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Parvalbumin in Fish Skin–Derived Gelatin: Is There a Risk for Fish Allergic Consumers?

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Abstract

The major allergen parvalbumin was purified from cod muscle tissues, and polyclonal antibodies were raised toward it. The antibodies were tested for specificity, and an enzyme-linked immunosorbent assay (ELISA) was developed using these antibodies. The ELISA was applied to measure parvalbumin in cod skin, the starting material for fish gelatin made from deep sea, wild fish. The ELISA was sufficiently sensitive (LLOQ = 0.8 ng ml^{-1} in extracts, corresponding to 0.02 μg of parvalbumin per g of tissue) and did not cross-react with common food constituents. Fish gelatin, wine, and beer, matrices for the potential use of this ELISA, did not cause disturbance of the assay performance. The data show that the parvalbumin content in cod muscle tissue is 6.25 mg g^{-1}, while the skins contained considerably less, 0.4 mg g^{-1}. Washing of the skins, a common industrial procedure during the manufacturing of fish gelatin, reduced the level of parvalbumin about 1000-fold to 0.5 mg g^{-1},

or 0.5 ppm. From 95 commercial lots of fish gelatin it is shown that 73 are below 0.02 mg g^{-1} parvalbumin. From the other 22 lots, the one with the highest concentration contained 0.15 mg g^{-1} of parvalbumin. These levels are generally assumed to be safe for fish-allergic individuals.

Keywords: allergen, fish, parvalbumin, fining, wine, beer

Introduction

Fish forms an important component of the human diet in Western and developing countries; it is rich in protein representing all essential amino acids and rich in some vitamins and minerals. The presence of omega-3 unsaturated fatty acids provides potential health benefits (Kris-Etherton et al. 2002; Riediger et al. 2009). In addition to the consumption of muscle tissue, fish-derived products are used in a variety of food products as ingredients with specific functionality. Gelatin from fish skin–derived collagen is an example of a value-added food ingredient widely used for several purposes in the food industry, including for clarification purposes in the beverage industry, for encapsulation of vitamins, and as a thickening agent. Fish gelatin also complies with kosher and halal requirements. Gelatin is obtained by the hydrolysis of collagen, which is the principal protein found in the skin and bones of many fish, including the commercially important cod.

Fish is an allergenic food source, and parvalbumin is considered to be the most important fish allergen. Parvalbumin is mainly found in muscle tissues where it plays a role in the muscle contraction/relaxation cycle, calcium buffering, and signal transduction (Kretsinger 1980). Parvalbumin from fish is considered to be a panallergen for fish-allergic patients, and parvalbumins from many different fish that are commonly consumed in Western countries share biochemical and immunochemical characteristics (Taylor et al. 2004; van Do et al. 2005). Evidence also indicates that parvalbumin is the major cross-reacting allergen in tropical fish commonly consumed in Asia-Pacific countries (Lim et al. 2008).

The development of an immunochemical assay to detect parvalbumin from cod was recently accomplished (Faeste and Plassen 2008). Faeste and Plassen (2008) demonstrated that the enzyme-linked immunosorbent assay (ELISA) platform is suitable for the sensitive detection of cod parvalbumin. Using similar assays, Kuehn et al. (2010) recently demonstrated that the parvalbumin content varied among different fish species, and that parvalbumin could be detected in processed fish products. Such analytical tools are valuable for quality assurance approaches in the food industry. Other techniques based on DNA amplification, such as PCR, are becoming available for speciation of fish in food products (Hildebrandt 2010). However, the sensitivity of DNA-based methods is above the threshold that is relevant for fish-allergic consumers, and it is a circumstantial demonstration of the presence of the fish allergen because DNA rather than proteinaceous allergen is being detected. This is particularly a problem when ingredients are prepared from fish by procedures potentially separating protein from DNA.

Parvalbumins are small acidic proteins (MW = 10–12 kDa, pI = 4.0–5.2) that are very soluble under various conditions (Elsayed and Aas 1971; Kretsinger and Nockold 1973). Therefore, parvalbumins may be found in fish-based ingredients other than muscle tissue and can potentially be present in ingredients like gelatin. Even though fish gelatin is used

in small amounts for fining of wine (10 g hl^{-1}; where hl is hectoliter), there is a concern that parvalbumin potentially present in fish gelatin poses a risk to fish-allergic consumers. However, the current practice for the manufacturing of gelatin involves extensive washing of the fish skins, which may serve to reduce the parvalbumin levels in this ingredient.

The aim of the current study is to develop an immunochemical assay suitable to detect and quantify parvalbumin in the various steps of fish gelatin production. It is shown that the washing process currently being applied in fish gelatin production by one manufacturer reduces the remaining parvalbumin in fish skin to very low levels so that the resulting gelatin is unlikely to trigger an allergic reaction in fish-allergic consumers. Furthermore, the levels of parvalbumin in commercial lots of fish gelatin were evaluated using the assay described herein.

Materials and methods

Purified parvalbumin for immunization

Parvalbumin was purified from frozen Atlantic cod (*Gadus morhua*) fillets using a protocol developed for the purification of parvalbumin from carp (Koppelman et al. 2010). Briefly, portions of 500 g of cod fillet were partially thawed and mixed with 500 ml demineralized water. After homogenization, 950 ml demineralized water, 40 ml 1M TRIS-HCl (pH 8.0) and 15 ml 1M NaOH were added, and the slurry was stirred for 10 min while the pH was kept at 9.0 by the addition of NaOH (1 M). The extract was clarified and subsequently applied to a Sephadex G-75 column (equilibrated with 20mM TRIS-HCl pH 8.0). Fractions were tested for the presence of parvalbumin (by means of SDS-PAGE and visual judgment of the presence of a 12 kDa protein band). Parvalbumin-containing fractions were collected, pooled, and applied to a Source-Q column equilibrated with 20 mM TRIS-HCl pH 8.0. After washing, the column was eluted by a linear gradient from zero to 0.15 M NaCl. The pooled parvalbumin fractions were desalted by means of a Sephadex G25 column, and the final protein batch was lyophilized. All chromatography steps were performed as described for the purification of carp parvalbumin (column dimensions, flow rates, purification apparatus and software). One final batch was prepared with a total yield of 2.8 g, with a purity of more than 95% as was checked by means of SDS-PAGE; the protein showed a single band on the gel (Fig. 1). MALDI-TOF analysis revealed molecular weights of 11364.5 ± 0.36 and 11462.1 ± 0.02 Da, matching with the two gene transcripts of Parvalbumin beta-*Gadus morhua* (Atlantic cod), i.e. Q90YL0_GADMO and Q90YK9_GADMO. Peptide mass fingerprinting revealed peptides with up to 50% sequence coverage for Q90YL0_GADMO (data not shown). These data confirm unambiguously that the purified protein is the desired allergen from cod.

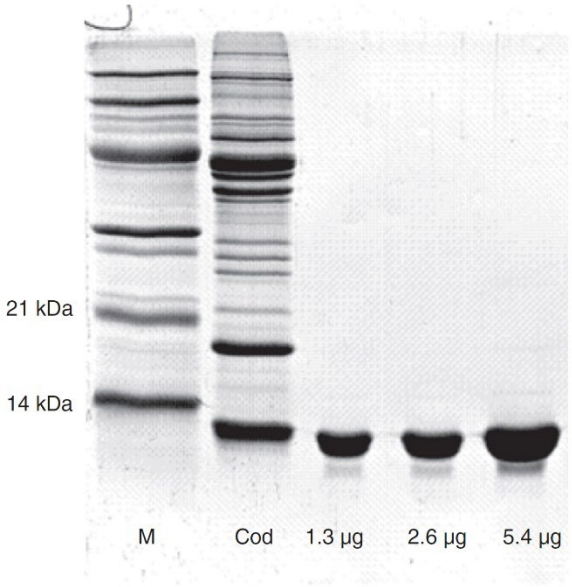

Figure 1. SDS-PAGE of cod parvalbumin. Lane M, molecular weight marker; Cod, cod extract. The following lanes show the purified cod parvalbumin analyses in different amounts (mg/lane).

Preparation of antisera

Polyclonal antibodies were produced at Covance Research Products, Inc. (Denver, Pennsylvania, USA) in three New Zealand White rabbits. The three rabbits received initial intradermal injections (back, two sites) with 200 mg purified parvalbumin protein mixed with Titermax Classic Adjuvant (Sigma-Aldrich Co., St. Louis, Missouri, USA). The subsequent scheduled boosts were performed approximately every 28 days with 100 mg parvalbumin protein at alternating subcutaneous injection sites (either neck or dorsal). The monthly boosts were performed with rotating adjuvant, mixing the purified parvalbumin protein with Titermax Classic Adjuvant for one scheduled boost followed by the next two scheduled boosts using the protein mixed with Freund's Incomplete Adjuvant (Thermo Scientific Pierce Protein Research Products, Rockford, Illinois, USA). Production bleeds were initiated 3 months after the commencement of the project when titers at 1 µg ml^{-1} (0.1 µg/well) parvalbumin coat rose to greater than 70,000 (70,000–190,000) in each of the three rabbits. Titers were sustained at more than 50,000 (54,000–270,000) for at least 12 months after initiation of the project.

Antibodies

Protein-G purified antibodies for coating ELISA plates

A total of 2 ml of antiparvalbumin serum was loaded onto a 1 ml Protein-G column (GE Healthcare Life Sciences, Diegem, Belgium; following the manufacturer's instructions). After washing with phosphate-buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl,

and a pH of 7.4, Fresenius Kabi, Schelle, Belgium) until the absorbance at 280 nm of the flow-through was lower than 0.02, about 10 mg IgG was eluted using glycine (100 mM, pH 2.7). Fractions were immediately neutralized with 10% (v/v) 1M TRIS, pH 9.0. This IgG fraction is total IgG, not only parvalbumin-specific IgG. The flow-through was deficient of antiparvalbumin antibodies, as determined by direct ELISA (data not shown) as was described by Koppelman et al. (1999).

Affinity-purified antibodies for detection in ELISA

A total of 4 mg of purified parvalbumin was coupled to 2.5 ml CNBr-activated Sepharose (GE Healthcare Life Sciences; following the manufacturer's instructions). This column was prestripped with glycine (pH 2.7) before use. After equilibration with PBS, 2 ml antiparvalbumin serum were loaded and the column was washed with PBS until the absorbance at 280 nm of the flow-through was lower than 0.02. The bound fraction was eluted with glycine (pH 2.7) and fractions were immediately neutralized with 10% (v/v) 1 M TRIS, pH 9.0. The eluted fraction indeed contained IgG as shown by the typical SDS-PAGE pattern (50 kDa and 25 kDa bands, representing the heavy and light chain of IgG, respectively; data not shown). The flow-through was deficient of antiparvalbumin antibodies, as determined by direct ELISA (not shown). The purified antibodies were dialyzed against 100 mM sodium acetate (pH 9.5), and labeled with horseradish peroxidase (Cedi-Diagnostics B.V., Lelystad, The Netherlands) using the protocol provided by the manufacturer. By measuring the ratio A403/A280, the number of peroxidase molecules per IgG was determined to be one to two on average.

Sample preparation

Fish samples and other food samples were ground with a kitchen blender or professional blender (Super Total Nutrition Center, stainless steel, Whole food machine, Vita-mix Corp., Cleveland, Ohio, USA) in the case of tough materials not sufficiently ground with the kitchen model. One part of the ground sample was mixed with 4 parts of PBS and stirred for 2 h at room temperature. Suspensions were clarified by centrifugation, and supernatants were collected to prepare dilutions for assaying.

Treatment of cod skins

Washing of cod skins

Thawed, unwashed skin from cod was cut into 1.5 cm^2 squares. Distilled water was added at a weight ratio of 1 part skin to 5 parts of water (20 g skin plus 100 ml water). The mixture was placed in a sealed bag and agitated for 10 min using a stomacher paddle blender (Seward Laboratory Systems Inc., Bohemia, New York, USA). The water (100 g) was drained and stored frozen at –20°C (wash water Sample 1). Another 100 ml of distilled water were added to the remaining tissue and again agitated for 10 min as described above. This procedure was repeated four more times, resulting in six wash water samples, numbered 1–6 in Table 3, that were tested for parvalbumin content via ELISA. The remaining tissue is referred to as washed cod skin. The experiment was performed in triplicate.

Extracting cod skins

Cod skins in the production facility were washed six times. Representative samples were removed and split into two portions. One portion was laboratory washed once more and the wash water was identified as wash water no. 7 (Table 3). From the other part, an extract was made to ensure the complete dissolving of all parvalbumin potentially present in the tissue. This was done by blending the tissue with distilled water in a 1:5 ratio using a kitchen blender operated at highest speed for 3 min. This resulted in a homogeneous gray mass. A supernatant was collected after the blended mass was allowed to settle. This sample is referred to as extract of washed cod skin (Table 3).

Preparation of cod skin gelatin

Thawed, unwashed skin from cod was cut into 1.5 cm^2 squares. Distilled water was added at a weight ratio of 1 part skin to 5 parts water (20 g skin plus 100 ml water) in a 250 ml beaker. Acetic acid (0.15 g 80%) was added and the contents were covered and heated in a hot water bath (87.5 ± 5°C) for 3 h. The beaker and contents were cooled in a 42°C bath for 10 min. The liquid gelatin was decanted from the insoluble material remaining in the beaker and stirred for 5 min with a magnetic stirrer. The gelatin solids were measured by refractometer, centrifuged at ambient temperature, and frozen in sterile plastic sample jars, referred to as cod gelatin. Washed cod skin (see above) after six laboratory-washing cycles was subjected to the same procedure resulting in gelatin referred to as refined cod gelatin.

ELISA techniques

A sandwich ELISA was constructed as follows. ELISA plates (Greiner Bio-One F-shaped, Microlon, Frickenhausen, Germany) were coated overnight at 4°C with 100 μl of 1 μg ml^{-1} Protein-G purified antibodies in 50mM sodium carbonate buffer, pH 9.6. Wells were blocked for at least 1 h at 37°C with PBS containing 3% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA; minimum purity of 98%). Samples and standards were diluted in PBS containing 0.5% BSA and 0.2% Tween 20. The following concentrations were used for the standard: 600, 200, 67, 22, 7.4, 2.5, 0.82 and 0.27 ng ml^{-1}. Samples and standards (100 ml per well) were applied in triplicate, and plates were incubated for 2 h at 37°C. Affinitypurified, peroxidase-labeled antibody diluted 500-fold in PBS containing 0.5% BSA and 0.2% Tween 20, was applied to wells (100 ml per well) and incubated for 2 h at 37°C. Between steps, plates were washed three times with 300 μl PBS containing 0.2% Tween 20. To develop the bound peroxidase label, the following procedure was used: 10 mg OPD (1,2-phenylenediamine dihydrochloride; Fluka Biochemica 78441, Buchs, Switzerland) were dissolved in 50 ml buffer composed of 100 mM phosphate and 50 mM citrate buffer (pH 5.0). A total of 20 μl of 30% hydrogen peroxide (MP Biomedicals LCC, Eschwege, Germany) was added shortly before use. Of this solution, 100 μl were added to each well, and ELISA plates were incubated for 7–15 min. The reaction was stopped by adding 50 μl 2N sulfuric acid, and ELISA plates were read by measuring absorbance at 490 nm using a microtiter plate reader.

A direct ELISA was constructed using a coat of different fish extracts. Fish extracts were prepared in triplicate and the protein content was determined by the Lowry method (Lowry et al. 1951). A total of 100 ml of 10 μg ml^{-1} fish extract were coated in triplicate in ELISA plates (NUNC-Immuno MaxiSorp 96-MicroWell plates, Nalge Nunc International,

Rochester, New York, USA), overnight at 4°C. The polyclonal antiparvalbumin serum was diluted 15,000-fold in PBS containing 0.1% BSA (RIA grade, Sigma Chemical Co.) and 100 µl/well were added and incubated for 1 h at 37°C. Bound IgG was detected by means of goat antirabbit IgG conjugated to alkaline phosphatase (Pierce Biotechnology, Inc., Rockford, Illinois, USA; diluted 4500-fold in PBS containing 0.1% BSA). An enzymatic reaction development was achieved by adding 100 µl/well of *p*-nitrophenyl phosphate substrate (Sigma Fast, Sigma-Aldrich) and incubated for 30 min at room temperature in the dark. The enzymatic reaction was then stopped by the addition of 1N sodium hydroxide at 100 µl/well. Wells were measured at 405 nm, and the experiment was repeated on 2 different days.

Results and discussion

Sensitivity of the sandwich ELISA

The sandwich ELISA constructed by coating with Protein-G-purified IgG and using peroxidase-labeled affinity-purified IgG as the detector antibody was tested for sensitivity. Dilutions of purified cod parvalbumin show that the ELISA can detect parvalbumin at a concentration of 0.27 ng ml^{-1}, with a quantitation range between 1 and 100 ng ml^{-1} (Fig. 2). The standard reaches saturation at the range of 200–600 ng ml^{-1}. These ranges are typical for immunochemical assays such as ELISA (Yeung 2006). Data points were fitted using the four-parameter logistic curve

$$y = b + \left((a - b)/(1 + xc)^d\right)$$

enabling recalculation of the standards. It appeared that the range from 0.8 and 22 ng ml^{-1} resulted in the most accurate results (Table 1). Standards with concentrations higher than 200 ng ml^{-1} could not be recalculated correctly, indicating that measuring in the plateau area of the calibration curve does not allow for quantification. The intra-assay variation as investigated by the standard deviation of triplicate measurements is low. Curve fitting showed high correlation coefficients (Table 1). The inter-assay variation was investigated by testing the reproducibility of the calibration curve on 4 consecutive days. Although the plateau reached at saturation differs somewhat, the shape of the curve and consequently the sensitivity remained the same. The four independent experiments resulted in calibration curves with high correlation coefficients and valid recalculated values for the standards (Table 1). The concentration of parvalbumin resulting in a signal amounting to twice the background was used as the lower limit of quantification (LLOQ) and appeared to be 0.8 ng ml^{-1}. Taking into account the extraction of 1:5 (w/v) and necessary dilutions (fivefold), this LLOQ corresponds to 20 ng g^{-1} of a food sample, or 0.02 ppm. The sensitivity reported here is in the same order of magnitude as the cod parvalbumin assay published by Faeste and Plassen (2008), who reported 10 ng g^{-1} of food sample. Kuehn et al. (2010), who published data on ELISA methods for the detection of parvalbumin of different fish species, reached sensitivities in the same range, or a little higher (2–6 ng ml^{-1}). The LLOQ reported here of 0.02 ppm refers to parvalbumin in a product. Considering a parvalbumin content in the order of magnitude of 1% in cod muscle tissues (Kuehn et al. 2010, and the

present work), this corresponds to 2 ppm fish solids in a food product. This achieved sensitivity is below 10 ppm, which is postulated as a sensible cut-off value for allergen detection in food (Taylor and Nordlee 1996).

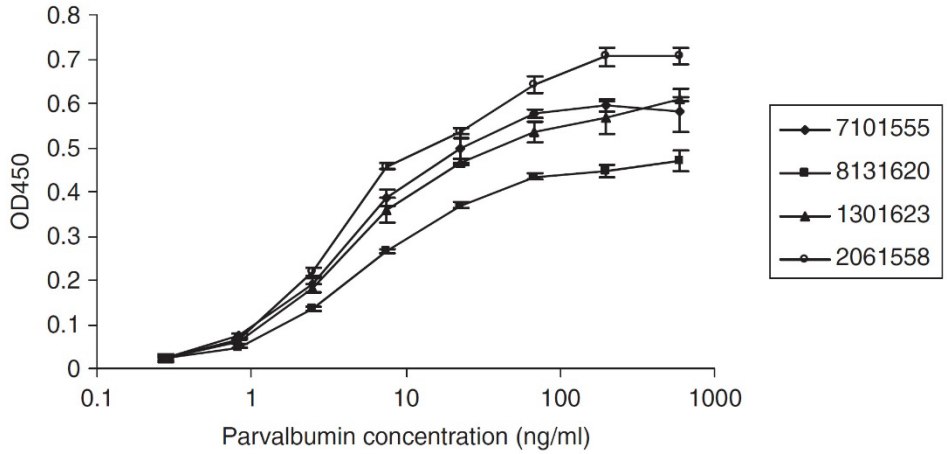

Figure 2. Calibration curves of four independent experiments. Standard deviations are indicated at each datum point as error bars. Curves represent independent experiments.

Table 1. Correlation coefficients and the recalculation of standards in four independent experiments

	Experiment 07101555 $r^2 = 0.998$		Experiment 08131620 $r^2 = 0.998$		Experiment 01301623 $r^2 = 0.994$		Experiment 02061558 $r^2 = 0.986$	
Standards (ng/ml)	Calculated value (ng/ml)	Deviation (%)	Calculated value (ng/ml)	Deviation (%)	Calculated value (ng/ml)	Deviation (%)	Calculated value (ng/ml)	Deviation (%)
600	?	n.a.	?	n.a.	?	n.a.	?	n.a.
200	?	n.a.	117.60	–4	127.21	–36	?	n.a.
66.67	92.79	39	72.54	9	51.47	–23	55.56	–17
22.22	19.20	–14	21.06	–5	21.16	–5	16.55	–26
7.41	7.81	5	7.68	4	8.36	13	9.64	30
2.47	2.40	–3	2.46	0	2.38	–4	2.29	–7
0.82	0.84	2	0.74	–11	0.68	–17	0.68	–18
0.27	0.28	1	0.33	21	0.36	31	0.36	31

Note: ? = infinite concentration calculated; n.a. = not applicable

By measuring dilutions of a cod extract, it is possible to estimate the amount of parvalbumin present in cod. The extract could be diluted 1,562,500-fold before losing reactivity. Taking into account the LLOD of 0.8 ng ml^{-1} and the 1:5 extraction ratio, this corresponds to 6.25 mg of parvalbumin g^{-1} of cod fillet, in line with the abundant parvalbumin band in the cod extract (Fig. 1). Kuehn et al. (2010) found 1.5–2.5 mg g^{-1} of cod tissue with their assay, which is in the same order of magnitude. The difference with our value may be due to different sampling methods. It has been described that the level of parvalbumin is not equally distributed over all parts of the muscle tissue and varies between ventral and

dorsal muscle as well as between anterior and posterior locations in the muscle (Kobayashi et al. 2006).

Specificity of the antibody

The intended use of the ELISA is to test fish skins and rinse water during the manufacturing of fish gelatin. Next to cod, pollock and haddock are other common sources of fish skin for gelatin production from deep sea, wild fish, although a variety of other sources are also used (Regenstein et al. 2010). Table 2 shows the reactivity of some of the individual fish extracts. The commercial source of fish gelatin used in this study is produced from skins of the species in Table 2. Using cod as the reference, the reactivity of pollock and haddock is more than 90% and the reactivity of less commonly used species varies between 37% and 108%, indicating that the species that might commonly be used for this commercial fish gelatin production are recognized by our antibody, although some species are somewhat less reactive. Other fish species are used to produce fish gelatin by other manufacturers; no attempt was made to evaluate all of these species.

Table 2. Reactivity of different fish commonly used to manufacture fish gelatin

Order	Fish species	Scientific name	Approved market name	Absorbance[a]	Relative percentage[b]
Gadiformes	Pacific cod	*Gadus morhua*	Cod	2.02 ± 0.13	100
	Purified cod parvalbumin	n.a.[c]	n.a.	1.72 ± 0.03	85
	Pacific pollock	*Pollachius virens*	Pollock	1.85 ± 0.12	92
	Haddock	*Melanogrammus aeglefinus*	Haddock	2.02 ± 0.13	100
	Cusk	*Brosme brosme*	Cusk	1.91 ± 0.13	95
	Hake	*Urophycis tenuis*	Hake	2.17 ± 0.14	108
Pleuronectiformes	Flounder dab	*Hippoglossoides platessoides*	Plaice	0.76 ± 0.10	37
Scorpaeniformes	Redfish	*Sebastes fasciatus*	Ocean perch	1.00 ± 0.11	49

Notes: [a]Data are mean absorbances ± standard deviation (n = 18 replicates).
[b]Relative recognition = (absorbance of fish – absorbance of cod) * 100.
[c]Not applicable.

Another intended use of the ELISA for parvalbumin is to analyze food ingredients and food products for the presence of parvalbumin. This may be done for quality assurance purposes on food products that do not contain fish as a constituent or ingredient but may contain traces of fish from the food manufacturing processes. We therefore investigated the cross-reactivity of common food ingredients with the ELISA. Fourteen food ingredients were extracted and tested at serial dilutions in the sandwich ELISA: shrimp, beef, pork, lamb, chicken, peanut, egg, corn, milk, wheat, soybean, soy milk, potato, and mixed tree nuts. Also, lager beer and red and white wine were tested for cross-reactivity. Dilutions starting at ten-fold and stepwise three-fold increases to 21,870-fold were tested, and all appeared to be negative (with two times the background signal) for all samples, while cod extract could be diluted over 1.5 million times before losing reactivity.

Recovery of a cod calibration curve in target matrices of beer and wine

Fish gelatin was spiked with parvalbumin in order to investigate potential matrix effects of gelatin on the parvalbumin ELISA. A calibration curve diluted in an extract of fish gelatin is shown in Figure 3. This calibration curve is, to a large extent, similar to the calibration curve prepared in buffer (Figs. 2 and 3). A similar approach was followed to investigate the potential matrix effects of red wine, white wine, and lager beer. These products do not react in the ELISA (see above). Calibration curves of parvalbumin were prepared in tenfold diluted wine and beer and were compared with a calibrator diluted in buffer (Fig. 3). None of the tested matrices affected the reactivity of the parvalbumin, indicating that the assay is suitable for analyzing parvalbumin in fish gelatin, wine, and lager beer.

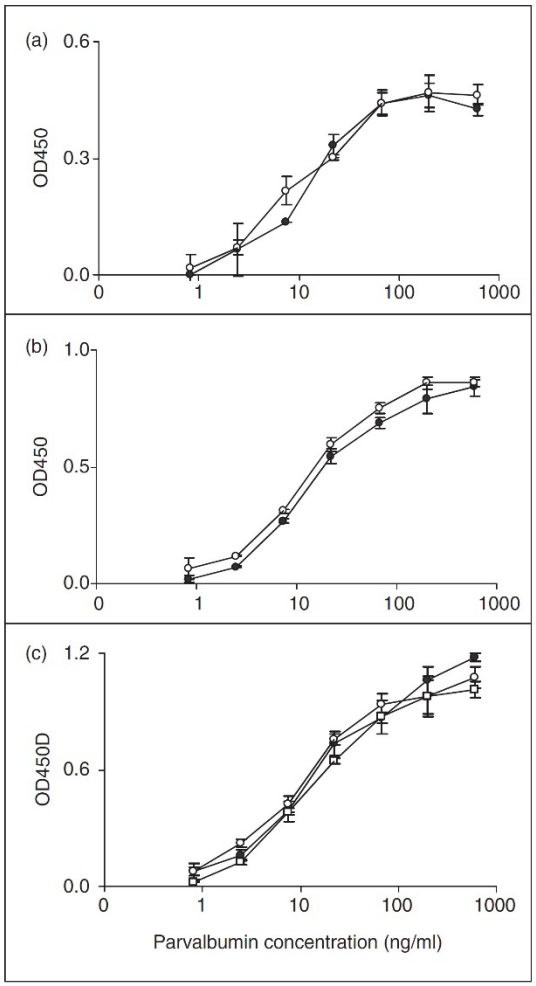

Figure 3. Spiking of parvalbumin in different target ingredients. Standard deviations are indicated at each datum point as error bars. Panel A, parvalbumin in buffer (closed symbol) and in gelatin (open symbol); Panel B, parvalbumin in buffer (closed symbol) and in lager beer (Heineken); and Panel C, parvalbumin in buffer (closed symbol) and in white wine and red wine (open symbols).

Effect of the washing of cod skin on the remaining parvalbumin content of cod skin

Unwashed cod skin was extracted in the laboratory and the parvalbumin determined to be 0.40 ± 0.09 mg g^{-1} skin. This value is over ten-fold lower compared with cod muscle (6.25 mg g^{-1}), which is as expected because the muscle is the primary source of parvalbumin. Washing the skin resulted in a high concentration of parvalbumin in the rinse water of approximately 70 μg ml^{-1} (Table 3). If all of the parvalbumin was washed out, this corresponds to 421 μg g^{-1} of skin, which is in line with the extracted unwashed skin. A second rinse water was prepared by draining the laboratory sample and adding fresh rinse water. In this rinse water approximately 9 μg ml^{-1} parvalbumin was found, indicating that the first rinsing did not completely remove the parvalbumin. The parvalbumin concentration decreased in subsequent rinse waters and leveled off at 0.2 μg ml^{-1} after five washing cycles. Similar data were obtained for other fish species whose skins were laboratory washed (pollock, hake, red fish, haddock, and grey sole; data not shown). An independent experiment was designed to quantify the amount of parvalbumin left behind in the remaining tissues after washing six times. The remaining skin sample was split in two portions. One part was washed once more, and the wash water contained 63 ± 11 ng ml^{-1} parvalbumin, corresponding to 0.38 ± 0.07 μg g^{-1}, or 0.38 ± 0.07 ppm in the unwashed tissue. From the other part of the split skin sample an extract was made, and it contained 83 ± 4 ng parvalbumin ml^{-1}, corresponding to 0.50 ± 0.03 μg g^{-1} or 0.50 ± 0.03 ppm parvalbumin in the unextracted tissue. This value is the same order of magnitude as the value based on the seventh washing, indicating that this washing procedure indeed solubilized the vast majority of the parvalbumin present in skins. Compared with the parvalbumin content of unwashed skin (0.4 mg g^{-1} of skin) the values for washed skin are about a 1000-fold lower. These skins were washed in the laboratory for a brief period using gentle agitation. Commercial production of fish gelatin utilizes longer washes with far greater agitation, suggesting that the remaining parvalbumin in commercially washed skins is lower.

Table 3. Parvalbumin in skin samples and rinse water samples and extract of skins

	Parvalbumin concentration in wash water (μg/ml)	Parvalbumin amount relative to original skin sample (μg/g)
Wash water No. 1	70 ± 16	421 ± 96
Wash water No. 2	8.7 ± 1.2	52 ± 7
Wash water No. 3	0.86 ± 0.20	5.2 ± 1.2
Wash water No. 4	0.34 ± 0.08	2.0 ± 0.5
Wash water No. 5	0.25 ± 0.08	1.5 ± 0.5
Wash water No. 6	0.21 ± 0.03	1.3 ± 0.19
Wash water No. 7	0.063 ± 0.011	0.38 ± 0.07
Unwashed cod skin	n.a.	400 ± 90
Washed cod skin	n.a.	0.50 ± 0.03

Note: n.a. = not applicable

Analysis of commercially produced fish gelatin samples

Samples from 95 batches of fish gelatin from one manufacturer and produced in the year 2010 were analyzed. Of these, 73 were below the detection limit (< 0.02 ppm), while the other 22 were slightly above (range = 0.02–0.15; mean = 0.058 ± 0.027 ppm). The highest positive samples contained 0.15 ppm parvalbumin or 0.15 mg parvalbumin kg^{-1} fish gelatin.

Trace amounts of parvalbumin in various fish skin-derived food ingredients: consequence for fish-allergic consumers

Some assumptions need to be made to relate the figure of 0.15 mg kg^{-1} to the amount of fish that can trigger allergic reactions. Fillets from white fish like cod contain 15–20% protein. Limited studies on minimal provoking doses for fish in fish-allergic individuals (reviewed by Taylor et al. 2002) indicate that the most sensitive fish-allergic individuals react to the consumption of 5–10 mg of fish protein (Hansen et al. 1992; Helbling et al. 1999), corresponding to 25–67 mg of fish fillet based on the average of 20% protein in fillets. In cod fillets we found 6.25 mg parvalbumin g^{-1} of wet weight, in the same order of magnitude reported by Kuehn et al. 2010 (1.5–2.5 mg g^{-1}). Using an arbitrary average of 5 mg g^{-1}, 25–67 mg fillet corresponds to 0.125–0.33 mg parvalbumin which led to reactions in the most sensitive subjects that were tested. Thus, such individuals would need to ingest 1–2 kg of fish gelatin to obtain a minimal provoking dose of parvalbumin. Therefore, the level of parvalbumin in fish gelation offers a large safety factor for fish-allergic, parvalbumin-allergic individuals. Not surprisingly, an oral challenge trial of fish gelatin derived from cod in cod-allergic subjects demonstrated that fish gelatin at levels up to 3.61 g were quite safe for such individuals (Hansen et al. 2004). A main application of fish gelatin is fining of beer and wine. Typically, 10–50 g are added to 100 L of wine, corresponding to 10–50 mg per glass of wine. However, most of the parvalbumin would be expected to precipitate with the fines so this level of exposure is likely unrealistic. Considering 0.15 ppm parvalbumin in the highest positive fish gelatin samples, this corresponds to 1.5–5 ng of parvalbumin per glass of wine. This highly conservative level of exposure is over 25,000-fold lower that the estimated minimal provoking doses of parvalbumin needed to provoke mild objective reactions in fish-allergic patients (Hansen et al. 1992; Helbling et al. 1999). For potent food allergens such as peanut, the thresholds for inducing subjective, transitory, very mild allergic reactions are in the range of 30–100 mg (Hourihane et al. 1997; Wensing et al. 2002), over 1000-fold higher than the parvalbumin levels we found in fish gelatin. Although lesser threshold data are available for fish allergic patients, the limited data indicated that fish allergic patients do not typically respond to such low levels. In fact, Rolland et al. (2006) tested the potential allergenicity of wines fined with isinglass, milk, or egg proteins in vivo. There was no correlation between reactions and the presence of fining agents. Similarly, using basophil histamine release assays, which are considered to be very sensitive for allergen-specific reactivity (Knol 2006), no correlation was found between the use of fining agents and histamine release (Rolland et al. 2006). Fining ingredients, including milk, egg, isinglass, and fish gelatin, were tested on their ability to induce a skin reaction in allergic patients. While milk- and egg-based fining ingredients induced a skin reaction in milk- and egg-allergic patients, respectively, it was shown that neither isinglass nor fish

gelatin was reactive in the skin (Kirschner et al. 2009). Our data on the low levels of parvalbumin present in fish gelatin are in line with these observations.

Another study investigated the allergenicity of fish gelatin by means of a double-blind, placebo-controlled food challenge. Here it was demonstrated that none of the tested patients ($n = 30$) reacted on a cumulative dosage of 3.6 g of fish gelatin. At an increased dosage (7.6 g), one patient experienced a mild, transitory subjective reaction (Hansen et al. 2004). It should be noted that the challenge material was fish gelatin, and because fish gelatin is normally used as a minor ingredient, the examined doses are considered to be very high.

Conclusions

Using the assay we developed for the quantification of parvalbumin in cod skin, it is shown that the washing of cod skins, reflecting the current practice in fish gelatin manufacturing, decreases parvalbumin content to a level of 0.5 μg g^{-1} of skin. This level is below the threshold for triggering allergic reactions in fish-allergic consumers. Consequently, in commercially produced fish gelatin the levels of detectable parvalbumin are very low as well. Using such fish gelatins for fining beer and wine does not represent a risk for fish allergic consumers when the manufacturer verifies that the parvalbumin content is very low, as was done in this case.

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