



Research paper

Quantitative sandwich ELISA for the determination of fish in foods

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Abstract

Allergy to fish represents one of the most prevalent causes for severe food-allergic reactions. Therefore, food authorities in different countries have implemented mandatory labeling of fish in pre-packed foods. Detection of fish proteins in food has previously been based on the use of patient serum. In the present study, a novel sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitation of fish in food matrixes has been developed and validated, using a polyclonal rabbit anti-cod parvalbumin antibody for capture and a biotinylated conjugate of the same antibody for detection. By employing the ubiquitous muscle protein parvalbumin as target the method succeeds to detect a variety of fish. However, the ELISA is specific for fish and does not cross-react with other species. Recoveries ranged from 68–138% in typical food matrixes, while the intra- and inter-assay precisions were <12% and <19%, respectively. The sensitivity of the cod parvalbumin ELISA with a limit of detection of 0.01 mg parvalbumin/kg food, about 5 mg fish/kg food, seems sufficient to detect fish protein traces in foods at levels low enough to minimize the risk for fish allergic consumers.

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Keywords: Cod parvalbumin; Fish; ELISA; Quantitative; Polyclonal antibody**1. Introduction**

Fish has always been an important source of dietary protein, especially in coastal regions. Recently, as transport by unbroken cold chain became routine and the demand for meager food increased, geographical restrictions are not longer an issue and fish is now consumed by a large part of the population. In parallel to this development, the number of persons with fish hypersensitivity has augmented, and allergy to fish is counted among the most common food allergies (Sampson, 2000). In industrialized countries, about 0.1–0.3% of the general population have fish allergy

whereas the prevalence in food allergic children and adults have been found to be at 2.3% to 30.4% depending on the study cohorts (Aas, 1966; de Martino et al., 1993). Threshold doses for eliciting immunologic reactions as a result of the ingestion of fish have been determined by double-blind placebo-controlled food challenge (DBPFC) in a range from 5 to 6000 mg fish (Taylor et al., 2002). Occupational exposure to fish allergens can likewise induce the typical immunoglobulin E (IgE)-mediated hypersensitive systemic, cutaneous, gastrointestinal, and respiratory reactions (Jeebhay, 2001; Sicherer et al., 2000). Fish allergy often is manifest already in small children and tends to be persistent, in contrast to some other food allergies with similar low ages of onset that are typically outgrown because of tolerance development (Eigenmann et al., 1998).

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The edible fish include more than 20000 species; however, the most commonly consumed belong to only a few orders of the ray-finned fish (*Actinopterygii*). Fish-sensitive patients are often allergic to multiple species of fish and are therefore advised to avoid consuming fish in general (Hansen et al., 1997). Clinical cross-reactivity was found to be about 50–70% (Helbling et al., 1999), and was strongest for taxonomically closely related species, due to a homologous major allergen (Bernhiesel-Broadbent et al., 1992). This protein has been identified as parvalbumin, an ubiquitous, well conserved, highly water-soluble sarcoplasmatic muscle protein of about 12 kDa with an isoelectric point (pI) of 4, containing in the tertiary structure three EF-hand motifs with two high-affinity calcium ion (Ca^{2+})-binding sites (Valenta et al., 1998). Parvalbumins are found at great amounts in the white muscles of low vertebrates (Wilwert et al., 2006) and in lower concentrations in the fast twitch skeletal muscles of higher vertebrates (Blum et al., 1977), but only the proteins from fish and frog, belonging phylogenetically to the β -lineage, are confirmed allergens (Elsayed, 2002; Hilger et al., 2004). Parvalbumins from fish are considered to be the major and sole allergens for 95 % of the patients suffering from IgE-mediated fish allergy (Swoboda et al., 2002).

The first fully characterized fish parvalbumin Gad c1, previously named Cod M, was isolated from Baltic cod, by then called *Gadus callarias* (Aas and Elsayed, 1975). It is very resistant to heating, extreme pH and denaturing agents, however, the allergenicity diminishes if bound Ca^{2+} is removed (Bugajska-Schretter et al., 1998). Therefore, the allergenic reactivity of fish is altered by the various food processing procedures like boiling, frying and canning in different ways (Chatterjee et al., 2006). In the mean time, parvalbumins from about 30 other fish species have been identified and molecularly characterized, e.g. Gad m1 (Atlantic cod, *Gadus morhua*), Sal s1 (salmon, *Salmo salar*), Cyp c1 (carp, *Cyprinus carpio*), and Ore m1 (tilapia, *Oreochromis mossambicus*) (Das Dores et al., 2002; Lindström et al., 1996; Bugajska-Schretter et al., 2000; Lee et al., 2006). In many species, e.g. zebrafish (*Danio rerio*), parvalbumin isoforms have been found (Friedberg, 2005).

The risk of severe anaphylactic reactions in highly sensitized individuals has induced food authorities in many countries to implement mandatory labeling of fish as an ingredient in pre-packed foods (European Union, 2003). Since avoidance of the offending food is the only cure available for food allergic consumers, great care and the prevention of cross-contaminations during the food manufacturing processes are required.

To support these efforts, analytical methods are needed (Poms et al., 2004). A number of techniques for the isolation and identification of parvalbumins by chromatography, isoelectric focusing, or mass spectrometry have been developed (Ross et al., 1998; Etienne et al., 2000; Carrera et al., 2006), and a radioimmunoassay using pooled human serum from patients with fish allergy was established for the determination of raw fish aeroallergens (Taylor et al., 2000). However, no quantitative method for the detection of fish is available so far.

Therefore, it was the aim of this study to develop a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for the determination of fish in foods.

2. Materials and methods

2.1. Purification of parvalbumin from Atlantic cod

Parvalbumin from Atlantic cod (*Gadus morhua*) was prepared by homogenizing muscle tissue from fresh fish in 0.1 M Tris(hydroxymethyl)aminomethane (Tris)/0.5 M glycine buffer pH 8.7/1 mM dithioerythriol (DTE) (Sigma-Aldrich) extraction buffer (w/v 1:2) with a rod homogenizer (Braun Vario, Kronberg, Germany). After extracting overnight at 45 °C in a shaking water bath (OLS 200, Grant, Cambridge, UK) and centrifugation for 15 min at 4 °C with 18,000 $\times g$ (J2-MC, Beckman Instruments, Palo Alto, CA), the supernatant was transferred to a glass beaker and heated almost to the cooking point, avoiding foam formation, for 30 min on a heatable magnetic stirrer (Gerhardt, Bonn, Germany). The supernatant of the subsequent centrifugation (10 min, 4 °C, 4000 $\times g$) was precipitated with 55% ammonium sulphate (Merck, Whitehouse Station, NJ), previously pounded in a mortar, at room temperature (RT) under continuous stirring. After further centrifugation (25 min, 4 °C, 4000 $\times g$), the supernatant was precipitated for a second time with ammonium sulphate, reaching a final concentration of 90%. The pellet obtained by a final centrifugation step (25 min, 4 °C, 9000 $\times g$) was resuspended in 50 mM Tris pH 8.0/1 mM DTE and separated by size exclusion chromatography on a 10 ml Sephadex G-50 (Sigma-Aldrich) gel filtration column which had been equilibrated with the same buffer. The column was eluted by gravitational flow, and 5 ml fractions were collected and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini Protean 3 cell, Bio-Rad, Hercules, CA) at 200 V for 40 min in 25 mM Tris/192 mM glycine buffer pH 8.3 under reducing conditions, using 12 % gels and ProSieve 10–190 kDa protein marker (Cambrex,

Karlskoga, Sweden). Fractions containing a single protein band at 12 kDa were joined together, extensively dialyzed against water in a dialysis tube with 6–8 kDa μ m pore-size (Spectra/Por, Spectrum Medical Industries, Los Angeles, CA), and freeze-dried (Heto, Allerød, Denmark). The protein was resuspended in phosphate-buffered saline (PBS) pH 7.4. The total protein content was determined with the Lowry Protein Assay (BioRad Laboratories, Hercules, CA), and adjusted to 2 mg/ml.

2.2. Production, purification and labelling of anti-cod parvalbumin antibody

A polyclonal antiserum was raised against the purified cod parvalbumin by injecting a chinchilla rabbit subcutaneously with 1.5 ml of a diluted (1:1 Freund's complete adjuvant) 200 μ g/ml protein solution. After a second immunization (1.5 ml, 1:1 Freund's incomplete adjuvant, week 4), and a third dose (50 μ l, week 5), the rabbit was finally bled in week 10, and anti-parvalbumin serum obtained by centrifugation. The crude serum was desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden), diluted 1:5 in 20 mM sodium phosphate buffer pH 7.0 (binding buffer), and then passed through a HiTrap r-Protein A FF-column (Amersham Biosciences) which had previously been washed with 0.1 M sodium citrate buffer pH 4.0 (elution buffer) and re-equilibrated with binding buffer. After washing with binding buffer, the bound IgG-antibodies were eluted with elution buffer, collected in 0.5 ml fractions, neutralized with 1 M Tris-base pH 8.5, and stored at 4 °C. Fractions were tested for binding activity using indirect ELISA, and total protein was determined by the Lowry method. The purity of the IgG was tested by SDS-PAGE, and pure antibody fractions were pooled. After a buffer exchange to 0.1 M sodium phosphate/0.15 M NaCl/0.1% sodium azide pH 7.4, the antibody was finally stored in aliquots at –80 °C until use.

For covalent conjugation with biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sigma-Aldrich), the buffer was changed to 0.05 M carbonate-bicarbonate buffer pH 9 and the antibody concentration adjusted to 2.8 mg/ml. The biotin salt was dissolved to 1 mg/ml in deionized water, and blended with the antibody solution at a ratio of 1 to 3.6. After vortexing, the reaction mixture was rotated at RT for 4 h, neutralized with 1 M NH_4Cl under rotation at RT for 10 min. After a further buffer change to 0.1 M sodium phosphate/0.15 M NaCl/0.1% sodium azide pH 7.4, the total protein concentration was measured and the biotinylated anti-cod parvalbumin antibody was stored at –20 °C until use.

2.3. Protein extraction and sample preparation

Fish and food samples for specificity testing were homogenized in a mechanical blender (Retsch GmbH & Co, Haan, Germany) and extracted (w/v 1:5) with Tris/glycine buffer pH 8.7 as described above. The total protein content was measured by Lowry method. Samples were stored in aliquots at –20 °C, and thawed only once.

2.4. Western Blotting

The NuPAGE Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS-PAGE, in accordance to the manufacturer's instructions. Protein extracts were diluted to 1 mg/ml, and prepared with lithium dodecyl sulphate (LDS) sample buffer and dithiothreitol (DDT) reducing agent (all from Invitrogen) for gel analysis. 1.5 μ g protein of each sample were loaded, and separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino) ethane sulfonic acid (MES) SDS running buffer, using 4–12% Bis-Tris gels and SeeBluePlus2 pre-stained reference standard. The proteins were either stained with SimplyBlue™ Safe Stain (Invitrogen) or electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad) for 60 min at 30 V with transfer buffer in an XCell II Blot Module (Invitrogen).

Tris-buffered saline (TBS) containing 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 pH 7.6 was used as washing buffer, and after addition of 3% BSA as blocking and incubation buffer for the Western blots. After blocking for 30–60 min, the blot was incubated under gentle shaking overnight at 4 °C with purified anti-parvalbumin antibody diluted 1:1000. All further steps were carried out at RT. The blot was washed (3 $\times$ 15 min) and incubated for 1 h with 1:5000 diluted HRP-conjugated goat anti-rabbit antibody (Zymed, San Francisco, CA). After a final washing step, the membrane was developed with Tetramethyl Benzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (5 min). All washing and incubation steps were performed at RT under gentle shaking.

2.5. Sandwich ELISA procedure

96-well flat-bottom polystyrene microtiter plates (Corning Inc., Corning, NY) were coated overnight at 4 °C with 100 μ L/well of purified polyclonal anti-cod parvalbumin antibody diluted to 5 μ g/mL in 0.05 M

bicarbonate buffer pH 9 (Sigma-Aldrich). The plates were washed three times with PBS/0.05 % Tween 20 pH 7.6 washing buffer using a programmable automatic plate washer (Skan 800, Skatron Instruments, Lier, Norway). All consecutive washing steps performed in the cod parvalbumin ELISA were the same. The wells were blocked with 200 μ L/well 1% BSA/ PBS pH 7.3 assay buffer (PBS: Oxoid, Basingstoke, UK) for 1 h at RT. The assay buffer was used for all dilution steps in the ELISA, and plates were sealed with plate-sealing tape (Greiner, Frickenhausen, Germany) during incubations which were performed for 1 h at RT under gentle shaking (Shuttler MTS 4I, IKA-Werke GmbH, Staufen, Germany). The blocked plate was washed, and 100 μ L/well of a 2-fold serial dilution of the purified cod parvalbumin standard were added in a concentration range of 500 to 0.49 ng/mL. Incubation of standards, assay buffer blanks, and sample extracts, diluted at minimum 1:20, were performed in triplicate (100 μ L/well). After washing, bound parvalbumins were

detected by incubation with 100 μ L/well biotinylated anti-cod parvalbumin antibody diluted 1:50,000. After another wash, 100 μ L/well of a horseradish peroxidase (HRP)-streptavidin conjugate (Zymed), diluted 1:5000, were added and incubated. A final wash was applied, and 75 μ L/well K-Blue TMB-substrate (Neogen, Lexington, KY) were added and incubated for 10 min at RT. The reaction was stopped by the addition of 50 μ L/well 2 M H_2SO_4 . Absorbance was read at 450 nm on a 1420 Victor² multilable plate counter (Wallac, Turku, Finland).

2.6. Assay validation: cross-reactivity between fish species, specificity, standard curve, limit of detection, limit of quantitation, precision, accuracy, robustness

The validation of the parvalbumin Sandwich ELISA followed generally the recommendations in the “Harmonized guidelines for single-laboratory validation of methods of analysis” (Thompson et al., 2002).

Table 1
Cross-reactivity of different fish species in the cod parvalbumin ELISA

	Fish species	Scientific name	Total protein [mg/mL] ^a	Protein recovered in ELISA [%]
1	Cod	<i>Gardus morhua</i>	2.0	100
2	Saithe	<i>Pollachius virens</i>	1.2	100
3	Mozambique tilapia	<i>Oreochromis mossambicus</i>	7.7	100
4	Gilthead seabream	<i>Sparus aurata</i>	5.0	98
5	Herring	<i>Clupea harengus</i>	3.0	71
6	Pollack	<i>Pollachius pollachius</i>	4.8	54
7	Wels catfish	<i>Silurus glanis</i>	7.1	53
8	Salmon	<i>Salmo salar</i>	6.5	50
9	Carp	<i>Cyprinus carpio</i>	15.8	48
10	Mackerel	<i>Scomber scombrus</i>	4.3	41
11	Yellowtail kingfish	<i>Seriola lalandi lalandi</i>	6.0	36
12	Sea trout	<i>Salmo trutta</i>	2.7	27
13	Flounder	<i>Platichthys flesus</i>	3.0	23
14	Bluefin tuna	<i>Thunnus thynnus</i>	4.6	5.1
15	Seawolf	<i>Anarhichas lupus</i>	1.0	4.8
16	Rainbow trout	<i>Onocorhynchus mykiss</i>	5.3	2.8
17	Halibut	<i>Hippoglossus hippoglossus</i>	3.0	1.5
18	Zander	<i>Sander lucioperca</i>	10.7	1.3
19	Zebra fish	<i>Danio rerio</i>	10.4	1.3
20	European John Dory	<i>Zeus faber</i>	5.1	1.1
21	Nile perch	<i>Lates niloticus</i>	10.7	0.79
22	European eel	<i>Anguilla anguilla</i>	2.9	0.31
23	Monkfish	<i>Lophius piscatorius</i>	3.3	0.29
24	Swordfish	<i>Xiphias gladius</i>	3.0	0.21
25	Redfish	<i>Sebastes viviparus</i>	2.1	0.16
26	Sturgeon	<i>Acipenser sturio</i>	8.7	0.15
27	Yellowfin tuna	<i>Thunnus albacares</i>	5.5	0.11
28	Blue marlin	<i>Makaira nigricans</i>	4.5	0.10
29	Red drum	<i>Sciaenops ocellatus</i>	3.0	0.03
30	Spiny dogfish	<i>Squalus acanthias</i>	10.8	0.03
31	Northern pike	<i>Esox lucius</i>	6.1	0.03
32	Anchovy	<i>Engraulis encrasicolus</i>	14.3	0.02

^a After extraction of 2 g fish muscle with 10 ml buffer.

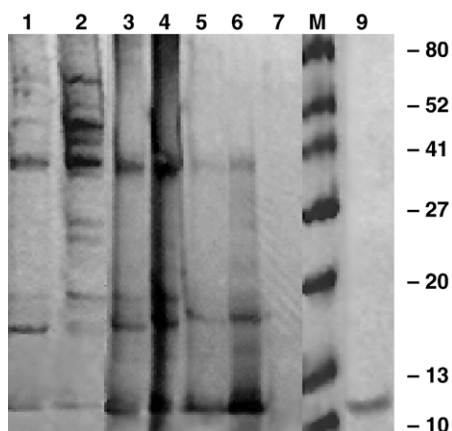


Fig. 1. Coomassie-stained SDS-PAGE of fractions from the cod parvalbumin purification. Lane 1: crude cod extract; lane 2: precipitate obtained by cooking; lane 3: supernatant of cooking precipitate; lane 4: precipitate by 55% $(\text{NH}_4)_2\text{SO}_4$; lane 5: supernatant of 55% $(\text{NH}_4)_2\text{SO}_4$ precipitation; lane 6: precipitate by 90% $(\text{NH}_4)_2\text{SO}_4$; lane 7: supernatant of 90% $(\text{NH}_4)_2\text{SO}_4$ precipitation; M: molecular weight marker, protein sizes (kDa) are indicated on the right side of the gel; lane 9: purified cod parvalbumin (after gel filtration of the 90% $(\text{NH}_4)_2\text{SO}_4$ precipitate).

The cross-reactivity of the cod parvalbumin ELISA with other fish species was assessed with a considerable number of fish extracts (Table 1). The experiment included salt- and freshwater ray-finned fishes from different temperature zones, and one cartilaginous fish (spiny dogfish). The concentrations measured were compared to the total protein content of the extracts, and retrieval rates were calculated.

The specificity of the method was tested by determining the recoveries for typical food ingredients in the cod parvalbumin ELISA, with focus on other seafoods and mammals. Extracts from shrimp (*Pandalus borealis*),

prawn (*Crangon crangon*), lobster (*Homarus gammarus*), spiny lobster (*Palinurus elephas*), edible crab (*Cancer pagurus*), European squid (*Loligo vulgaris*), blue mussel (*Mytilus edulis*), soft-shell clam (*Mya arenaria*), common scallop (*Pecten maximus*), edible frog (*Rana esculenta*), German cockroach (*Blattella germanica*), chicken (*Gallus domesticus*), pig (*Sus scrofa domestica*), cow (*Bos taurus*), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), peanut (*Arachis hypogaea*), lentil (*Lens culinaris*), soy (*Glycine max*), chick-pea (*Cicer arietinum*), common pea (*Pisum sativum*), lupin (*Lupinus albus*), milk casein and hen's egg ovomucoid were prepared as described before from food bought at local stores. The extracts were diluted 1:20 and measured according to the assay procedure, and recoveries of equivalents cod parvalbumin were calculated.

The limit of detection (LOD) for the cod parvalbumin ELISA was calculated from 34 experiments as the mean of the measured content of triplicate buffer blank samples plus three times the standard deviation (SD) of the mean value. The limit of quantification (LOQ) was calculated as the buffer blank mean value plus 10 times the standard deviation (SD) of the mean value in 34 experiments. Additionally, the LODs in five complex food matrices, i.e. bread, soy sauce, white sauce, mushroom soup, and wine, were determined in the same way on the basis of 8 experiments with matrix-assisted standard curves.

Precision was estimated with four control samples containing different amounts of fish protein, a white soup spiked with the parvalbumin standard (10 mg/kg), a fish soup (containing naturally about 150 mg/kg), soy sauce spiked with cod muscle (2000 mg/kg), and mushroom soup spiked with cod muscle (4000 mg/kg). The samples were extracted, stored in aliquots at -20°C , and each analysis was performed with freshly thawed extract. The

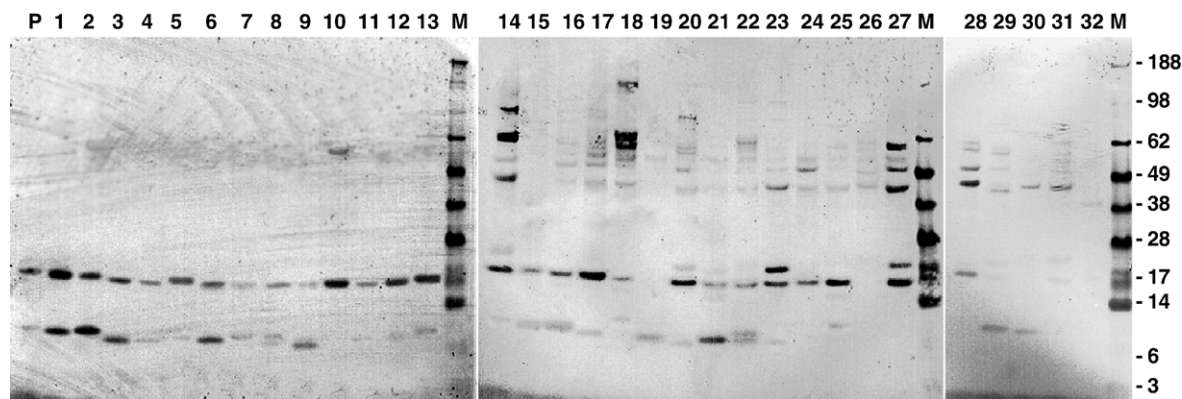


Fig. 2. Western blot analysis of extracts from various fish species with the purified polyclonal anti-cod parvalbumin antibody. P: purified cod parvalbumin; lane 1–32: fish species extracts in the same order as shown in Table 1; M: molecular weight marker, protein sizes (kDa) are indicated on the right side of the gel.

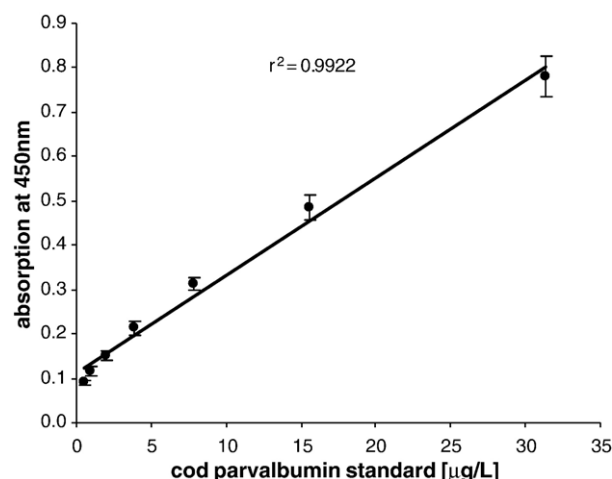


Fig. 3. Representative seven-point linear calibration curve, based on the standard curve obtained using the cod parvalbumin standard in the cod parvalbumin ELISA. The graph shows the average values of duplicates in four independent experiments, the standard deviations, and the linear regression coefficient r^2 .

intra-assay precision was determined as the mean coefficient of variation (CV) of 12 analyses in one assay, and the inter-assay precision was determined as the mean CV of triplicate analyses on 8 days.

The accuracy of the cod parvalbumin ELISA was evaluated by performing recovery studies using five different blank matrices. In ten independent experiments, bread, soy sauce, white sauce, mushroom soup, and wine were homogenised and spiked with the parvalbumin standard at levels of 0, 1, 10 and 100 mg/kg sample by adding 1 ml of suitably diluted standard solution to 2 g of the respective food. After equilibration for 15 min at RT, additional 9 ml extraction buffer were added, and the extraction was performed as described previously. The samples were analysed in triplicate, and the mean values for the recoveries and the standard errors of the means were calculated.

The robustness of the cod parvalbumin ELISA was tested applying small variations in incubation times or temperatures to the assay procedure. The stability of purified cod parvalbumin and fish extracts to repeated thawing and freezing was evaluated. Finally, the relevance of Ca^{2+} ions in assay buffer and samples was determined by performing duplicate analysis with concentrations of 6.5 μM CaCl_2 , 5 mM CaCl_2 (Fluka, Steinheim, Germany), or 5 mM ethyleneglycol-bis(2-aminoethylether)N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich) and comparing the results for standard curve and recovery from the fish soup reference sample with these from the unaltered cod parvalbumin ELISA.

3. Results

3.1. Cod parvalbumin purification

Crude Atlantic cod extract contained a number of distinct protein bands in the range from 12–75 kDa as found by SDS Page gel electrophoresis (Fig. 1). Two proteins at about 17 kDa and 39 kDa seemed to occur in high concentrations and were still present after the cooking and precipitation steps whereas all other proteins had been removed. However, the proteins could be separated by gel filtration, and relatively pure parvalbumin with a molecular weight of about 12 kDa was recovered. This was later confirmed by Maldi-ToF mass spectrometry and amino acid comparison with the cod parvalbumin sequence (Gad m1) published in the SwissProt/TrEmbl® database (PRVB_GADMO, Q90YK9) (data not shown).

3.2. Characterization of the anti-cod parvalbumin antibody

The binding characteristics of the purified polyclonal anti-cod parvalbumin antibody were determined on Western blot, using raw extracts of 32 fish species, including fresh- and saltwater fishes (Fig. 2). Parvalbumin-like proteins of about 12–14 kDa were detected in

Table 2
Specificity of the cod parvalbumin ELISA

Food/ingredient ^a	Recovery cod parvalbumin (%)
Shrimp	<0.00002
Prawn	<0.00002
Lobster	<0.00002
Spiny lobster	<0.00002
Edible crab	<0.00002
European squid	0.00006
Blue mussel	<0.00002
Soft-shell clam	<0.00002
Common scallop	<0.00002
German cockroach	<0.00002
Edible frog	<0.00002
Chicken	<0.00002
Pig	<0.00002
Cow	<0.00002
Hazelnut	<0.00002
Walnut	<0.00002
Peanut	<0.00002
Lentil	<0.00002
Soy	<0.00002
Chick-pea	<0.00002
Common pea	<0.00002
Lupin	<0.00002
Milk casein	<0.00002
Ovomucoid	<0.00002

^a After extraction of 2 g food with 10 ml buffer.

Table 3

Intra- and inter-assay variances (%CV) determined for the cod parvalbumin ELISA

Food	Fish protein [mg/kg]	Intra-assay variance [%CV] <i>n</i> =12	Inter-assay variance [%CV] <i>n</i> =8
White sauce	10 ^a	12	18
Fish soup	150 ^b	8	19
Soy sauce	2000 ^c	5	13
Mushroom soup	4000 ^c	4	11

^a Spiked with cod parvalbumin standard.

^b Naturally containing fish protein.

^c Spiked with raw cod muscle.

almost all species except in sturgeon, Northern pike, and anchovy. Additionally, several proteins of higher molecular weight were detected at 16–20 kDa and 40–90 kDa in some extracts.

3.3. Standard curve of the cod parvalbumin ELISA

The parvalbumin standard was used in a standard curve with concentrations from 0.49 to 500 µg/L. The working range of the assay was defined as the part of the curve which had a linear correlation coefficient of $r^2 > 0.99$. Generally, it included seven calibration points, ranging from 0.49–31.3 µg/l (Fig. 3). For the evaluation of possible matrix effects, standard curves were prepared in undiluted, and 1:20 or 1:100 diluted extracts of bread, white sauce, soy sauce, mushroom soup, and wine. The resulting standard curves were after a 1:20 dilution congruent to the calibration obtained in assay buffer (data not shown).

3.4. Cross-reactivity between fish species in the cod parvalbumin ELISA

Extracts of 32 fish species were tested for cross-reactions in the cod parvalbumin ELISA (Table 1). Using raw extracts only diluted 1:20 according to the

Table 4

Recovery (%) from selected blank food matrices spiked with 1, 10, and 100 mg/kg cod parvalbumin standard^a

Blank food	1	10	100
Bread	98±18	136±9	113±16
White sauce	90±9	100±14	106±14
Soy sauce	46±3	68±6	87±8
Mushroom soup	77±8	123±30	92±12
Wine	14±4	40±4	29±4

^a Values represent the average of ten spiking experiments and are reported as mean±standard error of the mean (S.E.M.).

assay procedure, recoveries were calculated in relation to the total protein contents. A group of nine fish species reached recoveries >50%, five fish species reached 20–50%, seven 1–20%, and twelve had recoveries <1%.

3.5. Specificity

Several seafoods and common food ingredients were analysed in the cod parvalbumin ELISA. The recoveries of equivalents to cod parvalbumin were calculated and were for all extracts <0.00002%, with the exception of European squid which caused a positive signal corresponding to 0.00006% (Table 2).

3.6. Limit of detection and limit of quantitation

The LOD of the cod parvalbumin ELISA was 0.11 µg/L measured in assay buffer, equivalent to 0.01 mg/kg in food, considering the dilutions. The LOQ was 0.02 mg/kg. Results of the matrix-assisted determinations were LODs of 0.12 µg/L in the ELISA, i.e. 0.01 mg/kg in bread, soy sauce, white sauce, mushroom soup, and wine. The LOQs were calculated as 0.02 mg/kg.

3.7. Intra- and Inter-assay precision

For the determination of the intra-assay and inter-assay precision of the cod parvalbumin ELISA, four

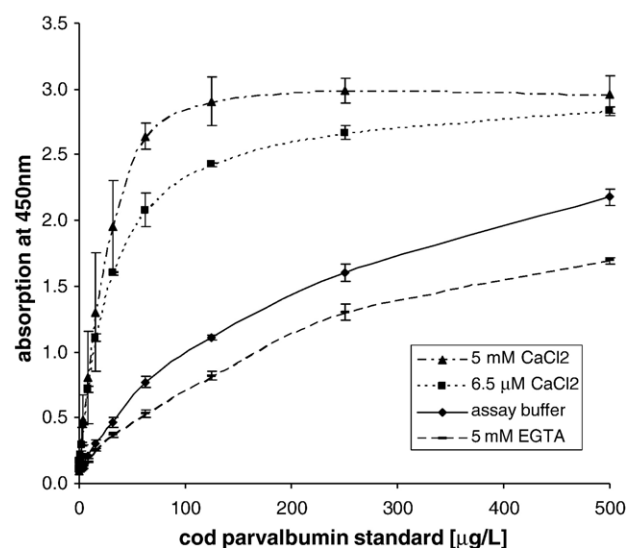


Fig. 4. Influence of the Ca^{2+} -concentration in the assay buffer on cod parvalbumin standard curves obtained in the cod parvalbumin ELISA. The graphs show the average values of four (6.5 µM CaCl_2 , assay buffer) or two (5 mM CaCl_2 , 5 mM EGTA) parallel samples, and the standard deviations.

typical food matrices containing different amounts of fish protein (10–4000 mg/kg) were analysed (Table 3). The intra-assay precision, as expressed by the coefficient of variation (% CV), ranged from 4 to 12%, and the inter-assay precision ranged from 11 to 19%.

3.8. Accuracy

Five blank matrices matching typically fish-containing foods were spiked with low, intermediate, and high amounts of the cod parvalbumin standard before sample extraction and used to determine recovery rates (Table 4). In bread, white sauce, and mushroom soup, the recoveries ranged from 77 to 136%. They were slightly lower in soy sauce, ranging from 46 to 87%. From wine, the recoveries were insufficient, reaching only 14 to 40%.

3.9. Robustness

Fish extracts degraded gradually if they underwent a number of freezing-thawing cycles. Therefore, they were stored in aliquots and used just once or twice. The cod parvalbumin standard was more stable, but nevertheless the same precautionary measures were taken. Minor time or temperature variations did not affect the cod parvalbumin ELISA. However, the method was sensitive to changes of the Ca^{2+} -concentration in the assay buffer (Fig. 4). Increasing the Ca^{2+} -content to 6.5 μM or even 5 mM led to higher affinities of the polyclonal anti-cod parvalbumin antibodies to the protein and resulted in steeper binding curves and faster saturation in the cod parvalbumin ELISA. In contrast, when the Ca^{2+} -ions were chelated by adding EGTA to the assay buffer, a more flattened curve was obtained.

4. Discussion

Fish is an important component of human nutrition. The world average yearly per capita consumption of fish has been increasing continuously during the last decades up to currently 16 kg. However, the world average consumption hides regional differences, and further overall growth is expected if countries with still low consumption increase their demand for fish (FAO, 2004). In parallel, the world fish production and fish processing industry have massively augmented. A large share of fish is now consumed not longer as entity but as ingredient in highly processed food products. This development involves that the risk for food cross-contamination due to elaborate manufacturing processes and differently handled hygiene practices has likewise increased. Contaminations may occur due to shared

equipment or production lines, re-circulating of intermediate products, inadequate cleaning routines or accidental introduction of fish under the preparation of a meal. Since already small amounts of fish protein can elicit anaphylactic reactions in sensitive persons with fish allergy (Aas, 1966), it is important to have analytical methods to control food products.

In the present study, a sensitive sandwich ELISA for the detection of fish in foods was developed and validated with four different matrixes. The method uses a polyclonal anti-cod parvalbumin antibody from rabbit for capture of the antigen and a biotin conjugate of the same antibody for detection. The Atlantic cod parvalbumin used as immunogen and standard in the ELISA was purified applying a procedure for carp parvalbumin (Bugajska-Schretter et al., 2000) with several modifications. As result, relatively pure cod parvalbumin, comparable to the published data for Gad m1, was obtained (Das Dores et al., 2002). Apparently, cod parvalbumin occurs in oligomeric forms, with a monomeric form at approximately 12 kDa, and dimeric, trimeric and tetrameric forms at respectively about 20, 40 and 50 kDa. In fish which contain two distinct parvalbumin isoforms like salmon (Lindstrøm et al., 1996) and European eel, both protein bands at 12 and 14 kDa were detected by the anti-cod parvalbumin antibody. For some of the fish species tested, e.g. sturgeon, Northern pike, and anchovy, only very weak signals were obtained, probably due to low cross-reactivity with the anti-cod parvalbumin antibody. Additionally, a protein of about 16 kDa was recognized on Western blot by the polyclonal anti-cod parvalbumin antibody in almost all fish species though it was undeterminable on SDS-gel (data not shown). Several explanations for this observation are conceivable: Either this band represented another parvalbumin complex, or otherwise the immunogen used to produce the antibody contained traces of the 16 kDa protein, or it possibly cross-reacted with the anti-cod parvalbumin IgG. Though the functionality of the ELISA is hereby apparently not influenced the identity of this protein remains to be solved which probably can be done by proteomic analysis.

In total, twelve fish species obtained recovery rates lower than 1% in the cod parvalbumin ELISA which might be unsuited to trace these kinds of fish. Among these species were several freshwater fish like Nile perch, European eel, sturgeon, and Northern pike, and one cartilaginous fish, the spiny dogfish. However, nine fish species reached recoveries above 50%, five fish species reached 20–50%, and seven still 1–20%. Among the fish with the best recovery rates were many of the most commonly eaten fish like cod, tilapia, salmon, carp,

mackerel and pollack. Therefore, the novel ELISA seems to be a promising tool for the determination of fish in foods. For a second step, it could be considered to generate new antibodies against a mixture of extracts from different fish, especially those which are not optimally detected under the current assay conditions.

For the present method, a polyclonal antibody was selected because of its high versatility, thus maximising the probability to detect fish proteins in processed foods because the antibody is directed against a multitude of peptides and epitopes. Different food manufacturing techniques may alter or denature proteins and destroy specific epitopes or expose hidden ones because of protein unfolding. Therefore, a polyclonal antibody has greater chances to retrieve the remaining protein than a monoclonal antibody that is directed against a single peptide target. Parvalbumin is known to resist rigorous treatment like cooking and extreme pH, and its peptides are at least equally resistant (Aas and Elsayed, 1975). The fish proteins' allergenicity is apparently partly conserved even after elaborate food processing techniques because IgE from fish allergic patients still bound to fish proteins after they had been boiled, fried or canned (Chatterjee et al., 2006; Yamada et al., 1999). It will be important to evaluate the novel cod parvalbumin ELISA's applicability to detect fish in differently treated food products in a follow-up study.

The key parameters of the cod parvalbumin assay were determined in an intra-laboratory validation. The standard curve was linear at concentrations levels of 0.5 to <50 µg parvalbumin/l, or 0.05 to <5.0 mg parvalbumin/kg food if the sample dilution in the assay is considered. Taking into account that fish muscle contains up to 2 g parvalbumin/kg, depending on the fish species (Kobayashi et al., 2006), the assay's working range covers about 25 to <2500 mg fish muscle/kg food. All samples with fish concentrations exceeding this amount have to be additionally diluted. The assay performed comparably well in 1:20 diluted extracts of bread, sauces, soup, and wine which were chosen because they are typical matrices for fish proteins. Fish sauces and soups are frequently consumed foods, and there are breads which use fish as an additional protein source. Wine, however, is often cleared with fish gelatine which might contain contaminating fish protein.

The specificity of the antibody used in the cod parvalbumin ELISA was studied by cross-reaction experiments with seafood and typical other food ingredients. With the exception of European squid that produced a signal equivalent to 0.6 mg/kg parvalbumin, no cross-reactions were observed. Even frog which contains parvalbumin belonging to the same β -lineage as the fish parvalbumins (Hilger et al., 2004) did not

cross-react in the assay. Other vertebrate species like chicken, pig, and cow were equally inert.

The sensitivity of the cod parvalbumin ELISA with an LOD of 0.01 mg parvalbumin/kg food, or respectively 5 mg fish/kg food, seems sufficient to detect fish protein traces in foods at levels in the range of the currently assumed threshold value of 5 mg/kg that is intended to minimize the risk for fish allergic consumers (Taylor et al., 2002). Dose-eliciting positive challenges were seen in DBPCFC studies at <500 mg fish in children (Sicherer et al., 2000) and 250 mg fish in adults (Helbling et al., 1999). Therefore, if e.g. 500 g of a meal containing 5 mg/kg fish are accidentally consumed by a person with fish hypersensitivity, the intake of fish would add up to about 2.5 mg fish, an amount which is probably low enough to grant a certain safety margin to the allergic consumer. For routine analysis, the LOD of the cod parvalbumin ELISA was set to 1 mg parvalbumin/kg food to reduce the probability for false positive results and to further increase the reliability of the assay, which is important for e.g. survey studies commissioned by food authorities.

The repeatability of the cod parvalbumin ELISA as measured by intra-assay precision was <12% and the reproducibility as measured by inter-assay precision was <19% in four typical food matrices. The method worked reliable both at low and high concentrations of fish protein and the performance precision was considered to be satisfactory.

The accuracy of the cod parvalbumin ELISA as determined for five different food matrixes in recovery experiments was good with variances (S.E.M.) <20% except for mushroom soup spiked with 10 mg/kg parvalbumin (S.E.M. = 30%). In all matrixes, the recoveries at the lowest spiking level 1 mg/kg were slightly lower than at 10 or 100 mg/kg. From conventional matrixes like bread, white sauce, and soup, 100% of the spiked parvalbumin was recovered whereas in a more difficult matrix like soy sauce the amount was reduced to about 80%. The recovery from wine with maximal 40% was considered insufficient. However, only one extraction buffer was tested during method validation, and further extraction experiments will probably indicate a buffer which is more suitable for these matrix types and lead to an increased recovery of parvalbumin.

The cod parvalbumin ELISA was robust with respect to minor temperature or time changes. However, as expected for a Ca^{2+} -binding protein with a K_D as low as 6.5 nM (Erickson and Moerland, 2006), the Ca^{2+} -concentration in the assay buffer had a considerable impact on the method performance. Ca^{2+} -depletion led to a strong loss of sensitivity in the cod parvalbumin ELISA, probably due

to reduced antibody-antigen binding. As previously has been shown, the removal of free Ca^{2+} -ions from the buffer by complexing with EGTA resulted in a substantial reduction of IgE-binding in sera of fish allergic patients which was explained by changes in conformational IgE-epitopes and/or unfolding of the protein (Swoboda et al., 2002; Bugajska-Schretter et al., 1998). In our study, we observed furthermore that a non-physiologically increase of the Ca^{2+} -concentration affected the affinity between the polyclonal antibody and the cod parvalbumin in the opposite way producing a much steeper binding curve in the assay. In the final procedure of the cod parvalbumin ELISA, Ca^{2+} was neither added nor depleted, so that only the naturally parvalbumin-bound Ca^{2+} -ions were present in the assay. These intrinsic Ca^{2+} -ions account for the parvalbumin's thermal stability (Bugajska-Schretter et al., 2000) which was utilized during the purification procedure by implementing a boiling step. However, considering the novel cod parvalbumin ELISA, further improvement might be possible by titrating the Ca^{2+} -concentration in the assay buffer to optimize the sensitivity for the desired working range.

In conclusion, a sensitive cod parvalbumin ELISA in sandwich format for the quantitative determination of fish in foods was successfully developed and validated in five complex food matrixes. The assay detects specifically fish but does not differentiate among fish species. It is therefore suitable to generally trace fish protein in foods or product ingredients with the aim to explain allergic incidents, to control the successful implementation of food laws or to support the food processing industry with the quality control of their production. Increased food safety is a current political issue, and protection of the increasing number of consumers with food allergy is a priority objective.

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