

Important Variations in Parvalbumin Content in Common Fish Species: A Factor Possibly Contributing to Variable Allergenicity

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Key Words

Allergen content • Allergen quantification • Fish • Fish allergen • Fish species • Food allergy • Parvalbumin • Skin prick tests

Abstract

Background: Although 95% of fish-allergic patients are sensitized to the major fish allergen parvalbumin, clinical reactions to different fish species vary considerably in symptoms, intensity and frequency in allergic subjects. This study aimed at the quantification of parvalbumin levels in salmon, trout, cod, carp, mackerel, herring, redfish and tuna. **Methods:** Fish muscle extracts were separated by SDS-PAGE and parvalbumin content was estimated by densitometric band quantification. Individual antisera were raised in BALB/c mice against parvalbumins purified from seven fish species. Parvalbumin content was quantified in fish (raw/processed) and skin prick test (SPT) solutions by ELISA using the corresponding anti-serum for detection and the purified parvalbumins as standards. **Results:** Using SDS-PAGE scanning, parvalbumin contents were <0.5 mg per gram tissue for mackerel, 0.5–2 mg for salmon and trout, and >2 mg for cod, carp, redfish and herring. Using ELISA, parvalbumin content ranged from <0.05 mg for tuna, 0.3–0.7 mg for mackerel, 1–2.5 mg for salmon, trout and cod to >2.5 mg per gram raw muscle for carp, herring and redfish. The parvalbumin con-

tent of processed samples (cooked/commercial) was 20–60% lower. Allergen content in SPT samples ranged from 20 to 70 µg parvalbumin/ml of extract. No parvalbumin was found in tuna SPT solution. **Conclusion:** The parvalbumin content of most commonly consumed fish species varies considerably. Differences range from severalfold to one hundredfold. This has to be taken into account when designing food challenge tests and advising fish-allergic patients.

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Introduction

Allergy to fish is common and sometimes severe [1]. The prevalence of fish allergy varies between 0.1 and 3% in the general population [2].

Parvalbumins are the major fish allergens. Over 95% of fish-allergic patients have IgE antibodies to parvalbumin [3–5]. Parvalbumins are calcium-binding muscle proteins of about 12 kDa. They are highly resistant to heat and enzymatic digestion [6]. Several fish parvalbumins have been characterized at DNA and protein level, such as Sal s 1 from salmon, Cyp c 1 from carp and Gad m 1 from cod [4–7]. The high protein identity and significant IgE cross-reactivity between different fish species were arguments to advise allergic individuals to avoid all fish [8]. Nevertheless, important variations in clinical sensi-

tivity to different fish species have been documented by double-blind, placebo-controlled food challenges [8, 9]. Monosensitivity to single fish species has also been reported [10].

It has to be considered that besides differences in IgE-binding epitopes the differences in parvalbumin content among different fish species play also a role. Bony fishes have two types of muscle tissue, white and dark, that have different physiological functions. In tuna, parvalbumin could only be detected in white muscle tissue [11]. For some Asian fish species, it has been shown that white muscle contains several times more parvalbumin than dark muscle tissue [12].

Up to now, no detailed data are available on parvalbumin content in most fishes consumed. In this study, we quantified and compared the parvalbumin content in raw tissues from eight fish species commonly consumed in Europe. We also determined parvalbumin levels in cooked and commercially processed fish and commercial skin prick test (SPT) samples. We used two different quantitative assays to determine the parvalbumin content in salmon, trout, cod, carp, mackerel, herring, red-fish and tuna.

Material and Methods

Parvalbumin Purification and Production of Polyclonal Mouse Antisera

Parvalbumins were purified from salmon (*Salmo salar*), trout (*Salvelinus fontinalis*), cod (*Gadus morhua*), carp (*Cyprinus carpio*), mackerel (*Scomber scombrus*), herring (*Clupea harengus*), tuna (*Thunnus albacares*) and redfish (*Sebastes marinus*) by ion exchange and size exclusion chromatography.

Protein was extracted from fish muscle in 10% (m/v) single detergent lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl and 1% Triton X-100) by grinding the muscle tissue in liquid nitrogen. Fish extracts were treated with a mix of proteinase inhibitors (Roche, Mannheim, Germany). The extracts were centrifuged for 10 min at 5,300 g to remove insoluble particles. The supernatants were dialyzed against 20 mM Tris-HCl, pH 8 (Nanosep 3 kDa; Pall, Zaventem, Belgium). Proteins were separated by anion exchange chromatography (Resource Q; GE Healthcare, Uppsala, Sweden) using an elution gradient of 0–500 mM NaCl in 20 mM Tris-HCl (pH 8). For salmon, trout, herring and tuna, a second ion exchange chromatography was added. Fractions containing parvalbumin were dialyzed against 20 mM Tris-HCl (pH 8) and applied to a further ion exchange column (Mono Q 5/50 GL; GE Healthcare). Parvalbumin-containing fractions were concentrated (Nanosep 3 kDa; Pall) and loaded on a high-performance gel filtration column (Superdex 75 10/300 GL; GE Healthcare) using 50 mM NaH₂PO₄ (pH 7.2) and 150 mM NaCl as running buffer. Eluted parvalbumin was pooled and dialyzed against phosphate-buffered saline (PBS) (pH 7.2).

Purity of parvalbumin preparations was analyzed in analytical sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie G-250 dye (Pierce, Erembodegem, Belgium) or silver staining (SilverSNAP kit; Pierce). Purified preparations of parvalbumin were separated by two-dimensional SDS-PAGE according to the manufacturers' instructions (Protean IEF system, ReadyStrip IPG pH 3–6; BioRad, Nazareth, Belgium), followed by silver staining (Pierce). Protein identity was confirmed by detecting parvalbumin bands or parvalbumin spots in immunoblotting using a polyspecific anti-carp parvalbumin antibody (Swant, Bellinzona, Switzerland). Calcium depletion assays were performed by co-incubation of parvalbumin and the commercial antibody in the presence of 5 mM ethylene glycol tetraacetic acid (EGTA).

Polyclonal sera were raised in BALB/c mice by intraperitoneal injection of 50 µg purified parvalbumin using Freund's complete adjuvant followed by three injections using Freund's incomplete adjuvant (Sigma, St. Louis, Mo., USA). Antibody titers were determined by immunoblotting and enzyme-linked immunosorbent assay (ELISA) using fish extracts and purified antigens. The mouse sera were diluted in a range of 1:10,000 to 1:100,000 in blocking buffer [3% bovine serum albumin (Sigma) in 0.05% Tween-PBS]. The titer value was assigned to the last dilution at which the antibody still detected the antigen. Anti-parvalbumin specificity of the mouse sera was tested by inhibition with the corresponding parvalbumin (0.5 mg/ml), followed by immunoblotting with fish extract or purified parvalbumin.

In all immunodetection experiments, primary antibodies and mouse sera were incubated in the presence of 0.5 mM CaCl₂. Parvalbumin was visualized by an anti-mouse IgG alkaline phosphatase-conjugated antibody diluted 1:10,000 (Sigma), followed by 5-brom-4-chlor-3-indolyl phosphate/nitro-tetrazolium-blue chloride color development in immunoblotting and *p*-nitrophenyl phosphate in ELISA.

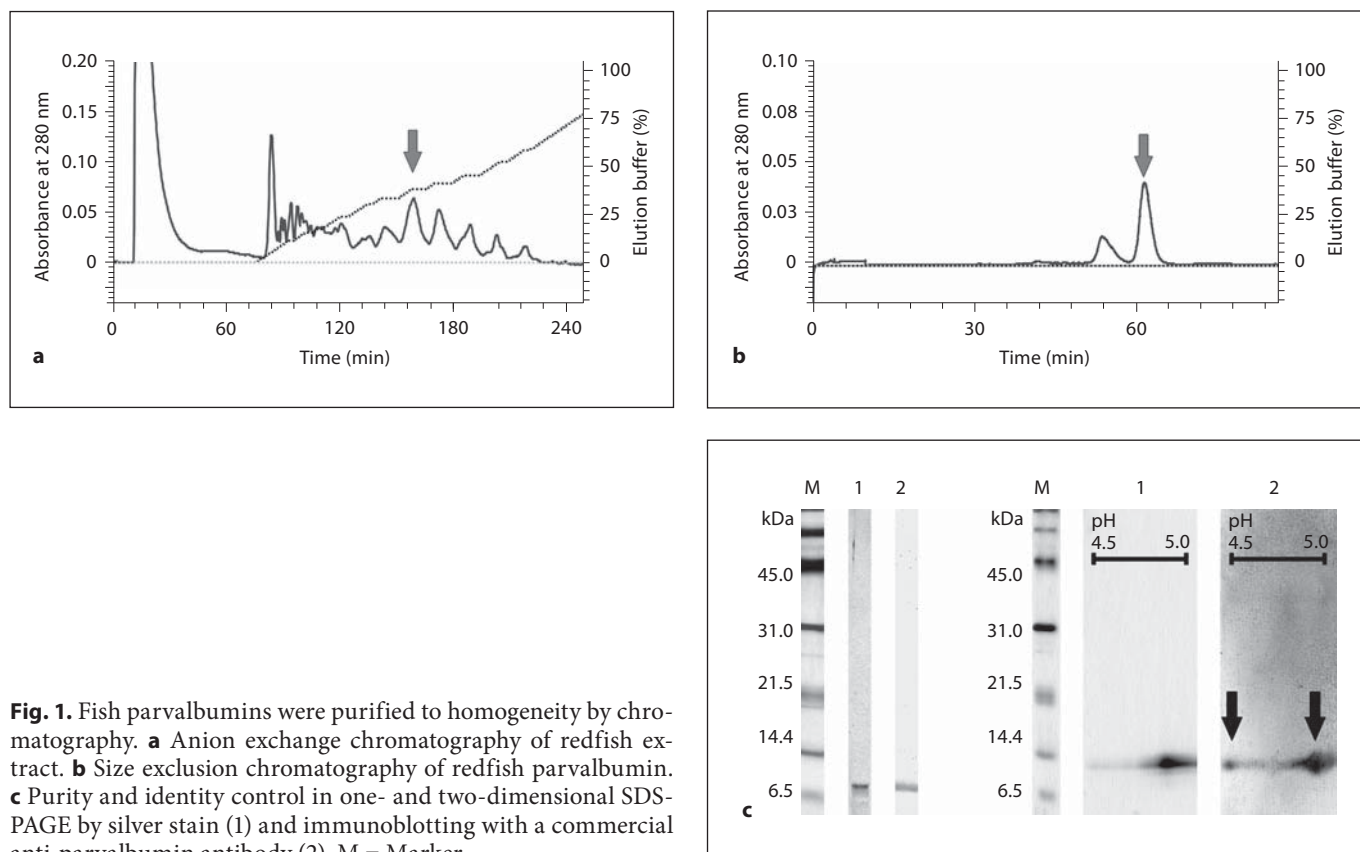
Sample Preparation

Fish tissues and processed samples were purchased from specialized trade. Raw fish samples were collected from different individuals (2 herrings, 2 carps, 3 redfish, 6 trouts, 2 salmons, 4 cods, 3 mackerels and 6 tunas). Two samples were taken from each fish at different longitudinal body positions. Processed fish samples were used from commercially available fish products (2 pickled herrings, 2 smoked trouts, 2 smoked salmons, 2 cured cods, 2 smoked mackerels and 2 canned tunas). Fresh fish fillets of 2 cm thickness were heated at 95°C for 10 and 20 min.

Each tissue sample (raw/cooked) and each commercial sample was divided into aliquots, and total protein was extracted in triplicate, as described elsewhere [13]. Insoluble proteins and cell debris, a byproduct from protein extraction, were re-extracted using 10% (m/v) 50 mM Tris-HCl (pH 8), 150 mM NaCl and 8 M urea.

SPT solutions were purchased from five commercial suppliers (ALK, Madrid, Spain; Allerbio, Varennes-en-Argonne, France; Allergopharma, Reinbek, Germany; HAL, Haarlem, The Netherlands, and Stallergènes, Antony, France).

Extracts and SPT samples were dialyzed against PBS buffer, pH 7.2, using 3-kDa Nanosep centrifugal devices (Pall). Protein concentrations were measured using the Bradford protein assay (BioRad). Samples were analyzed for presence of parvalbumins by immunoblotting using the respective mouse antisera (dilution 1:10,000).



Densitometric Band Quantification
Fish extracts and SPT solutions were separated in triplicate under denaturing conditions by analytical SDS-PAGE, loading 15 and 35 μg of each protein sample and, in a parallel lane, 2 μg of each purified parvalbumin. Proteins were stained with Coomassie G-250 dye (Pierce). Protein bands were analyzed using GeneTools software (Syngene, Cambridge, UK).

Quantitative ELISA
ELISA was performed in flat-bottom polystyrene 96-well plates (Nunc, Uden, The Netherlands). For ELISA calibration, purified parvalbumins were coated in duplicate in a concentration range of 2–600 ng parvalbumin/ml. Fish extracts and SPT samples were coated at serial dilutions of 0.5–5 $\mu\text{g}/\text{ml}$ to the ELISA plate. Parvalbumins were detected using the respective anti-parvalbumin mouse sera (dilution 1:10,000). Trout parvalbumin was quantified with salmon anti-serum as the protein identity between salmon and trout parvalbumin is >97%. All ELISA tests were performed in triplicate. The accuracy of ELISA was tested by performing recovery studies with blank matrices. Parvalbumin-free extracts from dark muscle tissue of tuna were mixed with 2–500 ng of each parvalbumin/ml. Parvalbumin contents were calculated per gram muscle as a species- and sample-specific concentration range.

Results

Parvalbumins were purified from eight commonly consumed fish species. Redfish parvalbumin is given as an example for chromatographic purification, and control of protein purity and identity (fig. 1). Purified parvalbumin appeared as a single/double protein band of 11 kDa in SDS-PAGE. A single protein band was observed for mackerel and tuna parvalbumin. No additional protein bands were detected by silver stain. Parvalbumin identity was confirmed using a commercial anti-parvalbumin antibody in immunoblotting. Addition of EGTA inhibited the detection of parvalbumin by the antibody. Isoelectric focusing of purified parvalbumin yielded distinct spots of pH 4–5 corresponding to the isoelectric points calculated for the parvalbumin isoforms (fig 1). These spots were detected by the anti-parvalbumin antibody in the corresponding immunoblotting.

Seven antisera were raised in mice using parvalbumins purified from fish muscle. High antibody titers were obtained after four immunizations. The IgG titers

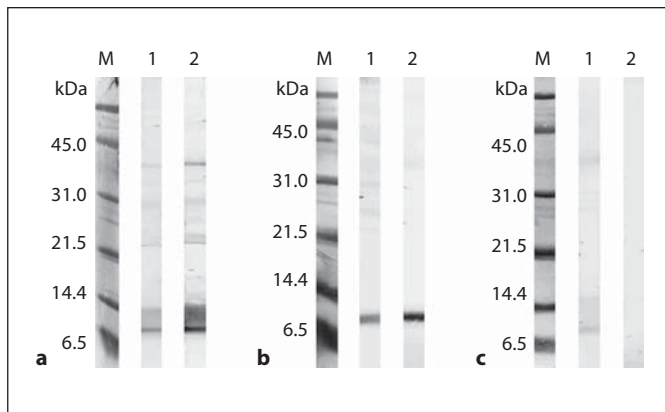


Fig. 2. Murine antisera were characterized for specific reactivity with parvalbumin in comparison to the commercial anti-parvalbumin antibody (1). This is shown on the example of anti-redfish parvalbumin serum (2). **a**, **b** IgG binding to redfish extracts (**a**) and purified redfish parvalbumin (**b**). **c** Inhibition of IgG binding to redfish extracts in the presence of redfish parvalbumin. M = Marker.

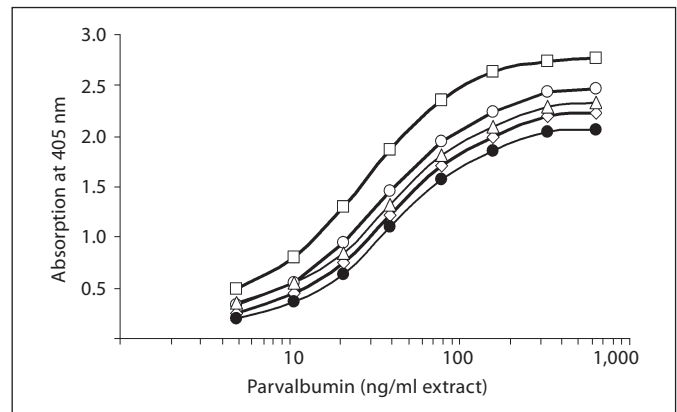


Fig. 3. Murine antisera were tested in ELISA calibration experiments with purified parvalbumins. Redfish parvalbumin detection is shown as an example using anti-redfish (□), anti-cod (○), anti-herring (△) and anti-salmon (◇) parvalbumin mouse serum, and the commercial antibody (●).

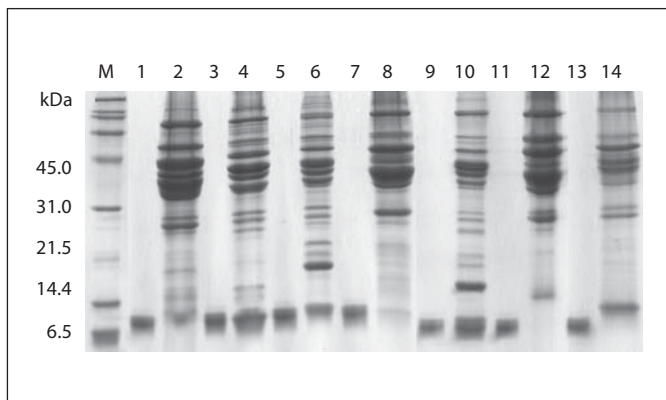


Fig. 4. Fish extracts and the corresponding purified parvalbumins migrated in parallel lanes in SDS-PAGE (15%) and stained by Coomassie blue for quantification: marker (M); salmon parvalbumin (1)/extract (2); carp parvalbumin (3)/extract (4); cod parvalbumin (5)/extract (6); mackerel parvalbumin (7)/extract (8); redfish parvalbumin (9)/extract (10); tuna parvalbumin (11)/extract (12), and herring parvalbumin (13)/extract (14).

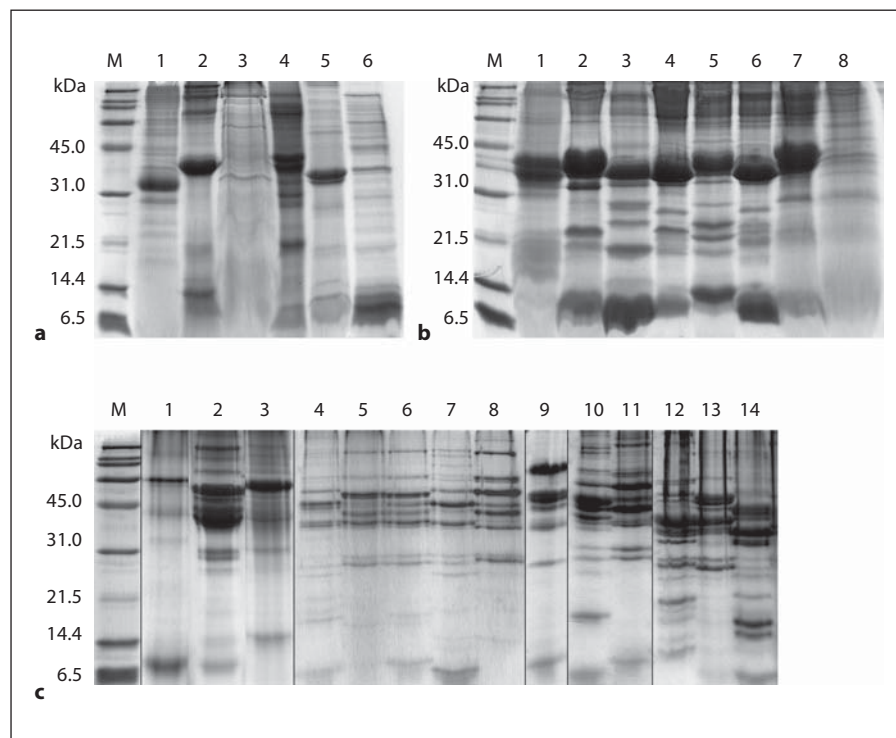
were determined at 1:50,000 to 1:70,000 in immunoblotting with fish extracts and 1:70,000 to 1:90,000 in ELISA with purified parvalbumins. The antisera specifically detected 11-, 23- and 35-kDa proteins in total extracts and 11-kDa proteins in purified antigen preparations (fig. 2). Antibody binding could be entirely abolished upon incubation with the corresponding purified native parvalbu-

min. The antisera were tested in ELISA with the eight purified parvalbumins in a concentration range of 2–600 ng protein/ml. The highest sensitivity was achieved when detecting the respective parvalbumin with the corresponding antiserum (fig. 3). This has been applied for calibration curves in parvalbumin quantification experiments.

The parvalbumin content was quantified for the eight fish species using several samples from different, individual fishes and processed specimens. Tuna samples were taken separately from dark and light muscle tissue. A total number of 60 samples from raw fish, 12 samples from commercially processed fish and 14 SPT solutions were analyzed. In addition, 16 samples from the eight fish species cooked for 10 and 20 min, respectively, were included. Insoluble residue was re-extracted with urea in a number of samples.

For densitometric band quantification in extracts from raw fish (fig. 4), the identity of parvalbumin bands was confirmed by immunoblotting using mouse sera raised against each specific parvalbumin. No parvalbumin was detected after re-extraction of insoluble residues. Parvalbumin oligomers were observed in salmon, cod, redfish and herring extracts in minor quantities at a higher molecular range as shown for the redfish parvalbumin (fig. 2). These bands were not considered in scanning experiments. Gel analysis allowed to classify fish species into three categories according to their allergen content: <0.5 mg for mackerel, 0.5–2 mg for salmon and

Fig. 5. **a** Migration of proteins of commercially processed fish samples in SDS-PAGE (15%) and staining with Coomassie blue: marker (M); pickled herring (1; 2); canned tuna (3); smoked mackerel (4; 5), and smoked trout (6). **b** Protein extracts from cooked fish fillets were separated by SDS-PAGE (Coomassie blue): marker (M); salmon (1); trout (2); carp (3); cod (4); herring (5); redfish (6); mackerel (7), and tuna (8). **c** SPT solutions were separated by SDS-PAGE (15%) and proteins were stained by Coomassie blue. M = Marker. SPT provider 1 – cod (1); salmon (2), and tuna (3); SPT provider 2 – cod (4); salmon (5); trout (6); carp (7), and tuna (8); SPT provider 3 – salmon (9); SPT provider 4 – cod (10) and herring (11), and SPT provider 5 – tuna (12); salmon (13), and cod (14).



trout, and >2 mg parvalbumin/g muscle for cod, carp, herring and redfish. This method did not allow an estimate of parvalbumin in tuna, although its presence could be detected by immunoblotting in concentrated extracts from white muscle.

For commercially processed fish and SPT samples, poor protein separation by SDS-PAGE did not allow quantification of parvalbumin bands. This was also the case for protein samples extracted from boiled fish fillets. Protein patterns differed from corresponding fresh extracts in number and density of bands (fig. 5). Using the specific mouse sera, parvalbumins were found in all samples from commercially processed fish, cooked fish fillets and SPT (data not shown). Minor quantities of parvalbumin were detected only in protein samples re-extracted from pellets of cooked fish after extraction. In contrast, no parvalbumin was detected in canned tuna or in tuna SPT samples by immunoblotting (data not shown).

Quantitative ELISA using specific mouse antisera and the corresponding purified parvalbumin gave reproducible results in a range of 2–600 ng parvalbumin/ml. The detection limit was calculated for each specific serum and given as the mean of the lowest parvalbumin concentration detected in 10 experiments. Detection limits were

2 ng parvalbumin/ml for cod, salmon, trout and 6 ng parvalbumin/ml for carp, herring, mackerel, redfish and tuna.

In raw fish, species-specific ranges of parvalbumin contents were calculated including values from different, individual fish, muscle sections and extractions (table 1). Values were expressed as mean values of parvalbumin per gram of raw muscle. In descending order, fish species were ranked from herring with 4.75 mg, carp with 3.75 mg, redfish with 2.5 mg, salmon/trout with 2.2 mg, cod with 2 mg, mackerel with 0.5 mg to tuna with 0.03 mg parvalbumin/g muscle. Parvalbumin contents in commercially processed samples were found to be about 60% lower in comparison to the corresponding raw fish sample. In protein samples extracted from boiled fish fillets, parvalbumin contents yielded the same ranking as in fresh fish, ranging from tuna with the lowest and herring with the highest parvalbumin level. Parvalbumin levels in cooked fish fillets were found to be up to 25% lower compared to the corresponding fresh fish sample. The ratio of parvalbumin to total soluble protein ranged from 0.03% (tuna) to 3.3% (herring) in raw fish extracts, but from 0.2% (tuna) to 16% (herring) in extracts from cooked fish. In protein samples re-extracted from protein pellets

Table 1. Parvalbumin contents in raw fish, and commercially processed and cooked fish samples by quantitative ELISA

	Fish sample	Fish n	Extracts n	Parvalbumin mg/g	Parvalbumin % ¹
Herring	raw	2	12	3.8–5.7	3.3
	pickled	2	6	1.2–2.8	5.6
	cooked	2	12	3.0–4.4	16
Carp	raw	2	12	2.5–5.0	3.0
	cooked	2	12	2.1–4.0	15
Redfish	raw	3	18	2.0–3.0	2.2
	cooked	2	12	1.7–2.3	14
Trout	raw	6	36	2.0–2.5	1.3
	smoked	2	6	0.9–1.1	9.2
	cooked	2	12	1.7–2.0	11
Salmon	raw	2	12	1.9–2.5	1.2
	smoked	2	6	0.7–1.0	8.9
	cooked	2	12	1.5–1.9	9.5
Cod	raw	4	24	1.5–2.5	1.7
	cured	2	6	1.0–1.3	1.3
	cooked	2	12	1.3–1.9	7.2
Mackerel	raw	3	18	0.3–0.7	0.1
	smoked	2	6	0.08–0.15	2.3
	cooked	2	12	0.2–0.5	5.3
Tuna, white	raw	6	36	0.01–0.05	0.03
	cooked	2	12	0.01–0.03	0.2
Tuna, dark	raw	2	12	ND	–
Tuna	canned	2	6	ND	–

Two tissue samples were taken from each raw fish at different longitudinal body positions. ND = Not detected.

¹ Percentage per total soluble protein.

of cooked fillet extraction, low amounts of parvalbumin were quantified. The sum of both parvalbumin contents (directly extracted and re-extracted) was found to be only up to 17% lower compared to the corresponding fresh fish sample.

In 14 SPT samples, concentrations varied in a range from 320 to 2,270 µg protein/ml extract and from 20 to 70 µg parvalbumin/ml extract (table 2). The ratio of parvalbumin to total protein content was calculated from 1 to 13.4% in SPT solutions. No parvalbumin was detected in tuna SPT samples from three manufacturers.

Discussion

In this study, we quantified parvalbumin contents in eight commonly consumed fish species using two different methods.

Table 2. Protein and parvalbumin concentrations in original SPT solutions from 5 different companies determined by quantitative ELISA

Sample	Protein µg/ml	Parvalbumin µg/ml	Parvalbumin % ¹
Salmon (1)	510	37	7.3
Cod (1)	390	45	11.5
Tuna (1)	380	ND	–
Cod (2)	320	41	12.8
Salmon (2)	340	25	7.4
Trout (2)	380	36	9.5
Carp (2)	410	55	13.4
Tuna (2)	400	ND	–
Salmon (3)	610	68	11.1
Cod (4)	910	61	6.7
Herring (4)	1,120	52	4.6
Cod (5)	1,560	70	4.5
Salmon (5)	1,920	20	1.0
Tuna (5)	2,270	ND	–

ND = Not detected.

¹ Percentage per total soluble protein.

Densitometric measurement of parvalbumin bands allowed to classify fish species according to their parvalbumin content. This rapid method was suitable to measure parvalbumin in the microgram range. The technique could not be used to measure tuna parvalbumin, parvalbumin oligomers and parvalbumin in processed food and SPT samples.

Using ELISA, standard curves were established using species-specific anti-parvalbumin sera and the corresponding parvalbumin. This allowed to detect parvalbumins with high sensitivity and accuracy in the samples. Previously, it has been shown that the use of non-species-specific antibodies carries pitfalls [12, 14, 15]. In this study, the calibration curves clearly demonstrate a difference in antibody sensitivity (fig. 3). This variability supports the importance of species-specific antibodies as reagents in quantitative assays for parvalbumin determination. Despite high sequence homologies among fish parvalbumins, not all epitopes recognized by polyspecific antibodies are present in all parvalbumins causing variable sensitivity in allergen detection [16]. In raw fish, parvalbumin levels decreased significantly in the following order: herring > carp > redfish > salmon/trout > cod > mackerel > tuna. Differences in herring and tuna parvalbumin levels were found to vary by a factor of 100. Mackerel and tuna have been reported as low-allergenic

fish species [17]. Tuna parvalbumin could be only quantified in the white muscle tissue, as described previously [10].

Lower parvalbumin contents were detected in commercial samples from processed fish (up to 60% less). Processing might result in a modification or destruction of parvalbumin epitopes preventing protein detection by murine antibodies. Denaturation of IgE epitopes has been considered as an explanation for reduced allergenicity of processed fish samples [18, 19]. Extensive protein denaturation can be caused by processing conditions like low pH, high pressure and high temperature [20]. In this study, we included such treated food samples from pickled, canned and smoked fish and could quantify significantly lower parvalbumin levels. The solubility of parvalbumins in processed food may also be reduced by interaction with other matrix ingredients and formation of high-molecular-weight complexes [21]. In contrast, parvalbumin contents in extracts prepared from cooked fish fillets were only up to 25% lower than in raw fish. This is consistent with the fact that parvalbumins have been described as relatively heat-stable proteins [6]. It seems that simple boiling is a relatively mild food processing reaction that does not change considerably the extractable and detectable parvalbumin contents. The predominant precipitation of heat-labile muscle proteins caused rather a relative accumulation of parvalbumin in the soluble fraction. From the heat-precipitated protein fraction, only minor parvalbumin quantities could be extracted,

re-solubilized and quantified. The sum of contents from directly extracted and re-solubilized parvalbumin was still up to 17% lower compared to the corresponding fresh fish sample. The missing 13% might be attributed to a modification of parvalbumin B-cell epitopes causing a decrease in protein recognition by the mouse antiserum. Overall cooking marginally diminishes parvalbumin content and serological recognition. Therefore it cannot be concluded that cooking considerably decreases parvalbumin allergenicity.

In commercial SPT samples, parvalbumins were well represented, except for tuna. Tuna SPT solutions do not seem to be suitable as standard skin test sample for the diagnosis of fish allergy.

In conclusion, parvalbumin content varies markedly in commonly consumed fishes. Besides differences in parvalbumin IgE epitopes in different fish species, variability in parvalbumin content is likely to contribute to the variation in clinical reactivity to different fish species. Parvalbumin levels should be taken into account when designing and interpreting challenge tests and advising fish-allergic patients.

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