Purification, biochemical, and immunological characterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin

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Abstract

***Background*—Almost 4% of the population suffer from food allergy which is an adverse reaction to food with an underlying immunological mechanism.**

***Aims*—To characterise one of the most frequent IgE defined food allergens, fish parvalbumin.**

***Methods*—Tissue and subcellular distribution of carp parvalbumin was analysed by immunogold electron microscopy and cell fractionation. Parvalbumin was purified to homogeneity, analysed by mass spectrometry and circular dichroism (CD) spectroscopy, and its allergenic activity was analysed by IgE binding and basophil histamine release tests.**

***Results*—The isoelectric point (pI) 4.7 form of carp parvalbumin, a three EF-hand calcium-binding protein, was purified to homogeneity. CD analysis revealed a remarkable stability and refolding capacity of calcium-bound parvalbumin. This may explain why parvalbumin, despite cooking and exposure to the gastrointestinal tract, can sensitise patients. Purified parvalbumin reacted with IgE of more than 95% of individuals allergic to fish, induced dose-dependent basophil histamine release and contained, on average, 83% of the IgE epitopes present in other fish species. Calcium depletion reduced the IgE binding capacity of parvalbumin which, according to CD analysis, may be due to conformation-dependent IgE recognition.**

***Conclusions*—Purified carp parvalbumin represents an important cross reactive food allergen. It can be used for in vitro and in vivo diagnosis of fish-induced food allergy. Our finding that the apo-form of parvalbumin had a greatly reduced IgE binding capacity indicates that this form may be a candidate for safe immunotherapy of fish-related food allergy.**

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Keywords: food allergy; parvalbumin; circular dichroism; epitopes; antibodies; immunochemistry

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While food intolerance can be caused by immunological, pharmacological, toxic, infectious, idiosyncratic, metabolic, and neuropsychological processes, food allergy is an adverse reaction to food or food additives with an underlying immunological mechanism.[1] IgE-mediated food allergy occurs in 1.5–6% of the population[2] with a higher prevalence in children less than three years of age compared with adults.[3 4] A study of 375 consecutive adult patients attending a gastroenterological outpatient clinic found that food allergy was a causative factor in 3.2% of patients suffering from inflammatory and functional gastrointestinal disease, as confirmed by endoscopic allergen provocation or elimination diet, or both.[5] Support for the importance of gastrointestinal symptoms in food allergy comes from a study reporting that 9.1% of a population of 383 patients with food allergy exhibited isolated gastrointestinal manifestations.[6]

Milk products, wheat, peanuts, eggs, and fish represent the most important sources for food allergens.[3 6] Parvalbumins from various fish species have been described as major food allergens which are recognised by IgE antibodies in more than 95% of patients allergic to fish.[7–14] In sensitised individuals who produce IgE antibodies against parvalbumin, contact with and consumption of fish and fish-related products can lead to severe health problems, ranging from urticaria and dermatitis to angioedema, diarrhoea, asthma and, at worst, systemic anaphylactic reactions and death.[9–11]

Parvalbumins represent a subfamily of closely related three EF-hand calcium-binding proteins.[15–19] They were first identified as small, acidic (10–12 kDa, isoelectric point (pI) 3.9–5.5) calcium-buffer proteins in fast muscle of lower and higher vertebrates.[20–26] Only two of the three helix-loop, helix EF-hand motifs of parvalbumin are functional and can chelate Ca^{2+} and Mg^{2+} while the first domain provides a cap that covers the hydrophobic surface of the pair of domains.[23 24] Based on amino acid sequence data, the parvalbumin family can be further subdivided into two evolutionarily distinct lineages: α type parvalbumins (109 amino acids) and β type parvalbumins (108 amino acids), which also includes oncomodulin.[27] Binding of Ca^{2+} and Mg^{2+} are known to induce conformational rearrangements in the parvalbumin structure[28] but to date no protein

Abbreviations used in this paper: CD, circular dichroism; pI, isoelectric point; RAST, radioallergosorbent test; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

ligands have been identified for the exposed domains. This is the reason why parvalbumins are termed Ca^{2+} buffer proteins as opposed to Ca^{2+} sensor proteins (e.g. calmodulin) which, on Ca^{2+} binding, interact with a variety of other proteins.[19] Parvalbumins were thought to be directly involved in the relaxation process in fast twitch muscle of lower and higher vertebrates as calcium buffers.[29] However, using immunohistochemistry, parvalbumin was also found in a variety of other tissues, including the central nervous system, where it was localised close to GABAergic neurones[30] and endocrine glands (pituitary, thyroid, adrenals, ovaries, testes).[31]

Evaluation of IgE-mediated reactions to foods depends on a careful clinical history and defined allergen preparations for in vitro and in vivo diagnosis.[32] Diagnosis of fish allergy is based on anamnesis, measurement of allergen-specific IgE antibodies and demonstration that allergen exposure induces clinical symptoms[33] (e.g. double-blind, placebo-controlled food challenge, colonoscopic allergen provocation[34]). Prevention of food allergy requires a detailed knowledge of the presence of food allergens in various sources and tissues. While allergen-specific immunotherapy is well established for the treatment of most allergic manifestations (rhinoconjunctivitis, asthma), it is rarely used for the treatment of IgE-mediated food allergy. This is because food allergen extracts are difficult to prepare and standardise.

In this study, we analysed tissue-specific expression and subcellular localisation of the major fish allergen carp (*Cyprinus carpio*) parvalbumin, as well as its occurrence as IgE reactive isoforms. The pI 4.7 form was purified to homogeneity, tested for IgE binding capacity, cross reactivity with parvalbumins from other fish species, and its ability to induce histamine release from basophils from a patient allergic to fish. The IgE binding capacity of the calcium-bound and apo-forms of parvalbumin pI 4.7 was investigated. Differences in conformation, thermal stability, and refolding capacity of the calcium-bound and apo-forms of the protein were analysed by CD spectroscopy. We discuss the conformation-dependent IgE recognition of parvalbumin as a paradigm for a conformational antigen-antibody interaction and propose the use of purified carp parvalbumin for the diagnosis and immunotherapy of fish-induced food allergy.

Materials and methods

SERA AND ANTIBODIES

Sera from 13 patients allergic to fish (10 adults, three children) with a positive case history of type I allergy to fish were selected. All patients were allergic to more than one fish species and experienced at least one of the typical clinical symptoms (dermatitis, urticaria, angioedema, diarrhoea, asthma, anaphylactic reaction) after contact with fish proteins. Diagnosis of IgE-mediated fish allergy was verified by determination of fish-specific IgE antibodies using the Pharmacia CAP-FEIA System (Pharmacia, Uppsala, Sweden). Serum from a healthy individual with a negative case history for any type I allergy and negative radioallergosorbent tests (RASTs) to fish allergens was included as a control. A monoclonal antibody directed against carp parvalbumin was purchased from Sigma (clone PA-235, Sigma, St Louis, Missouri, USA).[35]

IMMUNOGOLD ELECTRON MICROSCOPY

Small tissue blocks of carp (*Cyprinus carpio*) muscle and liver were fixed in 4% paraformylaldehyde and embedded in Lowicryl K4M (Plano, Wetzler, Germany) at −35°C. Ultrathin sections were cut and labelled with mouse monoclonal antibodies directed against parvalbumin (1:12 000 dilution of clone PA-235, Sigma) and goat anti-mouse IgG coupled to 10 nm colloidal gold particles (Plano, Wetzlar, Germany). An isotype matched mouse monoclonal antibody with no specificity for parvalbumin was used as a control (MAb 414, 1:10 000; BAbCo, Richmond, California, USA). Immunogold-labelled sections were stained with uranyl acetate and lead citrate, and analysed in a Hitachi H500 transmission electron microscope operated at 75 kV. Photographs were taken using Agfa-Gevaert 23D56 films.

ASSESSMENT OF SUBCELLULAR PARVALBUMIN DISTRIBUTION

Carp muscle was separated into subcellular fractions as described previously.[36] Briefly, 15 g of carp muscle were homogenised in saccharose solution I (sucrose 0.32 M, Tris-HCl 10 mM pH 7.4, $MgCl_2$ 5 mM, 2-mercaptoethanol 2 mM, and PMSF 1 mM) and filtered through gauze to remove cellular debris. Crude nuclei were pelleted at 800 *g* for 20 minutes at +4°C. Supernatant containing cytoplasm and organelles was centrifuged again at 8690 *g* for 15 minutes to remove mitochondria and then again at 110 000 *g* for 130 minutes to remove microsomes. Supernatant obtained after the last centrifugation step was termed the cytoplasmic fraction. Crude nuclei were further purified by dissolving the pellet in saccharose solution II (sucrose 2.4 M, Tris-HCl 10 mM pH 7.4, $MgCl_2$ 5 mM, 2-mercaptoethanol 2 mM, and PMSF 1 mM) and centrifuged at 50 000 *g* for 60 minutes at +4°C. Pelleted nuclei were dissolved in saccharose solution III (sucrose 0.25 M, Tris-HCl 10 mM, pH 7.4, $MgCl_2$ 5 mM, 2-mercaptoethanol 2 mM, and PMSF 1 mM) and centrifuged at 1000 *g* for 10 minutes at +4°C. Nuclei were then dissolved in PBS containing PMSF 1 mM and stored in aliquots at −20°C. The protein content of all extracts was estimated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (Coomassie Brilliant Blue R-250, Bio Rad Laboratories, Richmond, California, USA). Protein concentrations were determined using a Lowry assay (Bio-Rad DC Protein Assay, Bio Rad).

PURIFICATION OF CARP PARVALBUMIN

Raw fish muscle from carp (1 g) was homogenised under liquid nitrogen, dissolved in 8 ml of phosphate-buffered saline (PBS) containing

PMSF 1 mM and extracted overnight at +4°C at constant stirring. Total carp muscle extract (fraction E) was boiled for 30 minutes and precipitated proteins were removed by centrifugation at 4500 rpm for 10 minutes at +4°C (Hettich Rotanta TRC, Hettich, Tuttingen, Germany). Parvalbumin was enriched from supernatant (fraction A) by ammonsulphate precipitation of contaminating proteins. Ammonsulphate was removed by dialysis to water and the parvalbumin-enriched fraction B was lyophilised, redissolved in Tris 10 mM, pH 7.5, and applied to a DEAE Sepharose column (DEAE Sepharose Fast Flow column, Pharmacia Biotech, Uppsala, Sweden). Fractions containing purified parvalbumin isoforms were eluted with a linear salt gradient of Tris 10 mM and NaCl 0.5 M, pH 7.5.

SDS-PAGE ANALYSIS, ISOELECTRIC FOCUSING, IMMUNOBLOT ANALYSES

Proteins were separated by 12% SDS-PAGE, as described by Laemmli.[37]

IEF analysis was performed using agarose gels with a pH gradient 3–7 (FMC BioProducts, Rockland, Maine, USA) and a standard pI marker (pH range 3.6–10.2; FMC BioProducts) using a Multiphor II Electrophoresis System (Pharmacia Biotech).

Proteins separated by SDS-PAGE or IEF were blotted onto nitrocellulose membranes (Nitrocell, Pharmacia Biotech) or polyvinylidene difluoride membranes (Immobilon PVDF, Millipore, Bedford, Massachusetts, USA), as described by Towbin *et al*.[38] Nitrocellulose strips and PVDF membranes containing blotted proteins were blocked at room temperature in buffer C (PBS, pH 7.5, containing 0.5% v/v Tween 20). Strips were then probed overnight at +4°C with sera, diluted 1:10 in buffer C or with a 1:1000 diluted monoclonal anti-parvalbumin antibody (clone PA-235, Sigma Bio Sciences). Bound IgE antibodies were detected with ^{125}I-labelled anti-human IgE antibodies (Pharmacia) whereas bound mouse monoclonal antibodies were detected with ^{125}I-labelled sheep anti-mouse antiserum (Amersham) and visualised by autoradiography.

MALDI-TOF (MATRIX-ASSISTED LASER DESORPTION AND IONISATION-TIME OF FLIGHT) ANALYSIS OF PURIFIED PARVALBUMIN

Mass spectroscopic analysis of purified parvalbumin pI 4.7 was performed in a linear mode with a time of flight Compact MALDI II instrument (Kratos, Manchester, UK), operating at 20 kV acceleration voltage and equipped with a nitrogen UV laser (337 nm, pulse duration 3 ns; piCHEM, Research and Development, Graz, Austria). The m/z values were calibrated externally. Samples were dissolved in 10% acetonitrile (0.1% TFA). Alpha-cyano-4 hydroxy-cinnamic acid was used as a matrix dissolved in 60% acetonitrile (0.1% TFA). For sample preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air dried.

CIRCULAR DICHROISM (CD) ANALYSIS OF PURIFIED PARVALBUMIN

CD spectra of purified carp parvalbumin pI 4.7 were recorded on a Jasco J-710 spectropolarimeter fitted with a Jasco PTC-348WI Peltier type temperature control system and interfaced with a Fisons HAAKE GH water bath. The instrument was calibrated with a 0.1% aqueous solution of d-10-camphor-sulphonic acid. Results are expressed as mean residue ellipticity (Θ) at a given wavelength. Far ultraviolet CD spectra were recorded at +20°C in a 2 mm quartz cuvette (Hellma, Mullheim, Baden, Germany) at variable protein concentrations in the range 5–15 μM. Spectra were recorded with 0.1 nm resolution and resulted from 10 averaging scans. The final spectra were corrected by subtracting the corresponding baseline spectrum obtained under identical conditions. All measurements were performed in MilliQ water, pH 7.2.

Ca^2+-MODULATION OF IgE BINDING

The effects of depletion of parvalbumin-bound Ca^{2+} on IgE reactivity were investigated, as described previously.[14 39] Purified carp parvalbumin pI 4.7 was separated by SDS-PAGE (5 μg/cm gel) and transferred onto nitrocellulose membranes. Nitrocellulose strips containing equal amounts of blotted parvalbumin were exposed to sera from four patients allergic to fish and a monoclonal anti-parvalbumin antibody in the presence of $CaCl_2$ 0.5 mM or EGTA 5 mM, pH 7.5. Bound antibodies were detected as described for immunoblot analyses. Modulation of IgE binding to parvalbumin was also quantified by gamma counting (Wizzard, Automatic Gamma Counter, Wallac, Uppsala, Sweden) of the nitrocellulose membranes according to the following formula:

$$\%\ \text{inhibition} = \frac{\text{cpm}_{Ca^{2+}} - \text{cpm}_{\text{EGTA}}}{\text{cpm}_{Ca^{2+}}} \times 100$$

where $\text{cpm}_{Ca^{2+}}$ and cpm_{EGTA} indicate IgE binding to the calcium-bound and apo-forms, respectively.

QUANTITATIVE IgE ABSORPTION EXPERIMENTS

Sera from 11 individuals allergic to fish were preincubated with 5 μg of purified carp parvalbumin pI 4.7 or, for control purposes, 5 μg of an immunologically unrelated allergen (recombinant birch, *Betula verrucosa*, pollen allergen Bet v 1)[40] overnight at +4°C. Remaining serum IgE reactivity to cod, tuna and salmon extracts was measured using the CAP-FEIA System (Pharmacia). Percentage inhibition of IgE binding to fish extracts after preabsorption with purified carp parvalbumin pI 4.7 was calculated as follows:

$$\text{inhibition}\ (\%) = \frac{\text{cpm}_{\text{rBetv1}} - \text{cpm}_{\text{pI4.7}}}{\text{cpm}_{\text{rBetv1}}} \times 100$$

HISTAMINE RELEASE EXPERIMENTS

Granulocytes were isolated from heparinised blood samples from an individual allergic to fish containing parvalbumin-reactive IgE

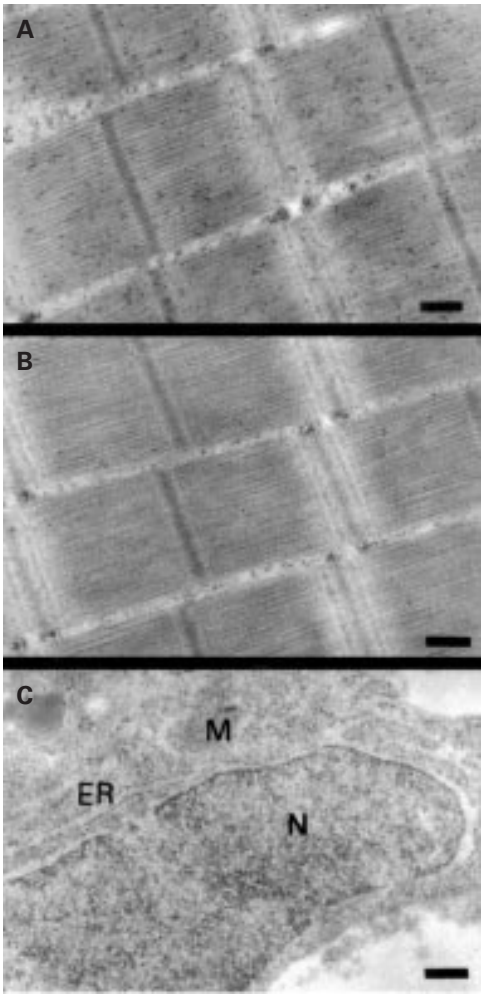

Figure 1 Subcellular localisation of parvalbumin in carp muscle and liver by immunogold electron microscopy. Bright field micrograph of ultrathin sections of carp muscle labelled with a monoclonal antibody against parvalbumin (A) and with an isotype matched control antibody without specificity for parvalbumin (B). (C) A section from carp liver labelled with the monoclonal antibody against parvalbumin. ER, endoplasmic reticulum, M, mitochondria, and N, nucleus. Magnification in all micrographs is 58 000×. The bars correspond to 0.25 μm. Black dots represent bound gold particles.

antibodies and, for control purposes, from a non-atopic individual by dextran sedimentation.[41] Washed cells were incubated with increasing concentrations (0.0001, 0.001, 0.01, 0.1, and 1.0 μg/ml) of purified carp parvalbumin pI 4.7 and with a monoclonal anti-human IgE antibody (E124.2.8 Dε2, Immunotech, Marseille, France). Total histamine released into the supernatant was measured after freeze-thawing of cells. Results are given as mean values of triplicate determinations expressed as percentage of total histamine.

Results

THE MAJOR FISH ALLERGEN, CARP PARVALBUMIN, REPRESENTS AN ABUNDANT CYTOSOLIC MUSCLE PROTEIN

Anti-carp parvalbumin immunoreactivity was detected in ultrathin sections of carp muscle fibres and fibre cells (fig 1A) but not in carp liver sections (fig 1C) by immunogold electron microscopy. However, no parvalbumin immunoreactivity was found in connective tissue (collagen fibres and cells) (data not shown). An isotype-matched mouse monoclonal antibody without specificity for carp proteins failed to stain ultrathin carp muscle sections (fig 1B). Subcellular localisation of parvalbumin was studied by exposure of total carp muscle extract, in addition to cytosolic and nuclear muscle extract fractions, to serum IgE from patients allergic to fish and to a mouse monoclonal anti-carp parvalbumin antibody. While SDS-PAGE and Coomassie blue staining showed that all three extracts (total, cytosolic, and nuclear) contained comparable amounts of fish protein, anti-parvalbumin immunoreactivity was detected only in the total and cytosolic fractions and not in the nuclear fraction (fig 2A). Sera from three individuals allergic to fish (Nos 1, 2, and 3) but not from the non-atopic individual (N) bound to parvalbumin at 12 kDa in total and cytosolic extracts (fig 2B). Successful separation of the nuclear fraction was confirmed by reactivity of an anti-histone antibody with this fraction and lack of reactivity with the cytosolic fraction (fig 2A). Serum IgE from patients allergic to fish and a parvalbumin-specific monoclonal antibody thus detected parvalbumin as an abundant cytosolic carp muscle protein.

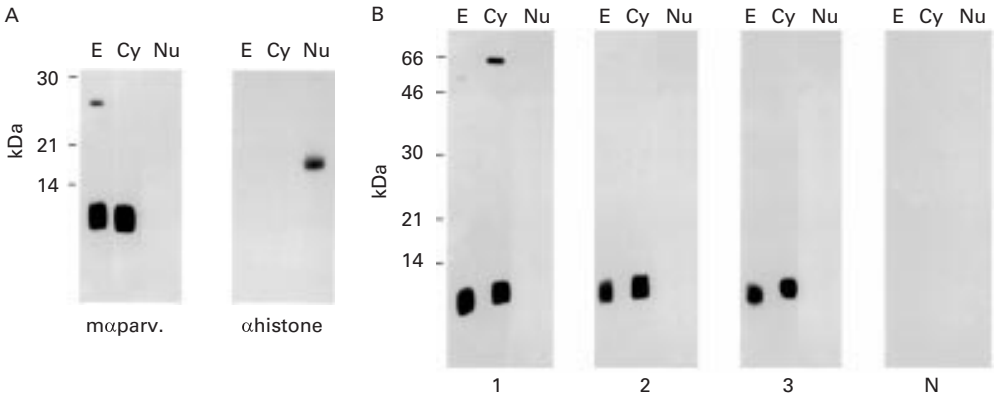

Figure 2 Detection of parvalbumin in subcellular carp muscle fractions. (A) Detection of parvalbumin (maparv.) and histone (ahistone) immunoreactivity in total (E), cytoplasmic (Cy), and nuclear (Nu) western-blotted carp muscle extracts with monoclonal antibodies. (B) Incubation of nitrocellulose strips containing total (E), cytoplasmic (Cy) and nuclear (Nu) carp muscle extracts with sera from three patients allergic to fish (Nos 1, 2, and 3) and from a non-atopic individual (N).

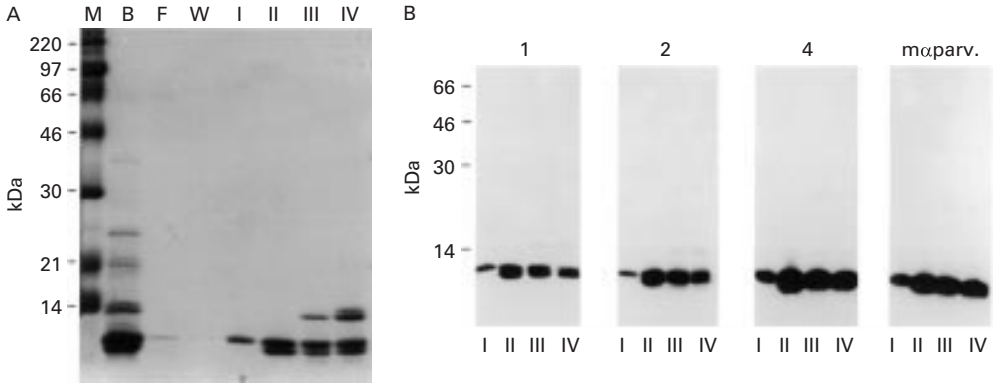

Figure 3 Purification of carp parvalbumin. (A) Purification of carp parvalbumin isoforms by DEAE chromatography monitored by SDS-PAGE and Coomassie blue staining. Parvalbumin-enriched soluble carp muscle protein fraction obtained by ammonsulphate precipitation (lane B) was applied to a DEAE column. Lanes F and W represent the flow through and column wash fractions, respectively. Lanes I–IV contain aliquots of parvalbumin fractions eluted from the DEAE column. (B) Nitrocellulose-blotted parvalbumin isoform-containing fractions I–IV were exposed to sera from three patients allergic to fish (Nos 1, 2, and 4) and to a mouse monoclonal anti-parvalbumin antibody (maparv.).

PURIFICATION OF THE IgE-REACTIVE CARP PARVALBUMIN pI 4.7 ISOFORM

Carp muscle parvalbumin was purified to homogeneity by several purification steps. Parvalbumin isoforms were enriched in the soluble fraction of an aqueous carp muscle extract by precipitation of heat-sensitive proteins and subsequent addition of ammonsulphate to a concentration of 60% w/v. The parvalbumin-enriched fraction thus obtained was applied to a DEAE column. The eluate fractions of the DEAE column (I–IV) contained one purified parvalbumin isoform (fig 3A, lane I), a mixture of two parvalbumin isoforms (fig 3A, lane II), a parvalbumin isoform mixture containing one additional 13 kDa moiety (fig 3A, lane III) and a mixture of the latter proteins and another 14–15 kDa moiety (fig 3A, lane IV). Serum IgE from patients allergic to fish and a monoclonal anti-parvalbumin antibody bound to the purified carp parvalbumin isoform of fraction I and to the parvalbumin mixtures in fractions II–IV but not to the 13 kDa and 14–15 kDa bands in fractions III and IV, respectively (fig 3B).

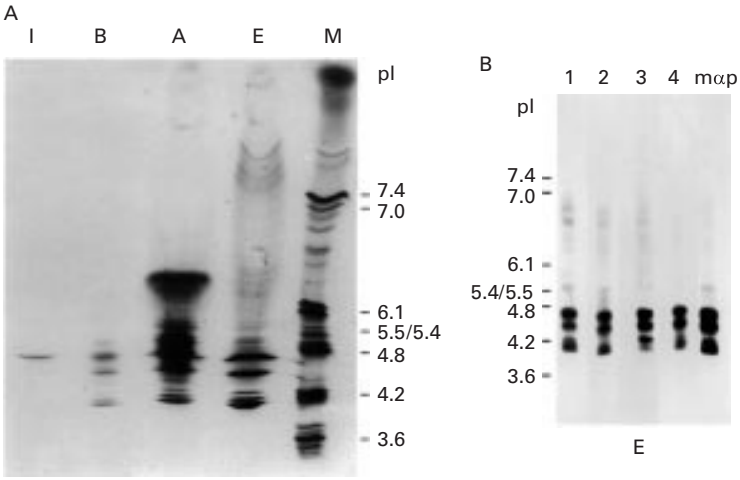

Figure 4 Characterisation of pI isoforms of carp parvalbumin by IEF. (A) Total carp muscle extract (lane E), soluble fraction after extract boiling (lane A) and after ammonsulphate precipitation (lane B), and a single purified carp parvalbumin isoform present in fraction I eluted from the DEAE column were separated by IEF in an agarose gel (pH 3–7) and stained with Coomassie blue. Lane M represents a standard pI marker (pI range 3.6–10.2). (B) Detection of pI isoforms of carp parvalbumin on membrane-blotted, IEF-separated carp muscle extract (E) with serum IgE from four patients allergic to fish (Nos 1, 2, 3, and 4) and with a mouse monoclonal anti-parvalbumin antibody (map).

Analysis of total carp muscle extract (E), the parvalbumin-enriched fraction obtained after boiling (A) and ammonsulphate precipitation (B), and the DEAE eluent (I) after IEF revealed the presence of several proteins in the acidic pH range (pI 3.9–4.7) (fig 4A). The DEAE eluent of fraction I contained pure carp parvalbumin pI 4.7. Exposure of membranes containing IEF-separated carp muscle extract to serum IgE from patients allergic to fish and a monoclonal anti-parvalbumin antibody revealed the presence of at least four antibody-reactive carp parvalbumin isoforms of pI 4.7, 4.5, 4.2, and 3.9 (fig 4B).

Mass spectroscopic analysis of the purified IgE-reactive carp parvalbumin pI 4.7 form and myoglobin (internal standard) revealed the presence of two peaks at 5740 Da and 11 487 Da molecular mass, corresponding to the M2H+ and MH+ forms of parvalbumin; the two peaks of 8476 Da and 16 951 Da represent the M2H+ and MH+ forms of myoglobin (fig 5). The molecular mass determined for purified IgE-reactive carp parvalbumin pI 4.7 is in agreement with its migration in SDS-PAGE and corresponds to the molecular weight deduced from the amino acid sequence of carp parvalbumin (11 489 kDa) determined by Coffee and Bradshaw.[22]

PURIFIED CARP PARVALBUMIN pI 4.7 ISOFORM CONTAINS MOST IgE EPITOPES PRESENT IN NATURAL FISH EXTRACTS

For quantitative estimation of IgE epitopes shared by purified carp parvalbumin pI 4.7 and natural extracts from cod fish, tuna, and salmon, immunoabsorption experiments were performed. Sera from 11 patients allergic to fish were preabsorbed with the purified pI 4.7 isoform and, for control purposes, with recombinant Bet v 1, the major pollen allergen, which does not share IgE epitopes with fish allergens. Table 1 shows the percentage inhibition of IgE binding to the various fish extracts after preabsorption of sera with purified carp parvalbumin pI 4.7. Mean inhibition of IgE binding of 81% to cod fish, 89% to tuna, and 78% to salmon extract was observed. The results of the immunoabsorption experiments showed that puri-

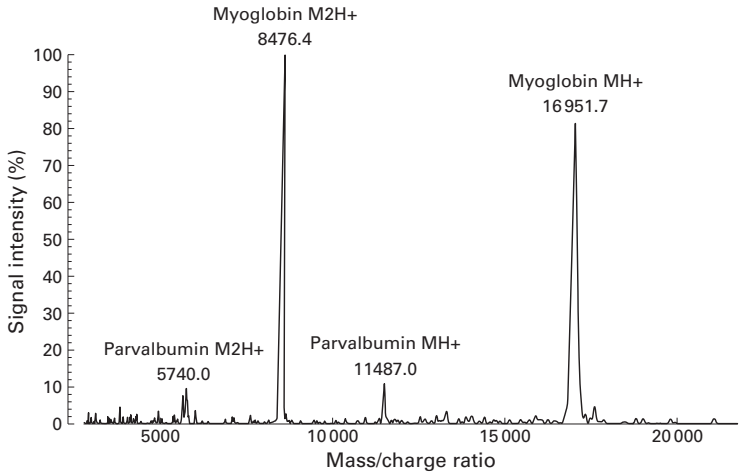

Figure 5 Mass spectroscopic analysis of purified carp parvalbumin pI 4.7. The x axis shows the mass/charge ratio; signal intensity is displayed on the y axis as a percentage related to the most intensive signal obtained in the investigated mass range.

Table 1 Inhibition of IgE binding to fish extracts after preabsorption of sera with purified carp parvalbumin pI 4.7. Sera from 11 patients allergic to fish were preabsorbed with purified carp parvalbumin pI 4.7. Percentage inhibition of IgE binding to extracts from cod fish, tuna, and salmon is displayed

Serum	*Cod*	*Tuna*	*Salmon*
1	88.6	86.0	77.4
3	67.8	78.2	38.4
5	55.0	60.2	55.0
6	91.0	93.8	89.1
7	91.0	93.0	93.0
8	80.0	95.1	80.8
9	78.6	90.6	78.4
10	89.9	97.3	86.0
11	89.3	98.3	88.6
12	85.7	96.5	83.8
13	78.3	86.4	84.2

fied carp parvalbumin pI 4.7 isoform contains most of the IgE epitopes which are present in natural fish extracts.

PURIFIED CARP PARVALBUMIN pI 4.7 INDUCES BASOPHIL HISTAMINE RELEASE

To investigate if IgE recognition of carp parvalbumin can lead to specific effector cell activation and release of biologically relevant mediators, granulocytes were isolated from a patient allergic to fish and from a non-atopic individual. Purified carp parvalbumin pI 4.7 induced dose dependent histamine release from basophils from the individual allergic to fish (fig 6A) but not from the non-atopic subject (fig 6B). Significant histamine release was observed at concentrations of purified pI 4.7 isoform of 0.001–0.01 μg/ml (fig 6A). Anti-human IgE antibodies induced basophil degranulation in the individual allergic to fish and in the non-atopic subject (fig 6A, B).

CALCIUM-DEPENDENT IgE RECOGNITION OF PURIFIED CARP PARVALBUMIN pI 4.7

IgE recognition of EF-hand pollen allergens can be modulated by the presence or absence of protein-bound calcium.[39] Using the purified carp parvalbumin pI 4.7 isoform, we demonstrated that IgE antibodies from patients allergic to fish bound preferentially to the calcium-bound form of parvalbumin. Three of four individuals allergic to fish (Nos 1, 2, and 4) showed greatly reduced IgE binding capacity to the calcium-free (apo-) form of parvalbumin (fig 7). Gamma counting demonstrated that the reductions in IgE binding of sera from subjects 1, 2, and 4 to the calcium-free parvalbumin form were 59%, 51%, and 79%, respectively. A less pronounced reduction in antibody binding was found for serum IgE of patient No 3 (27%) and for the mouse monoclonal anti-parvalbumin antibody (18%) (fig 7).

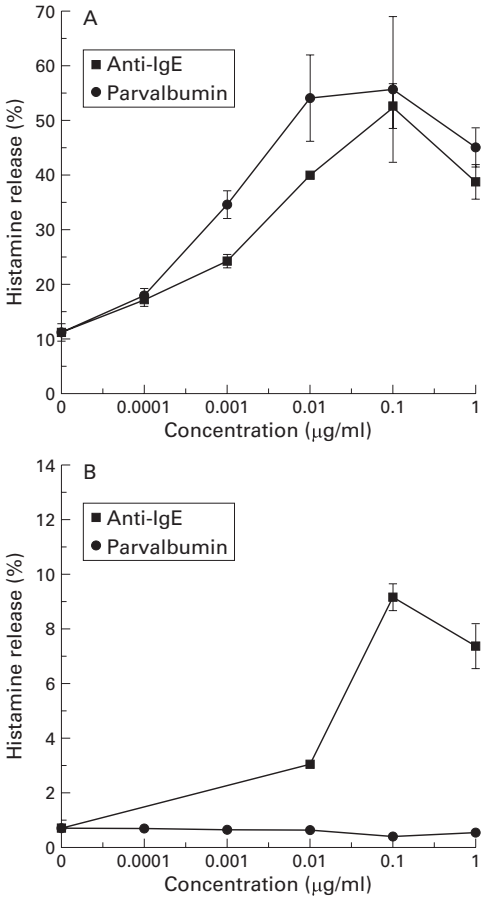

Figure 6 Induction of basophil histamine release with purified carp parvalbumin pI 4.7. Basophils from a patient allergic to fish (A) and from a non-atopic individual (B), were incubated with various concentrations of purified carp parvalbumin pI 4.7 and a monoclonal anti-human IgE antibody. The percentage of histamine release is shown.

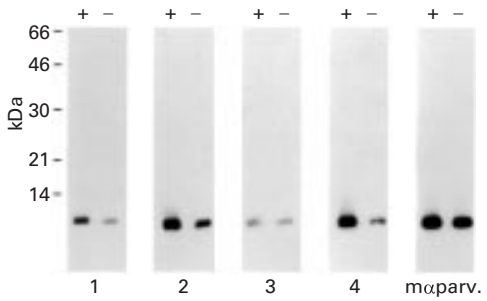

Figure 7 Calcium-dependent IgE recognition of purified carp parvalbumin pI 4.7. Nitrocellulose-blotted carp parvalbumin pI 4.7 was exposed to serum IgE from four patients allergic to fish (Nos 1, 2, 3, and 4) and to a mouse monoclonal anti-parvalbumin antibody (mαparv.) in the presence (+) and absence (−) of calcium.

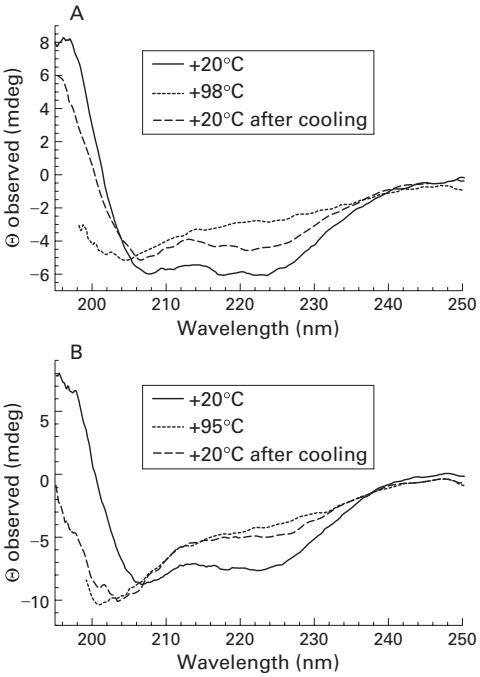

Figure 8 Far-ultraviolet circular dichroism analysis of purified carp parvalbumin pI 4.7. (A) Calcium-bound parvalbumin pI 4.7 at +20°C, +98°C and +20°C after cooling from +98°C. (B) Carp parvalbumin pI 4.7 in the presence of EGTA 5 mM at +20°C, +95°C and +20°C after cooling from +95°C. Spectra are expressed as observed ellipticity (Θ) at a given wave length.

CIRCULAR DICHROISM ANALYSIS OF CARP PARVALBUMIN pI 4.7

The far-ultraviolet CD spectrum of the purified calcium-bound carp parvalbumin pI 4.7 isoform recorded at +20°C was characterised by two broad minima at 208 nm and 222 nm (fig 8A). Comparing the spectra of the calcium-bound (fig 8A) and EGTA-treated pI 4.7 isoforms (fig 8B), significant differences in the shape of the spectra were noted. This, together with the decreased signal intensity observed for the apo-form, may indicate a decrease in the alpha helical content or a transition into a molten globule state, or both, as reported for other parvalbumins.[42 43] Thermal unfolding of parvalbumin was monitored as change in ellipticity at 222 nm in the absence and presence of EGTA 5 mM (data not shown). The unfolding transition of parvalbumin was monophasic and cooperative with a melting point of about +82°C for the calcium-bound form and 30°C for the apo-form (data not shown). At +98°C, the calcium-bound protein was found to be present mostly in a random coil conformation with a minimum at about 204 nm (fig 8A). For apo-parvalbumin, we observed a random coil conformation with a typical minimum at about 200 nm at +60°C (data not shown). Reversibility of folding was observed for calcium-bound parvalbumin, evident from the far-ultraviolet spectrum at +20°C after cooling from +98°C (fig 8A) and from the cooling profile monitored at 222 nm (data not shown). Refolding on cooling for the EGTA-treated protein did not occur (fig 8B). In conclusion, thermal stability of parvalbumin depends dramatically on the presence of protein-bound calcium ions.

Discussion

Parvalbumins belong to a family of Ca^{2+} buffer proteins which are thought to be involved in the relaxation process in muscle tissue.[29] While the physiological roles of parvalbumin are not completely established, fish parvalbumins represent major allergens for individuals who suffer from IgE-mediated hypersensitivity to fish products.[7–13] IgE antibodies of more than 95% of patients allergic to fish cross react with parvalbumins from a variety of fish species.[10 12]

In our study, we purified carp parvalbumin, a major food allergen, to homogeneity. Carp parvalbumin represents an abundant cytosolic muscle protein. As reported for parvalbumins from various species,[26] the IgE-reactive carp parvalbumin showed remarkable thermal stability. Boiling of carp muscle extract precipitated most other carp muscle proteins while IgE-reactive parvalbumin remained in the soluble fraction. Similarly, CD analysis revealed remarkable thermal stability of calcium-bound IgE-reactive carp parvalbumin. Both findings may explain why individuals allergic to fish experienced allergic symptoms after ingestion of cooked fish products while drying and canning were found to reduce the IgE binding capacity and the allergenic activity of fish extracts.[44] However, cooking parvalbumin leaves sufficient amounts of intact IgE-reactive allergens available that can be absorbed, recognised by IgE antibodies and that finally reach tissue-bound effector cells of the allergic reaction.

Carp muscle contained several IgE-reactive parvalbumin isoforms of which the pI 4.7 protein form was purified to homogeneity, as determined by mass spectroscopy and IEF. Preincubation of sera from patients allergic to fish with the purified pI 4.7 form of carp parvalbumin strongly inhibited IgE binding to the other isoforms (data not shown) and to extracts of cod fish, tuna, and salmon. The fact that the purified pI 4.7 isoform absorbed, on average, 83% of fish-specific IgE antibodies, indicates that it may replace crude fish extracts for diagnosis and perhaps therapy of fish allergy.

Diagnosis of fish allergy is currently hampered by the fact that the presence of specific IgE antibodies does not always correlate with clinical symptoms.[13 45] In contrast with serological determination of specific IgE antibodies, the basophil histamine release assay measures the IgE-dependent effector cell reaction and thus closely reflects the patient's symptoms. In fact, good agreement between results obtained from basophil histamine release and provocation testing was also reported when food extracts were used for testing.[46] However, in the case of fish allergy, biological tests (food challenge, skin testing, colonoscopic allergen provocation, basophil histamine release) are sometimes difficult to perform because crude fish extracts contain mediators which can cause false positive test results.[47] We showed that the purified carp parvalbumin pI 4.7 isoform

induced specific histamine release from basophils and thus suggest that the purified protein could be used in a cellular in vitro test for the diagnosis of fish allergy.

We also propose the use of purified carp parvalbumin for specific immunotherapy of fish allergy as it contains most of the relevant IgE epitopes present in fish extracts. Allergen-specific immunotherapy is performed by continuous injection of increasing allergen doses to yield non-responsiveness in the patient.[48] It should ideally be performed with purified allergens that are selected according to the patient's reactivity profile[49] and considerable effort is currently being spent on generation of hypoallergenic allergen variants which can be administered in higher doses with a reduced risk of anaphylactic side effects to patients (reviewed by Valenta and colleagues[50]). Our finding that most patients showed much stronger IgE binding to the calcium-bound form of parvalbumin suggests that they recognise epitopes which are more readily available on the calcium-bound form of parvalbumin. The latter could be explained by calcium-induced changes in the protein conformation which renders amino acids available that are buried in the apo-form of the protein. This hypothesis is supported by CD analysis experiments performed with the apo- and calcium-bound IgE-reactive pI 4.7 carp parvalbumin isoforms indicating significant differences in their secondary structures, and by the differences reported for the secondary structures of calcium-bound and apo-forms of type III cod parvalbumin being due to a conformational change or a transition to a molten globule state, or both.[42 43] The fact that the apo-form of carp parvalbumin had a greatly reduced IgE binding capacity indicates that it may be possible to engineer stable hypoallergenic variants of parvalbumin either by site-directed mutagenesis of the calcium-binding sites,[51] by mutational introduction of cysteines which creates a disulphide bond-linked apo-form[52] or by expression of parvalbumin fragments which, due to disruption of the protein conformation, lack their anaphylactic potential. Blocking IgG antibodies induced against the apo-form may perhaps also bind to the calcium-bound form, and thus protect the patient.

In conclusion, we purified a major IgE-reactive carp parvalbumin isoform which can be used for in vitro and in vivo diagnosis of fish allergy. Our finding that carp parvalbumin contains calcium-dependent conformational IgE epitopes may lead to the construction of genetically modified hypoallergenic parvalbumin variants which may be used for specific immunotherapy of fish allergy.

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