of numbers of allergenic members (www.meduniwien.ac.at/allergens/allfam/). The EF-hand corresponds to a helix-loop-helix motif of 30 residues in length. Both helices E and F, with 10 residues each, are flanking a central loop that contains the metal-binding residues [4]. Parvalbumins are important for the relaxation of muscle fibers by binding free intracellular

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Abbreviations: CD, circular dichroism; **ESI-QTOF,** ESI-Quadrupole TOF

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calcium [5]. The binding of the calcium ligand was found to be necessary for maintaining the parvalbumin in a conformation that is able to bind IgE. Loss of calcium results in a change in conformation together with an associated loss of the protein's IgE binding capacity [6–8]. Parvalbumins with bound calcium also possess a remarkable stability to denaturation by heat, denaturing chemicals, and digestive enzymes [8–10] which influences their allergenic activity.

Parvalbumins can be subdivided into two distinct evolutionary lineages based on the comparison of their amino acid sequences [11]. α-Parvalbumins comprise 109 amino acid residues and are less acidic with p*I*s at 5.0 or higher. β-Parvalbumins consist of 108 amino acid residues and are more acidic with p*I*s at 4.5 or lower [12]. In general, α-parvalbumins are not allergenic with the exception of representatives from two frog species [13, 14]. In contrast, many allergenic β-parvalbumins are found in a variety of fish species [15, 16]. Some of them have been shown to display more than two parvalbumin isotypes [17]. Today, allergenic β-parvalbumins are considered as crossreactive pan allergens in fish [18–20]. Sufficient IgE-reactive epitopes remain after cooking to trigger allergic reactions in susceptible individuals as has been demonstrated by double blind placebo controlled food challenge [21]. CD analysis of carp parvalbumin revealed a remarkable stability and refolding capacity of the calcium-bound form [7]. However, there is one contradictory report which questions the stability of parvalbumins to pepsin [22]. The authors reported a recombinant allergenic carp parvalbumin (rCyp c 1) to be completely degraded after 30 s treatment with pepsin.

In the present study, the structural and immunological properties of natural and recombinant allergenic fish parvalbumins were investigated and compared. In addition, the impact of thermal denaturation on purified parvalbumins was studied. We have selected cod as a sea water and carp as a fresh water fish species for our studies.

2 Materials and methods

2.1 Sera and antibodies

Fish allergic patients from Spain, The Netherlands, and Greece ($n = 26$; including children and adults) were identified according to convincing case histories and positive CAP values (Phadia Diagnostics, Uppsala, Sweden; Table 1) to fish. Sera were stored at −20°C until use. The mouse monoclonal antiparvalbumin clone Parv-19 antibody from Sigma (St. Louis, MO, USA) and a rabbit polyclonal anti-Gad m 1 antibody (Tepnel BioSystems, Deeside, Flintshire) were used in this study.

2.2 Preparation of crude fish extract

Fresh filet of Atlantic cod (*Gadus morhua*) was purchased from a local market. Fish muscle (500 g) was homogenized with three volumes of 20 mM Bis-Tris buffer pH 6.5. Proteins were extracted by stirring the homogenate for 3 h at 4°C. After centrifugation at $17\,000 \times g$ for 45 min at 4°C the supernatant was collected and filtered through Miracloth® (Merck Biosciences, Nottingham, UK) and filter papers, subsequently, to remove cellular debris. Cod extract was further used for purification and immunological assays. For IgE inhibition assays 4 g of carp muscle were homogenized with five volumes of double distilled H_2O containing 3 mM NaN_3, and extracted by stirring for 3 h at 4°C. The total extract was cleared by centrifugation at $20\,000 \times g$ for 15 min at 4°C. The protein concentration was determined by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, Ireland), according to the manufacturer's instructions. The extracts were stored at 4°C.

2.3 Purification of natural cod parvalbumin

As a first purification step the cod extract was cleared by filtration then incubated with Biocryl BPA-1000 (Supelco, Bellefonte, PA) to remove nucleic acids and then centrifuged at $20\,000 \times g$ for 10 min at 4°C. The supernatant was applied to a DEAE Sepharose Fast Flow column (GE Healthcare, Little Chalfont, UK) and washed with 20 mM Bis-Tris buffer, pH 6.5. Bound protein was eluted with a linear salt gradient from 0 to 25% elution buffer (20 mM Bis-Tris, 1 M NaCl, pH 6.5) and parvalbumin was detected by SDS-PAGE and immunoblotting using the mouse monoclonal antiparvalbumin clone Parv-19 antibody. Fractions containing parvalbumin were then loaded onto a HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare) which had been equilibrated at room temperature with 20 mM Bis-Tris, 150 mM NaCl, pH 6.5. Parvalbumin was eluted as a single peak from the column.

2.4 cDNA synthesis and RT-PCR amplification

Total RNA was isolated from 100 mg cod muscle tissue using the RNeasy® Protect Midi Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Five micrograms of total RNA was used for cDNA synthesis. The reverse transcription was performed with an oligo-dT primer T_{25}NN. Gene-specific primers (5′-ATG GCA TTC GCT GGA ATT CTC G-3′ for rGad m 1.01 and 5′-ATG GCT TTC GCC GGA ATT CTG A-3′ for rGad m 1.02) were synthesized according to the published N-terminal sequences of cod parvalbumins [23]. These primers were used in conjunction with the oligo-dT primer T_{25}NN to amplify Gad m 1 encoding cDNAs. The PCR reaction was performed as described elsewhere [24].

2.5 cDNA cloning and DNA sequencing

Amplified Gad m 1 cDNA was ligated into the pCP2.1-TOPO vector (Invitrogen, Carlsbad, USA) and competent

Table 1. Patients' characteristics

No.	Country	Age	Sex	CAP	Symptoms
1	E	11	f	Cod 3.18	OAS, AD
2	E	11	m	Cod 17.2	U, V, AD
3	E	11	m	Haddock 32.9	OAS, U, AD
4	E	5	m	Cod 8.91	U, V, AD
5	E	6	m	Cod 2.74	OAS
6	E	27	m	Cod 2.42	AE, D, CU, AD
7	E	15	m	Cod 0.52	OAS, AE, DY, AD
8	E	27	m	Cod 55.4	U, A
9	E	4	f	Cod 0.72	OAS
10	E	23	m	Cod 9.6	AN
11	E	10	m	Sole < 0.78	V, AD
12	E	7	f	Cod 6.38	U, AD
13	E	29	m	Cod 7.17	DY
14	NL	29	f	Cod 37.0	R, D, AD
15	NL	22	m	Cod 53.0	R, ST, AD
16	NL	41	m	Cod 11.0	OAS, AD
17	NL	39	f	Cod 3.2	AD
18	NL	15	m	Cod 6.3	OAS, U, AD
19	NL	17	m	Cod 12.0	OAS, TT, AD
20	GR	5	f	Cod 5.22	AN
21	GR	10	m	Cod >100	A
22	GR	14	m	Cod 99.3	U, G
23	GR	ND	m	– ND	G
24	GR	12	f	Cod 1.99	U
25	GR	13	m	Cod 9.09	AN, A, U
26	GR	4	m	Cod 11.2	AN

Patients' sera used for immunoblotting are indicated in bold. E, Spain; NL, Netherlands; GR, Greece; f, female; m, male; CAP, capsulated hydrophobic carrier polymer (kU/mL); A, asthma; AD, atopic dermatitis; AE, angioedema; AN, anaphylaxis; C, cough; CU, contact urticaria; D, dyspnoea; DY, dysphagia; OAS, oral allergy syndrome; R, rash; ST, swelling throat; TT, tightness in the throat; U, urticaria; G, gastrointestinal; V, vomiting.

TOPO10F' *E. coli* cells were transformed with the pCP2.1-Gad m 1 plasmids. Sequencing of the inserts was performed by VBC-Biotech Service (Vienna, Austria). Sequence analysis was performed using the BLAST Program of the National Center of Biotechnology Information (Bethesda, MD) and ClustalW program of EMBL-EBI.

2.6 Expression and purification of rGad m 1.02 and rCyp c 1.01

The cDNA coding for Gad m 1.02 was PCR amplified from the respective pCP2.1-plasmid and subcloned into the *Hin*dIII/*Bam*HI sites of expression vector pET17b (Novagen, Madison, WI). The primers for subcloning were designed in a reading frame avoiding the expression of the T7-Tag. This was achieved using the following oligonucleotide primer for the 5′ end of the clone: 5′-AC AAG CTT ATG GCT TTC GCC GGA ATT CTG A-3′, which contained a *Hin*dIII site and a primer for the 3′ end with a *Bam*HI site: 5′-AT CGG ATC CTA TGC CTT GAT CAT GGC-3′. pET17b containing the Gad m 1.02 cDNA was expressed in *E. coli* BL21 (DE3)-RIPL cells. Single colonies were grown overnight at 37°C in LB medium containing 0.1 mg/mL ampicillin and 50 μg/mL chloramphenicol. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for 5 h at 30°C. Cells were harvested and disrupted by repeated cycles of freezing in liquid nitrogen and thawing in a water bath. Cell pellets were resuspended in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Digestion with DNase I (0.1 μg/g cell pellet) and RNase A treatment (0.01 μg/g cell pellet) were performed at room temperature for 30 min under constant stirring. The lysate was centrifuged at 13 000 × *g* for 30 min at 4°C. Recombinant Gad m 1.02 was purified from the supernatant using two anion exchange columns, a DEAE Sepharose Fast Flow (buffer A: 20 mM Bis-Tris, pH 6.5, buffer B: A + 1 M NaCl) and a MonoQ 5/50 GL Tricorn high performance column (GE Healthcare, buffer A: 20 mM Tris, pH 8.0, buffer B: A + 1 M NaCl).

Recombinant Cyp c 1.01 was expressed from the pET17b-Cyp c 1 plasmid [25, 26]. *E. coli* lysate containing rCyp c 1.01 was heated up to 75°C for 40 min and centrifuged 13 000 × *g* for 30 min at 4°C. Subsequently, ammonium sulfate (75%) was added and the protein extract was centrifuged. Recombinant carp parvalbumin was obtained from the supernatant and was applied onto a Phenyl-Cellufine column (GE Healthcare) which was equilibrated with 1 M NaCl, 200 mM NaH_2PO_4 pH 5.0, 1 mM β-mercaptoethanol (buffer A). Bound protein was eluted by a linear gra-

dient (0 – 100%) of buffer B (25 mM Tris/HCl pH 9.3, 8% 2-propanol). Recombinant Cyp c 1.01 enriched fractions were further purified by an anion exchange chromatography (DEAE Sepharose Fast Flow column). The column was equilibrated with buffer A (20 mM imidazol pH 7.4, 2 mM β-mercaptoethanol), the bound recombinant Cyp c 1.01 was eluted by a gradient with buffer B (buffer A + 0.4 M NaCl). A final dialysis step was performed against 5 mM NaH_2PO_4, 2 mM β-mercaptoethanol, pH 7.4.

2.7 SDS-PAGE, Western blotting, IgE immunoblotting, and IgE inhibition

Purified nGad m 1, rGad m 1.02, and rCyp c 1.01 were separated by 15% SDS-PAGE as described by Laemmli [27] under reducing conditions and either visualized by CBB R-250 staining or transferred to nitrocellulose membranes (pore size 0.2 µm, Pall Corporation, Pensacola, USA) for immunodetection.

Blots were incubated with the mouse monoclonal anti-parvalbumin clone Parv-19 antibody (1:4000 diluted in TBST [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 v/v]) or the rabbit polyclonal anti-Gad m 1 antibody (1:12 000 diluted in TBST) under constant shaking at room temperature for 2 h, respectively. Subsequently, alkaline phosphatase (AP)-conjugated swine antimouse (DAKO, Glostrup, Denmark, 1:1000 diluted in TBST) and antirabbit Igs (Jackson ImmunoResearch Laboratories, West Grove, USA, 1:5000 diluted in TBST), respectively. Development was performed with BCIP/NBTC reagent solutions. In addition, blotted proteins were incubated with fish allergic patients' sera diluted 1:5 in buffer B (42 mM Na_2HPO_4, 7 mM NaH_2PO_4, 0.05% w/v Na-azide, pH 7.5, 0.5% Tween-20) overnight at 4°C and detected by ^{125}I-labelled rabbit anti-human IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany, 1:20 diluted in buffer B). Autoradiography was performed at room temperature for 2 – 48 h with intensifying phosphor screens and imaged on the Storm 860 Imager (GE Healthcare) or with a BioMax Ms film (Kodak, Sigma – Aldrich, St. Louis, USA) at –70°C. IgE inhibition assays were performed using purified nGad m 1 (50 µg/mL), rGad m 1.02 (10, 50, and 100 µg/mL) and rCyp c 1.01 (50 µg/mL) as inhibitors respectively over night at 4°C. Incubation of the blot strips and detection were performed as above.

2.8 IgE ELISA and inhibition assays

Purified nGad m 1, rGad m 1.02, and rCyp c 1.01 (1 µg/well diluted in 25 mM $NaHCO_3$, pH 9.6) were coated on microtiter plates (Nunc Maxisorp, Nalge Nunc International, Roskilde, Denmark) overnight at 4°C. Nonspecific binding sites were blocked with 3% milk in TBST. The coated allergens were incubated with sera from 26 fish allergic patients (1:4 diluted in TBST containing 0.5% BSA) overnight at 4°C. Bound IgE was detected with AP-conjugated mouse antihuman IgE antibody (BD-Biosciences Pharmingen, San Diego, CA, USA) and developed with the SIGMA FAST™ *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). Color development was measured using an ELISA reader (Spectra Max Plus 384; Molecular Devices, Munich, Germany) at 405 and 510 nm as reference wavelength. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs. IgE inhibition assay was performed using cod or carp fish extracts (10 µg/mL) diluted in coating buffer (25 mM $NaHCO_3$, pH 9.6) and purified recombinant parvalbumin of cod (10 µg/mL) and carp (10 µg/mL) as inhibitors.

2.9 N-terminal sequencing

Fifty picomoles of purified proteins was diluted into 100 µL of 0.1% TFA and immobilized on a polyvinylidene difluoride (PVDF) membrane (ProSorb sample preparation cartridges, Applied Biosystems, Foster City, CA, USA), which was soaked in 10 µL of methanol before. The PVDF membrane was washed with 0.1% TFA. After drying at room temperature membrane was subjected to the automated gas-phase Procise 491 sequencer (Applied Biosystems). For nGad m 1, the N-terminal acetyl group was cleaved off with TFA prior to sequencing.

2.10 Circular dichroism (CD) and stability studies

Far UV CD spectra of purified parvalbumins were recorded with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK) at 20°C in aqueous solutions. Protein samples were concentrated to 0.1 µg/µL and measured in quartz cuvettes (Hellma, Mullheim, Baden, Germany) of 0.1 and 0.2 cm path length. All spectra were corrected for the proper baseline using the corresponding aqueous solution. Absorption between 190 and 260 nm was monitored at 0.5 nm intervals. The obtained spectra represent an average of three consecutive scans. Results of each sample were averaged, and the mean residue ellipticity (θ) was expressed as $deg \cdot cm^2 \cdot dmol^{-1}$. The mean residue molecular weight of each parvalbumin was calculated from the amino acid composition. For monitoring the stability to heating, samples were incubated for 10 min at 95°C.

2.11 NMR analysis

The allergens rCyp c 1.01 and nGad m 1 were analyzed by NMR, according to the following protocol. Before and after the NMR experiments, the allergens were stored at –20°C. A solution of each allergen was prepared in 0.45 mL of H_2O plus 0.05 mL of D_2O. The concentrations were 0.52 mM for rCyp c 1.01 and 0.05 mM for nGad m 1. The solutions were placed into high-quality NMR tubes with Ar as head-space

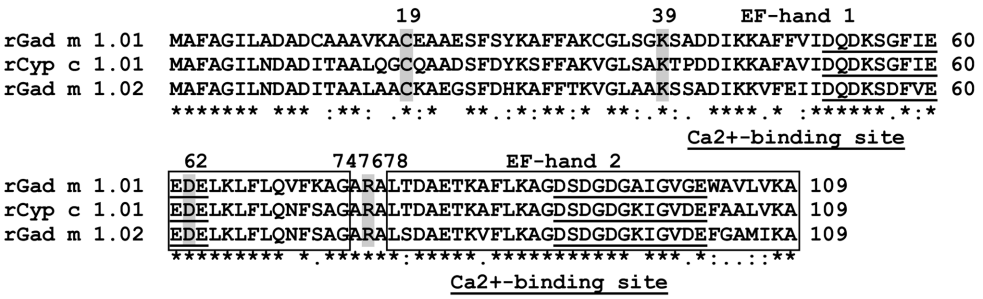

Figure 1. Comparison of amino acid sequences and analysis of the two cod β-parvalbumins (rGad m 1.01 and rGad m 1.02) and carp β-parvalbumin (rCyp c 1.01). The sequences are available under accession numbers AM497927 for rGad m 1.01, AM497928 for rGad m 1.02, and AJ292211 for rCyp c 1.01. Two EF-hand repeats including the calcium binding sites are boxed and the calcium binding sites are underlined. Characteristic amino acid residues for currently known parvalbumins are highlighted. Stars indicate conserved, ":" highly conserved, and "." weakly conserved amino acid residues.

gas. Two High Resolution NMR experiments were carried out, by a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla) at 25°C. The two experiments were different in the method to manage the water signal: the zgpr experiment minimizes the water peak, while the zgesgp experiment suppresses it. For each experiment 1024 scans were programmed to analyze nGad m 1 and 256 scans for rCyp c 1.01.

2.12 Mass determination

For mass determination of intact Gad m 1, approximately 2 µg of protein was reduced with the reagents of the Proteoextract™ Trypsin Digestion Kit (Calbiochem, San Diego, USA). Prior to the mass analysis, salts and reagents were removed using C18 ZipTips™ (Millipore, Billerica, MA, USA), following the manufacturer's protocol, except that trifluoro acetic acid was replaced by formic acid. Proteins were eluted from the RP material with aqueous 50% v/v HPLC-grade ACN and 0.1% v/v formic acid and directly infused into an ESI-Quadrupole TOF (ESI-QTOF) mass spectrometer (Ultima Global, Micromass-Waters, Milford, MA, USA) at an infusion rate of 1 µL/min. The intact mass of rCyp c 1.01 was determined without prior Ziptip purification at a concentration of approx. 500 fmol/µL and an infusion rate of 0.5 µL/min. The Waters Nanoflow spray head was used with nitrogen as desolvation gas and a capillary voltage of 3.4 kV. The instrument was calibrated with the fragment ions of [Glu]-Fibrinopeptide B (Sigma). Spectra were recorded for 3 min in a mass/charge range from 400 to 1900. More than 200 mass scans of 1 s each were combined for optimal S/N. Multiply charged peaks were processed using the MaxEnt1™ algorithm of the MassLynx™ software package (Waters).

For nano-LC-MS/MS-based peptide mapping 50 µg aliquots of rCyp c 1.01 each were digested overnight at 37°C with 2 µg trypsin or V8 protease (Roche, Basel, Switzerland). Present cysteine residues were reduced by pretreatment of the protein with a ten-fold molar excess of DTT and a 20-fold molar excess of iodoacetamide, both incubations lasted for 30 min at room temperature. Alternatively, 5 µg of Gad m 1 was reduced, alkylated, and digested with the Proteoextract Trypsin Digestion Kit (Calbiochem). Proteolytic digests were diluted 1:20 in 0.1% formic acid and 5 fmol was injected to RP capillary HPLC (Nanoease Symmetry 300™ trap column and 0.075×15 mm^2 Nanoease Atlantis dC18™ separating column on CapLC, Micromass-Waters) directly coupled to ESI-QTOF in data-dependent analysis mode. Tandem mass spectra were analyzed using the ProteinLynx Global Server 2.2.5™ software (Waters) with both automatic and manual data verification. For MS/MS-based sequencing a combined Swiss-Prot/TrEMBL database was used and automatic validation was enabled. Therefore, positive identification of rCyp c 1.01- or rGad m 1.02-derived peptides by CID was based on at least four consecutive unequivocally identified *y*-ions in MS/MS mode.

3 Results

3.1 Cloning and sequence analysis

Two full-length clones of 599 and 797 bp (named Gad m 1.01 and Gad m 1.02), coding for cod β-parvalbumins were obtained. The sequences were submitted to EMBL Genbank Database (accession numbers AM497927 and AM497928). Gad m 1.01 had one amino acid exchange at position 101 (E/D) compared to cod parvalbumin with the accession number AY035584 [28]. Gad m 1.02 had four nucleotide differences with one different deduced amino acid at position 41 (S/P) compared to cod parvalbumin with the accession number AY035585 [28].

The coding regions of the two parvalbumin cDNAs each encompass 330 bp coding for 109 amino acid residue proteins both with a theoretical p*I* of 4.58 (Fig. 1). The deduced amino acid sequences of the two cod β-parvalbumin isoforms are 71% identical. Both Gad m 1 isoforms share 80–81% sequence identity with Cyp c 1.01. Like all the other

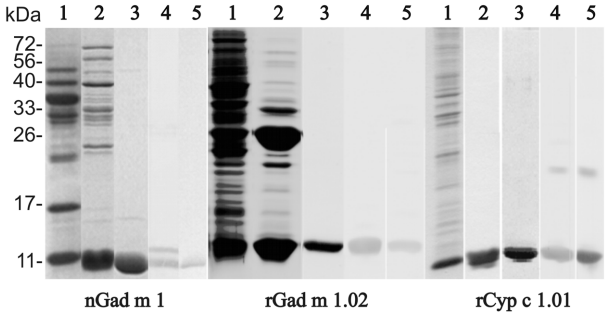

Figure 2. Purification of parvalbumins: nGad m 1: Lane 1: cod extract. Lane 2: protein purification by anion exchange followed by size exclusion chromatography (lane 3). Lane 4: detection of two Gad m 1 isoforms by a rabbit polyclonal anti-Gad m 1. Lane 5: detection of one Gad m 1 isoform by the mouse monoclonal antiparvalbumin Parv-19 antibody. rGad m 1.02: Lane 1: cell lysate of *E. coli* expressing rGad m1.02, lane 2: protein purification by anione exchange followed by size exclusion chromatography (lane 3), lane 4: detection of purified rGad m 1.02 by a rabbit polyclonal anti-Gad m 1 antiserum. Lane 5: detection of purified rGad m 1.02 by the mouse monoclonal antiparvalbumin Parv-19 antibody. rCyp c 1.01: cell lysate of *E. coli* expressing rCyp c 1.01. Lane 2: purification of rCyp c 1.01 by hydrophobic interaction chromatography followed by anion exchange chromatography (lane 3). Detection of purified rCyp c 1.01 by polyclonal (lane 4) and monoclonal antibodies (lane 5) showing dimers at around 20 kDa.

known fish parvalbumins, the Gad m 1 encoding sequences contain two characteristic EF-hand repeats which bind calcium and contain conserved amino acid residues (Fig. 1). An invariant aspartic acid residue is present at position 62 which is common to all currently known parvalbumins. In addition, a cysteine at position 19 and an arginine at position 76, conserved characteristics of the β lineage parvalbumins [28], were also identified.

3.2 Extraction and purification of nGad m 1, expression and purification of rGad m 1.02 and rCyp c 1.01

Natural cod parvalbumin was purified from cod muscle protein extract. The SDS-PAGE profile of the extract showed a prominent band at approximately 12 kDa (Fig. 2, nGad m 1, lane 1). Natural Gad m 1 was purified by a combination of anion exchange (Fig. 2, nGad m 1, lane 2) and size exclusion chromatography (Fig. 2, nGad m 1, lane 3). Purified nGad m 1 representing two isoforms was detected by immunoblotting with polyclonal and monoclonal antibodies (Fig. 2, nGad m 1, lanes 4 and 5, respectively). The total yield from 500 g fish muscle was 25 mg pure nGad m 1.

Recombinant Gad m 1.02 and rCyp c 1.01 were expressed in the pET17b expression vector and produced as nonfusion proteins in BL21 (DE3)-RIPL cells. Recombinant parvalbumin proteins were mostly found in the soluble fraction of the cell cultures (Fig. 2, rGad m 1.02 and rCyp c 1.01, lane 1). After precipitation (rCyp c 1.01), proteins were purified by several chromatographic steps (Fig. 2, rGad m 1.02 and rCyp c 1.01, lanes 2 and 3). Purified proteins were detected by immunoblotting using the mouse monoclonal antiparvalbumin clone Parv-19 antibody and the rabbit polyclonal anti-Gad m 1 antibody (Fig. 2, rGad m 1.02 and rCyp c 1.01, lanes 4 and 5, respectively).

3.3 N-terminal sequencing

N-terminal sequence analysis of all three purified proteins nGad m 1, rGad m 1.02, and rCyp c 1.01 revealed that the initiating methionine was cleaved off, resulting in the following first five amino acid residues AFAGI.

3.4 IgE binding activity and crossreactivity

Sera from 26 fish allergic patients from Spain, The Netherlands, and Greece were used for IgE ELISA. The IgE binding activities of purified natural and recombinant β-parvalbumins of cod and recombinant β-parvalbumin of carp were comparable for most sera (19/26, Fig. 3A). A serum pool from five fish allergic patients (patient no. 4, 8, 10, 18, and 21) was used for IgE inhibition experiments. The serum pool was preincubated with cod or carp muscle extract (10 μg/mL) purified rGad m 1.02 (10 μg/mL) or rCyp c 1.01 (10 μg/mL). IgE binding activity was inhibited by 91% to nGad m 1 and rGad m 1.02 and by 76% to rCyp c 1.01 (Fig. 3B). IgE binding to rGad m 1.02 was inhibited 98% by rCyp c 1.01 and IgE binding to rCyp c 1.01 was reduced 86% by rGad m 1.02 (Fig. 3C).

Sera from 10 fish allergic patients (patient no. 1, 3, 4, 8, 10, 14, 15, 16, 18, and 21) were used for IgE immunoblotting of the three parvalbumins (Fig. 3D). Serum no. 21 was used for the inhibition assay (Fig. 3D, lane 21i). The band recognized by the IgE antibodies from patients' sera was at 12 kDa. The identity of this band as parvalbumin was confirmed by immunoblots with antiparvalbumin antibodies. Most samples displayed equal IgE reactivity to all three proteins. The immunoblot inhibition experiments were performed with cod and carp muscle extract, purified nGad m 1, rGad m 1.02, and rCyp c 1.01. IgE binding to nGad m 1 and rGad m 1.02 was almost completely inhibited, and IgE binding to rCyp c 1.01 was inhibited by more than 50% with fish extracts (Fig. 3D, lane 21i). These results were in agreement with the results of ELISA inhibitions (Fig. 3B). The immunoblot inhibition experiments with purified natural and recombinant proteins showed that the IgE binding capacity of rCyp c 1.01 was completely inhibited by carp extract (Fig. 3E). The IgE binding capacity of nGad m 1 and rGad m 1.02 was inhibited more than 90 and 80% by cod extract (Fig. 3E), which was measured using ChemiImager™ 400 (Alpha Innotech Corporation). Normal human

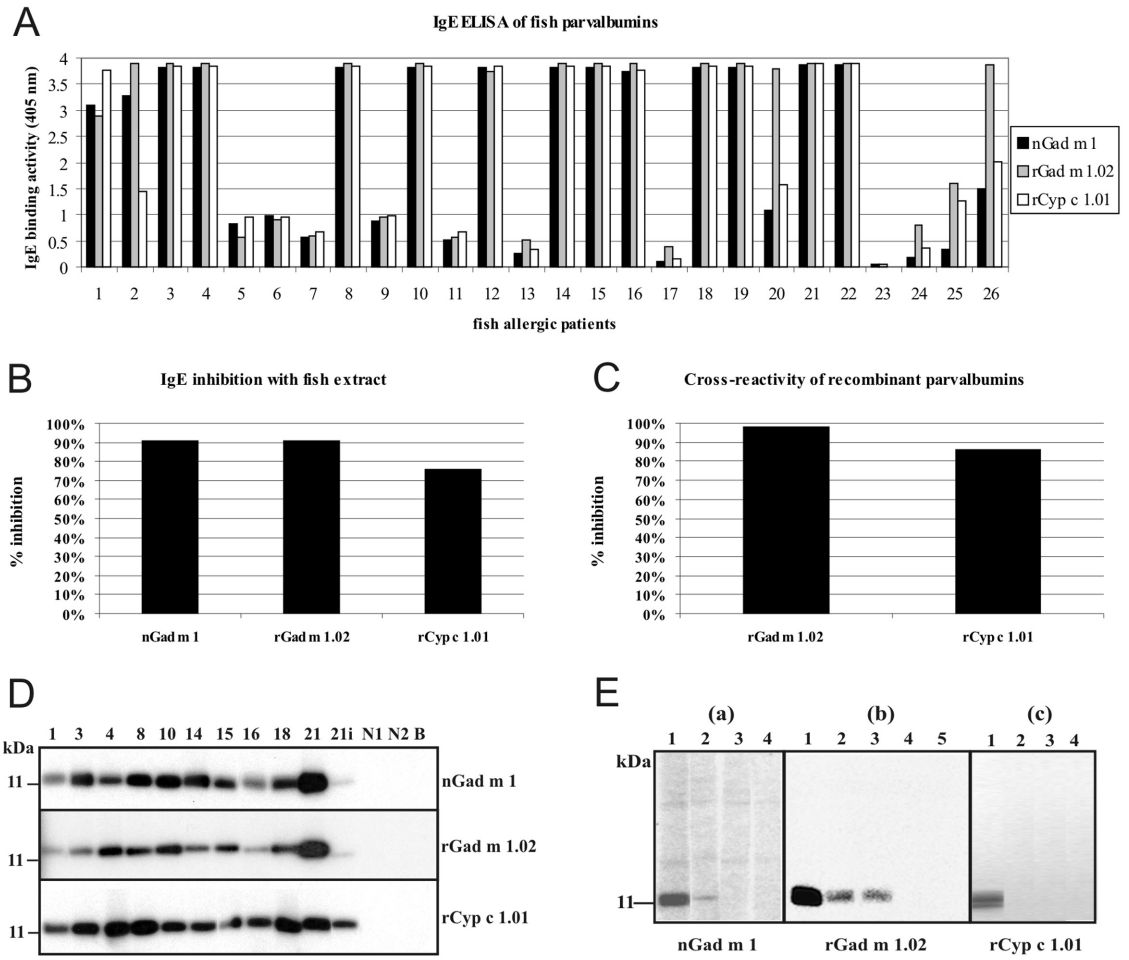

Figure 3. Immunological analysis of nGad m 1, rGad m 1.02 and rCyp c 1.01. (A) IgE binding to purified parvalbumins of sera from fish allergic patients from Spain, The Netherlands, and Greece. (B, C) IgE inhibition assays were performed using cod or carp extract or purified recombinant parvalbumin of cod or carp as inhibitors. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs. (B) Residual IgE binding to rGad m 1.02, nGad m 1, and rCyp c 1.01 after preincubation with cod and carp protein extract, respectively. (C) Crossinhibition assay: Residual IgE binding to rGad m 1.02 after preincubation with rCyp c 1.01, IgE binding to rCyp c 1.01 after preincubation with rGad m 1.02. D: IgE immunoblot and IgE inhibition analysis. 1–21, sera of fish allergic patients; 21i, serum was preincubated with cod extract for nGad m 1 (top) and rGad m 1.02 (middle), or with carp extract for rCyp c 1.01 (bottom). N1 and N2, healthy nonallergic individuals; B, buffer control. (E) Immunoblot inhibition of IgE binding to cod or carp extract by purified nGad m 1, rGad m 1.02 or rCyp c 1.01. nGad m 1: Lane 1: IgE binding to cod extract, lane 2: Immunoblot inhibition of IgE binding to cod extract by 50 μg/mL nGad m 1. rGad m 1.02: Lane 1: IgE binding to cod extract, lane 2: Immunoblot inhibition of IgE binding to cod extract by 100 μg/mL rGad m 1.02, and by 50 μg/mL rGad m 1.02 (lane 3). rCyp c 1.01: Lane 1: IgE binding to carp extract, lane 2: inhibition of IgE binding to carp extract by 50 μg/mL rCyp c 1.01. Lane 3 of (a) and (c) and lane 4 of (b): healthy nonallergic individuals. Lane 4 of (a) and (c) and lane 5 of (b): buffer controls.

sera and buffer used as controls were negative in all experiments.

3.5 NMR analysis

For both rCyp c 1.01 (Fig. 4A left) and nGad m 1 (Fig. 4A right) the amide region (7–9 ppm), the aromatic region (6–8 ppm), the H-α region (below and above 4.4 ppm), and the aliphatic region (0–5 ppm) were crowded with clearly separated, narrow peaks. Moreover, aromatic and amide protons showed shifts above 9 ppm and below 7 ppm, α protons showed shifts above 5 ppm. All these facts gave evidence of a complete folding of rCyp c 1.01 (Fig. 4A left). The same evaluation applied to the spectra of nGad m 1 with some difference (Fig. 4A right). The peaks were slightly broader for nGad m 1, and some part of the protein appeared not to have a rigid tertiary structure.

3.6 Mass determination

The identity of rCyp c 1.01 with the UniprotKB/TrEMBL entry Q8UUS3 for a parvalbumin of *Cyprinus carpio* could

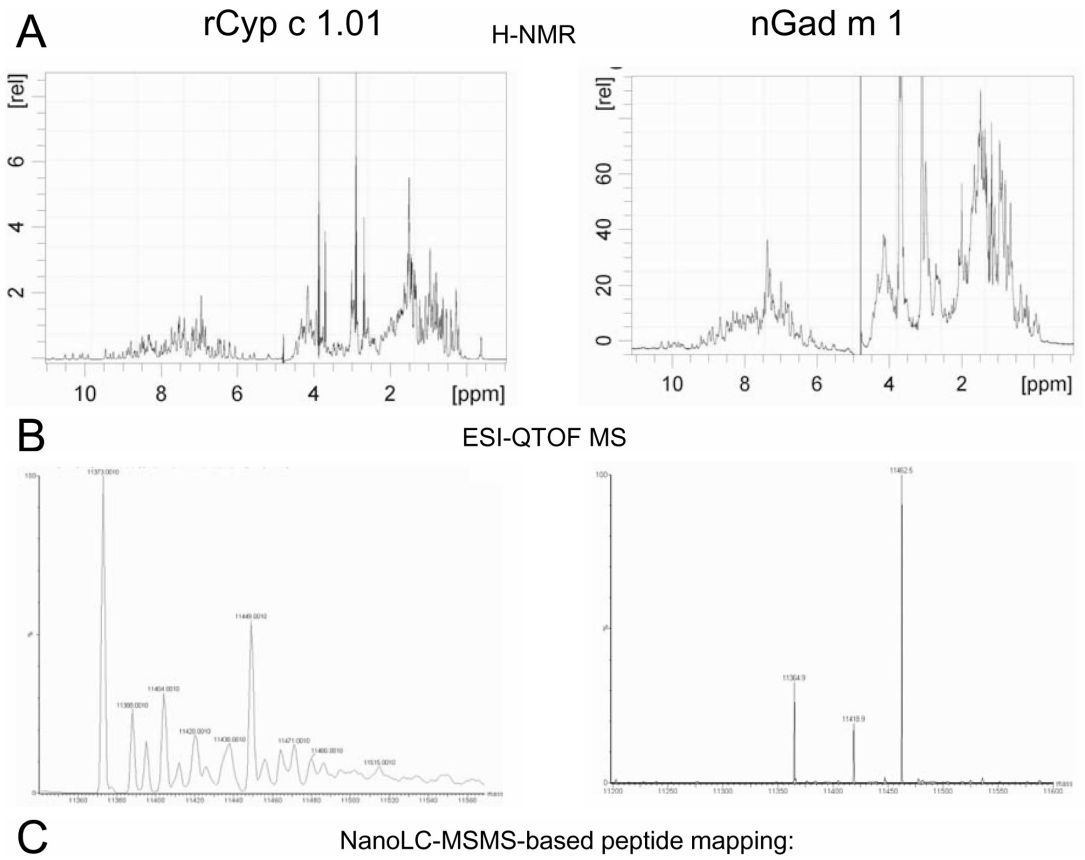

Figure 4. NMR and mass spectrum analysis. (A) ^{1}H-NMR analysis: left: ^{1}H 700 MHz spectrum of rCyp c 1.01, zgesgp experiment, 256 scans, 25°C. Right: ^{1}H 700 MHz spectrum of nGad m 1, zgesgp experiment, 1024 scans, 25°C. (B) MS analysis: rCyp c 1.01 (left) and nGad m 1 (right). (C) Results of nano-LC-MS/MS-based peptide mapping for rCyp c 1.01 and nGad m 1, black: sequence coverage of obtained peptides, gray: no coverage.

be shown by ESI-QTOF MS (Fig. 4B left). The determined intact mass of 11 373 ± 1 Da indicated cleavage of N-terminal methionine, however, a second less prominent peak at 11 449 ± 1 Da pointed at a partial addition of β-mercaptoethanol to the free sulfhydryl groups of the single cysteine residue in position 18 (Fig. 4B). By nano-LC-MS/MS-based peptide mapping a sequence coverage of 69.4% was reached (Fig. 4C).

Mass spectroscopic analysis of the purified natural nGad m 1 revealed the presence of three peaks at 11 462.5, 11 364.9, and 11 419 Da molecular mass (Fig. 4B right). Nano-LC-MS/MS-based peptide mapping resulted in sequence fragments of two different isoforms, which showed a sequence coverage of 90% as well as 88% to previously published parvalbumin sequences from *G. morhua* (Acc No: Q90YK9, Q90YL0, [23]) (Fig. 4C). In both protein sequences, the acetylated N-terminal alanine was included. The intact mass of the least intense peak (11 419 Da) was in good agreement with the theoretical mass of the already published Gad m 1 isoform Q90YK9 (11 420 Da) [23], whereas 11 462.5 Da corresponded to the form with the deacetylated N-terminus. The mass of 11 364.9 was assigned to the N-terminal acetylated Gad m 1 isoform Q90YL0 (11 323.8). The difference of 42 Da compared to the theoretical masses are due to modification by acetylation of the N-termini. Mass analysis showed a higher amount of the isoform corresponded to the previously published Q90YK9 [23].

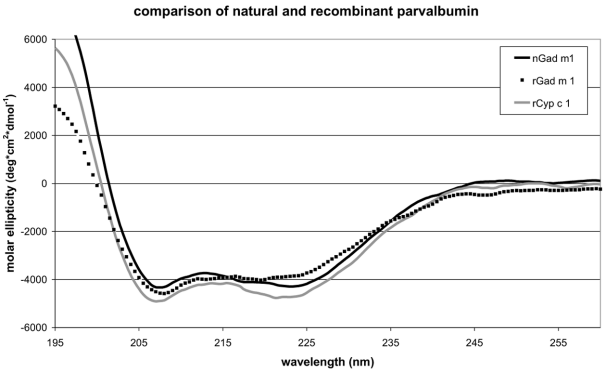

Figure 5. Comparison of Ca^{2+}-bound nGad m 1, rGad m 1.02 and rCyp c 1.01. CD spectra analysis revealed conformational differences between the natural and recombinant parvalbumins. Black: nGad m 1, black dotted: rGad m 1.02, gray: rCyp c 1.01.

3.7 CD and stability studies

The natural cod parvalbumin showed a far UV CD spectrum typical for α-helical proteins characterized by two broad minima at 208 and 222 nm at 20°C (Fig. 5), as previously described [29]. The recombinant carp parvalbumin was more similar to the natural than to the recombinant cod protein. The spectrum of recombinant cod parvalbumin showed a reduced minimum at 222 nm (Fig. 5).

CD spectra of nGad m 1 were recorded at pH 2.5, 7.0, and 8.5 (Fig. 6A). The spectra revealed conformational changes of the natural protein under acidic conditions. The minimum of around $-8000\ \mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$ measured by 208 nm drifted to $-10\,000\ \mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$. No change was caused under neutral or basic conditions. Thermal stability values were monitored at different pH values (Fig. 6B). The spectrum of nGad m 1 heated to 95°C at pH 7 showed an increase of negative dichroism. Whereas the heating of nGad m 1 to 95°C at pH 2.5 and 8.5 resulted in one broad minimum and changes in ellipticity at 208 nm to a higher negative dichroism (Fig. 6B). The spectra of unheated and heat-treated rCyp c 1.01 at pH 8.5 showed no shifts, similar to the ones of nGad m 1 (data not shown). In contrast to nGad m 1, rCyp c 1.01 changed its conformation to an irregular structure after heating at pH 2.5 (Fig. 7).

After Ca^{2+}-depletion by addition of 5.0 mM EGTA a decreased signal intensity was measured in the spectrum of rCyp c 1.01, as previously described [7]. In contrast, nGad m 1 displayed no change in the signal intensity, but a complete loss of the peak at 222 nm was monitored (Fig. 6C). Thermal stability of natural cod and recombinant carp parvalbumin after Ca^{2+}-depletion was observed after heating samples. Whereas, natural Gad m 1 treated with 5.0 mM EGTA showed a remarkable increased negative dichroism with a minimum at 208 nm after the heat treatment (Fig. 6C).

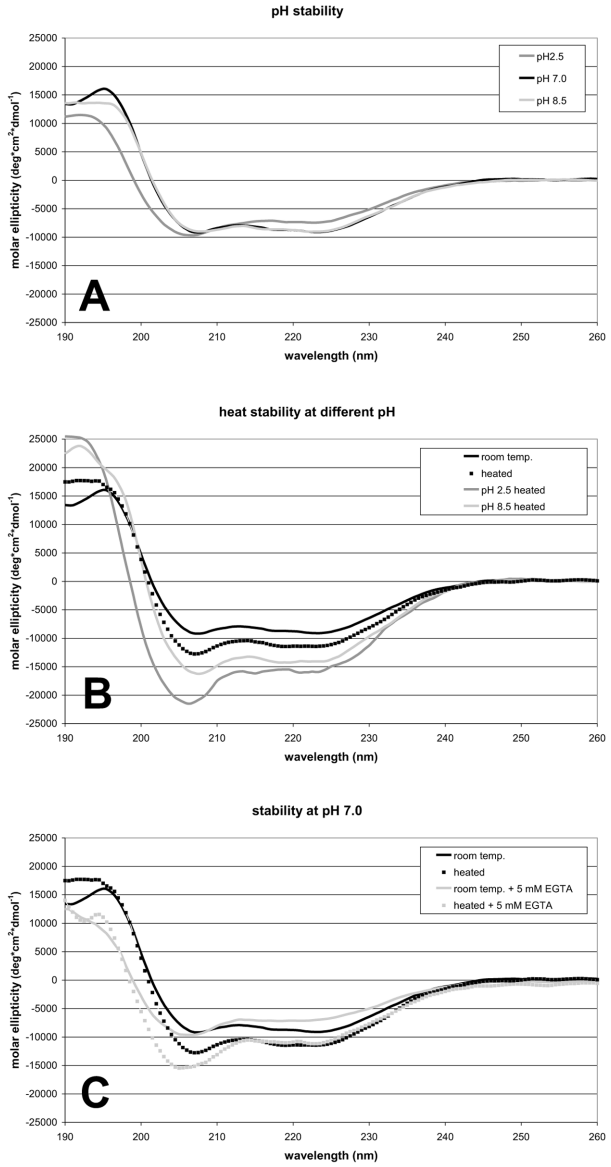

Figure 6. CD spectra of stability measurements of nGad m 1. (A) pH stability. No significant conformational changes at pH 7.0 and 8.5, but a complete loss of the minimum at 222 nm at pH 2.5. (B) heat stability. An increased dichroism and a loss of the minimum at 222 nm were monitored for each heated sample. (C) Ca^{2+}-depletion. Only one minimum at 208 nm was observed for the Ca^{2+}-depleted form. An increase of negative dichroism was revealed in the samples after heating.

4 Discussion

This work reports the comparison of natural and recombinant forms of parvalbumin of Atlantic cod and carp. Parvalbumins have previously been identified as major and cross-reactive allergens in various fish species [6, 30]. As the major cod allergen Gad m 1 is well studied and regarded as a representative for fish allergen, it is used for the develop-

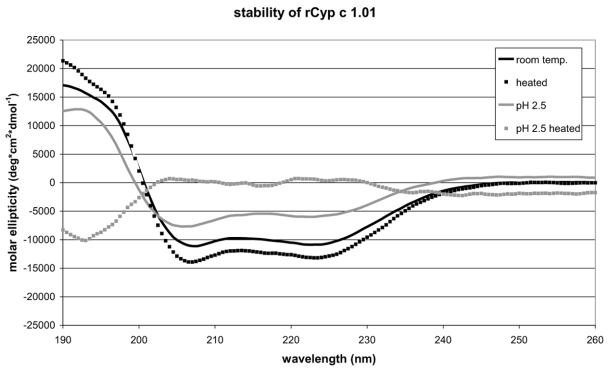

Figure 7. Stability of rCyp c 1.01. An increased negative dichroism after heating was monitored. Conformational change of the heated protein to an irregular structure at pH 2.5. Black line: room temperature; black dotted line: heated at 95°C; gray line: pH 2.5; gray dotted line: heated to 95°C at pH 2.5.

ment and validation of novel diagnostic tools [15, 31]. In our study, we established new purification protocols for natural and recombinant cod parvalbumin and recombinant carp parvalbumin. Cod muscle contains several IgE-reactive parvalbumin isoforms including at least two β-isoforms, which were purified and characterized by mass spectroscopy and NMR. In addition, we cloned two β-parvalbumins (rGad m 1.01 and rGad m 1.02) and produced rGad m 1.02 from cod.

IgE ELISA, IgE immunoblotting and inhibition experiments revealed a high crossreactivity between cod and carp parvalbumins (Fig. 3). Almost all sera of fish allergic patients (25/26) in this study, which had IgE reactivity to native and recombinant cod parvalbumin, also had IgE reactivity to recombinant carp parvalbumin (Fig. 3A). This result supports the assumption that cod and carp parvalbumins share at least some B-cell epitopes. The high amino acid sequence identities between the two parvalbumins of cod (71%) and between parvalbumins of cod and carp (80–81%) support this finding. The results of immunoblotting performed with the mouse monoclonal antifrog parvalbumin antibody Parv-19 and a rabbit polyclonal anti-Gad m 1 antiserum further illustrate the crossreactivity of parvalbumins (Fig. 2). The IgE binding capacity of rGad m 1.02 was comparable to nGad m 1. Interestingly, some serum samples (sera 2, 13, 17, 20, 24, 25, and 26) showed stronger IgE binding to rGad m 1.02 than to nGad m 1 (Fig. 3A). Hence, standardized batches of recombinant cod parvalbumin can replace nGad m 1 for diagnostic assays. The second band detected in immunoblot of nGad m 1 with the polyclonal anti-Gad m 1 antiserum (Fig. 2, nGad m 1, lane 4) represents a second isoform of cod parvalbumin according to the results from MS. An approximately 22 kDa band present in both lanes 4 and 5 (Fig. 2) of the rCyp c 1.01 blot could be a protein dimer according to the results of immunoblots with fish allergic patients' sera (data not shown).

CD analysis revealed that purified rGad m 1.02 and rCyp c 1.01 were present in solution as a folded protein with a predominantly α-helical secondary structure similar to that of the native cod parvalbumin, as was previously described for the recombinant carp parvalbumin rCyp c 1.01 [26]. However, we could observe a similarity between the CD spectra of rGad m 1.02 and the Ca^{2+}-depleted form of natural cod parvalbumin (Figs. 5 and 6C). In contrast to nGad m 1, which did not show conformational differences after adding Ca^{2+} (data not shown), we could detect a conformational change of rGad m 1.02 after dialysis against a Ca^{2+}-containing buffer (data not shown). These data indicate that the native parvalbumin contained bound calcium ions even after undergoing the purification procedure. This may not be the case with the protein produced in *E. coli*. The Ca^{2+} depletion experiments for carp parvalbumin described in the literature [7] were performed with the recombinant protein rCyp c 1.01. In contrast, our Ca^{2+} depletion experiments for cod parvalbumin were performed with the natural protein. For carp parvalbumin no comparison was made between the natural and the recombinant proteins regarding their stability depending on bound Ca^{2+}. The conformation of natural cod parvalbumin appears to be more resistant to calcium depletion than the conformation of recombinant carp parvalbumin. Recombinant Cyp c 1.01 seems to be stable at pH 7.0 even after heating to 95°C as previously described [7]. Additionally, we could observe an effect of pH 2.5 on conformation and the stability after heating the sample (Fig. 7). As the glutamate residues of the Ca^{2+}-binding site [32] are uncharged at pH 2.5, the binding of the chelate ion is hindered. The similarity of the spectra of EGTA treated rCyp c 1.01 and at pH 2.5, as well as the instability of rGad m 1.02 under basic conditions (data not shown) are most likely due to the reduced amount of bound Ca^{2+}. In contrast, natural cod parvalbumin was stable at each pH value tested and we could observe only a loss of the broad minimum at pH 2.5, and at pH 2.5 after heating (Figs. 6A and B). These observations illustrate that the binding of the chelate ion plays an important role for the conformation and for the stability of the recombinant proteins.

The mass data obtained for rCyp c 1.01 showed a second less prominent peak at 11 449 ± 1 Da (Fig. 4B left). This pointed at a partial addition of β-mercaptoethanol to the free sulfhydryl groups of the single cysteine residue of the protein in position 18. This addition in a minor component of the recombinant protein might be a likely leftover from the purification procedure involving trace amounts of the reducing agent. The mass data for nGad m 1, in addition to the least intense peak showed two additional peaks with masses of 11 462.5 and 11 364.9, respectively (Fig. 4B right). Noticeably, both masses showed a difference of 42 Da compared to the theoretical masses of isoform Q90YK9 (11 420.9) and isoform Q90YL0 (11 323.8). We therefore speculate that the majority of nGad m 1 molecules was post-translationally modified by acetylation. This

assumption was confirmed by MS/MS analysis of the peptides obtained by tryptic digest of nGad m 1 (Fig. 4C). When 92% of the Q90YK9 sequence could be confirmed, the acetylated N-terminal alanine was included.

Our data indicate that natural and recombinant parvalbumins can be used equally well for *in vitro* diagnostic assays to detect parvalbumin-specific IgE. Nevertheless, the various batches of recombinant proteins need to be characterized by several physico-chemical methods to assure their consistent quality. However, when the natural and recombinant allergens are compared in more detail, differences do emerge.

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The authors have declared no conflict of interest.

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