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ARTICLE

Competitive Indirect ELISA for the Determination of Parvalbumins from Various Fish Species in Food Grade Fish Gelatins and Isinglass with PARV-19 Anti-parvalbumin Antibodies

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Parvalbumins are well-known as major fish allergens. However, no quantitative analytical method is currently available for the determination of parvalbumins from different fish species. The aim of this study was the isolation of the various parvalbumins by the application of gel chromatography and dialysis and the development and validation of a competitive indirect ELISA for the determination of parvalbumins from various fish species. This ELISA method was applied to several fish gelatins and isinglass samples used in food production. The competitive ELISA was capable of detecting all tested parvalbumins within a range of 0.1−0.5 mg/L. No parvalbumin was detected in any of the investigated fish gelatins or in a fish skin used as raw material for fish gelatin production. Contrarily, isinglass was found to contain parvalbumin amounts of up to 414.7 ± 30.6 mg/kg.

KEYWORDS: Parvalbumin; ELISA; PARV-19; isinglass; fish gelatin; cod; hake; haddock; tilapia; tuna; sturgeon; pollock; salmon

INTRODUCTION

Parvalbumins are 10−13 kDa in weight, acidic, water-soluble, and calcium-binding proteins that appear mainly in the white muscle of fish but also in the muscle tissues of all other vertebrates. More than 20 parvalbumins from fish species such as cod (*1*), pollock (*2*), Atlantic salmon (*3*), and mackerel (*4*) have been classified as major fish allergens. These parvalbumins are highly cross-reactive due to the conserved amino acid sequences located in the calcium-binding domains (*5*, *6*). Thus, individuals allergic to fish are usually advised to avoid all kinds of fish and fish products in their diet because even low amounts of fish muscle of only a few milligrams can trigger an allergic reaction (*7*).

Although products composed of fish muscle are obviously allergenic, no information is available on whether products derived from other tissues can present a risk to the affected humans. Important products are fish gelatin and fish swim bladders, which are widely used in the food and pharmaceutical industries (*8*). Fish swim bladders for commercial uses are mostly designated "isinglass". Fish gelatins are used as a thickener, stabilizer, dietary supplement, carrier for flavors and dyestuffs and as a processing aid in the production of various beverages, such as beer, wine, sparkling wine, and juices. Isinglass is commonly used in the production of some beverages, particularly beer, wine, sparkling wine, cider, and juice. It contributes to the organoleptic properties and stability against proteinogenic haze due to the adsorption of polyphenolic compounds and proteins.

Fish gelatin is basically derived from skins of various species such as cod, pollock, haddock, hake, tilapia, tuna, perch, cusk, flatfish, and redfish. Isinglass is derived from the swim bladders of the same and other genera, such as sturgeon, catfish, croaker, and threadfin (*8*). Usually, these products are composed of 80−95% collagen or its fragments. Although collagen from fish does not seem to present a risk for humans sensitive to fish (*8*, *9*), the adherence of or contamination with fish muscle tissue may lead to significant and hazardous amounts of parvalbumin. If parvalbumin is not present, then these products may be considered safe for consumption by fish allergic individuals (*9*).

Until now, no quantitative analytical method has been available for the determination of parvalbumins from various fish species. Chen et al. presented an effective method for the qualitative detection of parvalbumins from carp, catfish, cod, and tilapia by immunoblotting, using a commercial anti-parvalbumin PARV-19 antibody (*10*). Faeste et al. developed a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the determination of fish muscle using self-made anti-cod parvalbumin antibodies (*11*). However, no information is given whether this method will sufficiently detect parvalbumins from various fish species. Van Do et al. developed three qualitative indirect ELISA methods by using different polyclonal antibodies (*6*). Native parvalbumins were recognized by these antibodies, but no information was given about the extent of cross-reactivity and which native parvalbumins were investigated. A recent study from Gajewski et al. used fish extracts for their qualitative ELISA that were not characterized with regard to the parvalbumin

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content (*12*). This group used the same commercial anti-parvalbumin PARV-19 antibody as Chen et al.

The aim of the present study was the isolation of parvalbumins from various fish species important for the production of fish gelatins and isinglass by applications of gel chromatography and dialysis. These purified parvalbumins were used in the development and validation of a competitive indirect ELISA using commercial PARV-19 anti-parvalbumin antibodies raised against frog muscle parvalbumin. The ELISA method was applied to various food grade fish gelatins and isinglass samples to detect residues of parvalbumins.

MATERIALS AND METHODS

Fish Samples. Eight samples of fish muscle were purchased filleted and frozen from local stores: cod (*Gadus* ssp.), pollock (*Pollchius virens*), hake (*Merluccius capensis*), haddock (*Melanogrammus aeglefinus*), tuna (*Thunnus* ssp.), salmon (*Salmo salar*), sturgeon (*Acipenser* ssp.), and tilapia (*Oreochromis* ssp.).

Fish Gelatins and Isinglass Samples. Between 2005 and 2008, 12 samples were purchased directly from a total of 7 different manufacturers from Germany (Erbslöh GmbH, Gustav Permentier GmbH, SIHA Begerow GmbH, Naumann GmbH), France (Rousselot SAS), and the United States (Norland Products Inc., Sigma Aldrich Co.). Fish gelatins ($n = 5$) were derived from fish skins and consisted of yellow powders or pellets. Hydrolyzed fish gelatins ($n = 2$) were also derived from fish skins and consisted of white powders. Isinglass samples ($n = 4$) were provided as brown-yellow, yellow, or white sheets or, in one case, as a white powder. Two isinglass samples were derived from sturgeon (*Acipenser*). Finally, one dried fish skin derived from cod and used as a raw material for the fish gelatin production was provided.

Purification of Parvalbumins. Fish muscle tissues were extracted as described elsewhere (*13*). Briefly, the fish muscle tissue was homogenized with 3 volumes of distilled water using an Ultra Turrax T25 at 8000 rpm (IKA Labortechnik, Staufen, Germany). The homogenate was shaken for 20 min and, with shaking continued, subsequently heated for 30 min at 70 °C. The heated homogenate was cooled with ice water and centrifuged for 30 min at 5 °C and 10000*g* (Sigma 3K12, Sigma Laborzentrifugen, Osterode, Germany). The supernatant containing the heat-stable parvalbumin was freeze-dried (Christ Alpha 1-4 LD, Osterode, Germany), and 40−100 mg of the dry matter was used for gel chromatography. A 100 × 2.5 cm glass column was filled with a Sephadex G-50 medium (Sigma-Aldrich, Schnelldorf, Germany) previously swollen in a 0.15 M NaCl solution. The dry matter was dissolved in 4 mL of the 0.15 M NaCl solution, filtered through a 0.2 μm PTFE membrane filter (CS Chromatographie Service, Langerwehe, Germany), and injected onto the column. Parvalbumin was eluted with 0.6 mL/min of the 0.15 M NaCl solution using a LaChrom L-7100 HPLC pump, an L-7400 UV detector (Merck Hitachi, Tokyo, Japan), and a Foxy Jr fraction collector (Teledyne Isco, Kreuztal, Germany). The fraction size was set to 15 mL (corresponding to 25 min), and parvalbumin was detected at 220 nm. Fractions containing the parvalbumin (controlled by SDS-PAGE) were united, freeze-dried, redissolved in double-distilled water, filtered through a 0.2 μm PTFE membrane filter, and dialyzed against the 75-fold volume of double-distilled water in a 3500 MWCO Slide-A-Lyzer dialysis cassette (Peribo Science, Bonn, Germany) to remove any salts. Dialysis was performed for 48 h, and the water was changed three times during this period. Finally, the cassette containing the parvalbumin was emptied, the solution was freeze-dried, and the residue representing the pure parvalbumin was stored at −24 °C.

SDS-PAGE. SDS-PAGE was performed on Invitrogen equipment (Invitrogen, Karlsruhe, Germany) using a Powerease 500 power supply, an XCell Surelock chamber, and an MES running buffer as the electrolyte: 2.5 mM 4-morpholineethanesulfonic acid (MES), 2.5 mM tris(hydroxymethyl)aminomethan (Tris) base, 0.005% SDS, and 0.05 mM EDTA, pH 7.3. The proteins were separated with 1 mm thick, 8 × 8 cm, precast NuPAGE Novex gels with 12% acrylamide and Bis-Tris buffer system (Invitrogen). Serva Mark12 (Invitrogen) served as the molecular weight ladder. Electrophoresis was performed at 200 V for 45−50 min. Gels were silver stained according to the procedure described by Heukeshoven et al. (*14*) or were blotted onto nitrocellulose membranes according to the procedure described by Weber et al. (*15*). The immunostaining of the membranes was performed on the basis of the competitive indirect ELISA procedure with a 1:3000 dilution of the anti-parvalbumin antibody.

Sample Preparation. Fish gelatins were dissolved in a 20-fold excess of a Tris−Ca solution (pH 7.4), composed of 50 mM Tris-HCl, 150 mM NaCl, and 0.5 mM $CaCl_2$ in double-distilled water, and adjusted to a pH of 7.4 with sodium hydroxide. If necessary, to achieve a clear solution, the suspension was heated for 20 min at 40 °C. Recovery experiments were performed by the addition of definite amounts of cod parvalbumin to this solution before heating.

Isinglass and the fish skin derived from cod were cut into small pieces and ground in a mortar. A 20-fold excess volume of the Tris−Ca solution was added, and the suspension was homogenized using an Ultra-Turrax T25. The extraction was performed overnight under shaking conditions. Insoluble material was removed by centrifugation for 30 min at 8000*g*, and the supernatant was stored at −80 °C.

Competitive Indirect ELISA. The following solutions were prepared: coating buffer, substrate reagent, and citric buffer were prepared as described elsewhere (*16*). Tris−Tween 20 solution contained 50 mM Tris, 150 mM NaCl, and 0.5% polyethylene-sorbitan monolaurate (Tween 20) in double-distilled water (*17*). The Tris−Tween 20−Ca solution, pH 7.4, was composed of a Tris−Tween 20 solution supplemented with 0.5 mM $CaCl_2$ and adjusted to a pH of 7.4 with hydrochloric acid.

For the competitive ELISA, 0.4 μg/mL of cod parvalbumin was dissolved in the coating buffer, and 250 μL/well of this solution was coated to a certified Maxisorp F96 polystyrene microtiter plate (Nunc, Wiesbaden, Germany) overnight at 8 °C. The plate was washed three times with 300 μL of the Tris−Tween 20 solution. Afterward, free binding sites of the wells were blocked with 250 μL/well of the Tris−Tween 20 solution for 2 h at room temperature. Finally, the plate was washed three times with 300 μL of the Tris−Tween 20−Ca solution.

The competitive ELISA procedure was performed by adding 75 μL/well of the sample solution and 125 μL/well of a monoclonal PARV-19 mouse anti-frog parvalbumin antibody solution (Sigma, Schnelldorf, Germany), diluted to 1:42000 in the Tris−Tween 20−Ca solution, into the coated wells in succession. After incubating for 1.5 h at room temperature, the plate was washed three times with 300 μL/well of the Tris−Tween 20−Ca solution. Thereafter, 200 μL/well of a goat anti-mouse peroxidase conjugated IgG solution (Sigma), diluted to 1:2000 in the Tris−Tween 20−Ca solution, was added and incubated for another 1.5 h at room temperature. The wells were washed four times with 300 μL/well of the Tris−Tween 20−Ca solution and finally filled with 200 μL/well of the substrate reagent. The enzymatic colorimetric reaction was performed for 20−40 min at 8 °C in darkness and stopped by the addition of 100 μL of 2 M sulfuric acid. The optical density (OD) values were measured at 450 nm against a reference wavelength of 630 nm using an MRX microtiter plate reader (Dynex Technologies, Chantilly, VA). The plate was covered with a plate lid during each incubation step (Nunc).

The attained curves were evaluated by AssayZap software (Biosoft, Cambridge, U.K.) using a four-parametric regression. Therefore, the ODs were plotted against the logarithm of the parvalbumin concentration. The limit of decision (LODC; 50% probability that the measured values lie within the spread of the blank values B_0) was defined as the mean B_0 minus 3-fold the standard deviation of B_0. The limit of detection (LOD; 99.8% probability that the measured values are larger than the spread of B_0) was defined as the mean B_0 minus 6-fold the standard deviation of B_0 (*18*, *19*).

RESULTS

Purification of Various Parvalbumins. Seven of the eight fish muscle extracts were found to contain parvalbumin by SDS-PAGE (**Figure 1**). Parvalbumins were detected between 8 and 11 kDa in cod, pollock, hake, haddock, salmon, sturgeon, and tilapia. No parvalbumin was detected in the white muscle of tuna, which is in good accordance with the findings of Chen et al. (*10*) and may be explained by the diverse distribution of parvalbumin between different muscle tissues in tuna (*20*).

Sufficient yields of parvalbumin were achieved by gel chromatography and dialysis from cod, pollock, hake, haddock, sturgeon,

and tilapia. Salmon contained very low amounts of extractable parvalbumin, and tuna was not found to contain any extractable parvalbumin at all. Thus, neither species was used in the subsequent experiments. Cod parvalbumin was eluted in the gel chromatography between approximately 455 and 590 min as illustrated in **Figure 2**. Chromatograms of the other fish species were similar. Three fractions collected between 500 and 575 min were found to contain no proteins apart from the parvalbumin discovered by SDS-PAGE with silver staining and immunostaining (**Figure 3**). After all, about 2−4 mg of pure parvalbumin was achieved per injection.

Competitive Indirect ELISA. All purified parvalbumins were recognized by the monoclonal PARV-19 anti-parvalbumin antibody in the competitive indirect ELISA with moderate to strong cross-reactivities among the various parvalbumins. Antibody binding was strongly inhibited between 0.1 and 100 mg/L as illustrated in **Figure 4**. Fifty percent inhibition was achieved with 0.7 mg/L parvalbumin from tilapia and 2.4−5.4 mg/L parvalbumin from the remaining five fish species. Thus, cross-reactivities related to cod parvalbumin were 614% for tilapia, but between 80 and 179% for the remaining parvalbumins. LODC and LOD values were determined between 0.1 and 0.5 mg/L (LODC) and between 0.2 and 1.2 mg/L (LOD). Coefficients of variation (CVs) were < 15% in almost every case (**Table 1**).

Matrix effects caused by the various fish collagen-based products were investigated by spiking the experiments with cod parvalbumin. Therefore, two fish gelatins and one hydrolyzed fish gelatin sample were spiked with cod parvalbumin at levels of 2, 20, 60, 200, and 400 mg/kg of fish gelatin. Recovery rates were found between 79 and 140% within the range of 60−400 mg/kg of fish gelatin. The mean LODC was determined to be 10.6 mg/kg of fish gelatin (7−13.8 mg/kg) and the mean LOD at 18.7 mg/kg of fish gelatin (14.4−21.8 mg/kg). In any case, the CVs were all < 10%. The results are shown in **Table 2**.

Sample Investigation by Competitive Indirect ELISA. Neither fish gelatin nor hydrolyzed fish gelatin revealed inhibitions significantly different from the background noise in the competitive indirect ELISA. Even the fish skin from cod was found to contain no detectable amount of parvalbumin.

Contrarily, two of the four investigated isinglass samples showed a strong inhibition in the competitive indirect ELISA. One isinglass sample was derived from sturgeon and the other sample from non-sturgeon fishes. The parvalbumin content of the isinglass sample derived from sturgeon was determined by

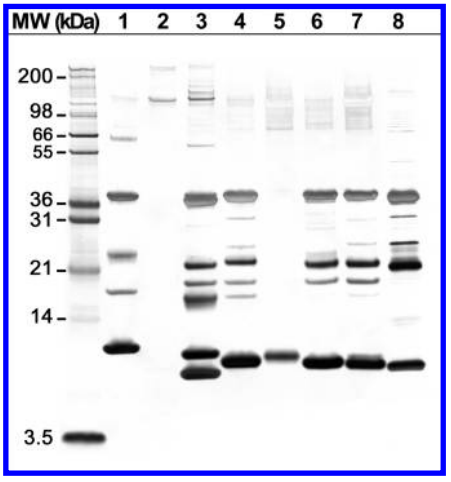

Figure 1. SDS-PAGE with silver staining of various fish muscle extracts. Lanes: MW, molecular weight ladder; 1, sturgeon; 2, tuna; 3, tilapia; 4, pollock; 5, salmon; 6, cod; 7, haddock; 8, hake.

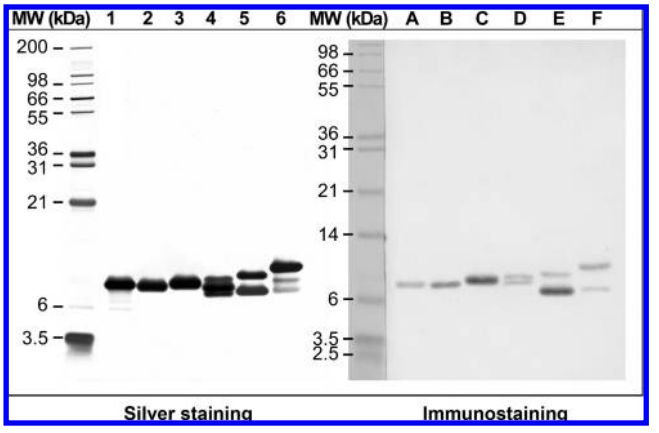

Figure 3. SDS-PAGE with silver staining and immunostaining of the purified parvalbumins. Lanes: MW, molecular weight ladder; (silver staining) 1, cod; 2, haddock; 3, pollock; 4, hake; 5, tilapia; 6, sturgeon; (immunostaining with PARV-19 antibody) A, cod; B, haddock; C, pollock; d, hake; E, tilapia; F, sturgeon.

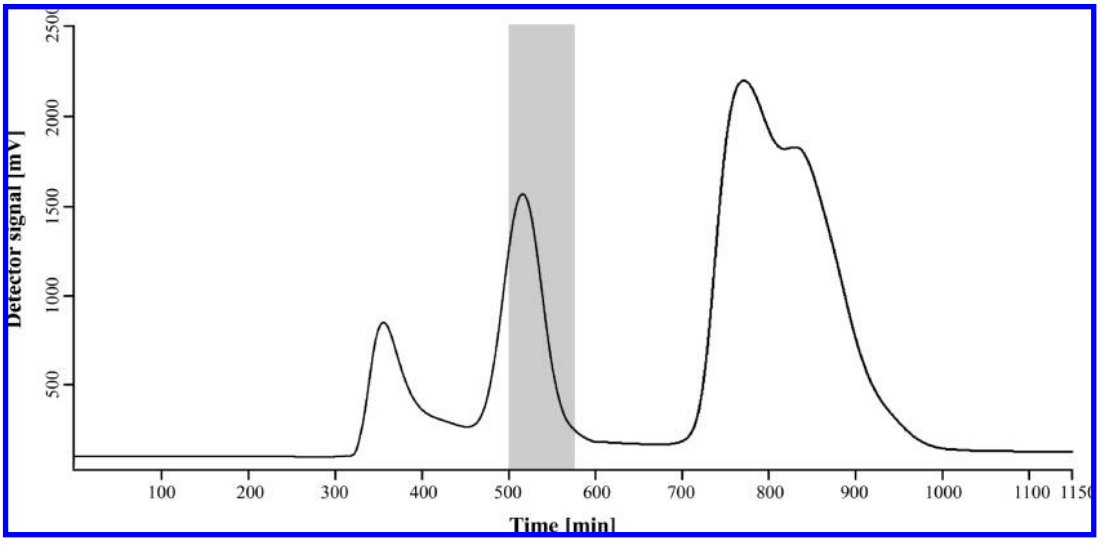

Figure 2. Gel chromatography of the dry matter derived from cod muscle tissue. The gray box indicates the fractions used for the further parvalbumin purification.

calibration against the sturgeon parvalbumin, resulting in a content of 414.7 ± 30.6 mg/kg of isinglass. The mean parvalbumin content of the non-sturgeon-derived isinglass was determined to be 59.9 ± 6.0 mg/kg of isinglass. However, the content largely depended on which of the five non-sturgeon parvalbumins had been used for calibration (from 14.5 ± 1.3 to 113.0 ± 10.5 mg/kg; **Table 3**). Interassay variation of these results was demonstrated with a calibration against cod parvalbumin on four different plates and on four different days, and this revealed a value of 8.8%. All positive findings were confirmed by repeated tests and SDS-PAGE (**Figure 5**).

DISCUSSION

The developed competitive indirect ELISA was suitable to detect parvalbumins from all investigated fish species. Four of the five parvalbumins revealed high cross-reactivities between 80 and 179% in relation to the cod parvalbumin shown in **Table 1**. Cod, pollock, haddock, and hake belong to the same order, *Gadiformes*, whereas tilapia belongs to the order *Perciformes*. This may explain the significantly greater affinity of parvalbumin from tilapia to the PARV-19 antibody, which resulted in a cross-reactivity of 614%. Generally, sequence identities of various fish species in relation to cod parvalbumin are between 60 and 70%. Parvalbumin from hake (*Merluccius merlussic*) was found to present a sequence identity of 65% and tilapia (*Oreochromis mossambicus*) an identity of 64% in relation to cod parvalbumin [*Gadus morhua*; National Center of Biotechnology Information BLAST (NCBI Blast), www.ncbi.nlm.nih.gov]. Chen et al. already discussed that the calcium-binding motifs of the parvalbumin proteins are the highest conserved regions and that the PARV-19 antibody binding epitope lies within these calcium-binding motifs or, at least, is strongly associated with them (*10*). Consequently, the appearance of cross-reactivities was not surprising. The competitive ELISA was able to detect all of the investigated fish parvalbumins within a narrow range of 0.1−0.5 mg/L (LODC) with a suitable degree of precision and accuracy even in fish gelatin matrices. Different results for isinglass are unlikely because they are practically composed of the same chemical components. These results are in accordance with the qualitative findings of Chen et al. (*10*). Contrarily, the study of Gajewski et al. found that PARV-19 is not able to detect parvalbumin from cod, haddock, and pollock by indirect ELISA (*12*). However, the use of fish extracts rather than purified parvalbumin must be noted. Fish extracts from various fishes or from various muscles within the same individual may contain very different amounts of parvalbumin and calcium (*20*). Calcium is of high importance for the binding of PARV-19. Additionally, an indirect ELISA appears to be problematical for this kind of standard material because the surface of the ELISA wells may be blocked by other matrix proteins rather than by the parvalbumin in the coating step. In this case, parvalbumin cannot bind properly to the well surface. Both the use of nonstandardized material and the use of an indirect ELISA may cause various problems for the proper detection of parvalbumin and may have led to the different findings.

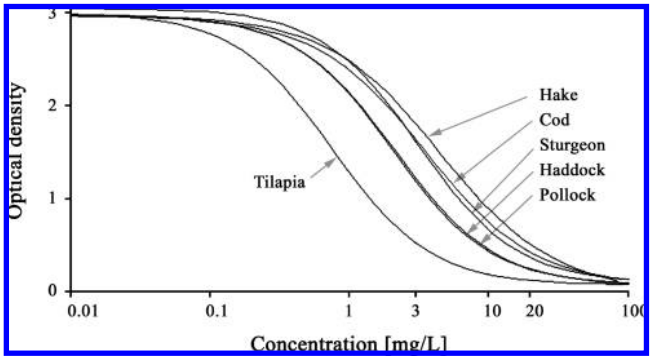

Figure 4. Competitive indirect ELISA curves of various parvalbumins.

In this study, no parvalbumins or antigenic parvalbumin fragments were detected in commercial fish gelatins and a fish skin from cod by the competitive indirect ELISA. With regard to the LODC of cod parvalbumin in the different fish gelatin matrices, amounts of antigenic compounds could be considered lower than approximately 10 mg/kg in these substances. Fish skin, used as a raw material for the production of fish gelatins, is closely associated with muscle tissue. However, the results of this study indicated that fish muscle tissue is properly separated from the fish skin during production. Usually, fish skins are thoroughly washed to remove remaining muscle tissue, fish bones, salts, and off-flavors. This step obviously contributes to the removal of the parvalbumin from the raw material. Nevertheless, Andre et al. found immunoreactivities of some sera from fish allergic humans against parvalbumins in a self-prepared tuna fish skin. No information is given in that study as to whether the investigated fish skin had been treated comparably to commercial fish skins, particularly by washing. Thus, the comparability with the results of this study is limited (*21*).

Intensive processing, especially acidic and enzymatic hydrolysis and heating, is needed to derive fish gelatins from washed fish skins (*22*). These steps most likely lead to a degradation of potential parvalbumin traces in the raw material so that parvalbumins were detected neither in the raw nor in the finished material. Accordingly, it appears to be reasonable to expect potential parvalbumin residues in commercial fish gelatins in the range of a few milligrams per kilogram or lower. Considering the low intake of these substances, it appears unlikely that fish gelatins can represent a risk to individuals allergic to fish. They usually do not appear as major ingredients in foodstuffs and are not ingested in high quantities with a few exceptions. Thus, the intake of potential parvalbumin residues due to these substances appears to be negligible. This assumption is supported by two independent double-blind placebo-controlled food challenges (DBPCFC) performed by Hansen et al. and Andre et al. with gelatin derived from cod and tuna (*21*, *23*). Neither group observed allergic reactions with clinical relevance in a total of 33 humans allergic to fish for cumulative dosages of 5 g (3 patients) and 14.6 g of fish gelatin (30 patients), respectively.

Table 1. LODC, LOD, CV, 50% Inhibition, and Cross-Reactivity Related to Parvalbumin from Cod for Parvalbumins from Various Fish Species

	LODC (mg/L)	LOD (mg/L)	CV (%)	50% inhibition (mg/L)	cross-reactivity related to cod (%)
cod	0.3	0.7	1.1−8.1	4.3	
hake	0.2	0.5	2.8−5.9	5.4	80
tilapia	0.1	0.2	2.5−12.6	0.7	614
pollock	0.4	1.2	4.4−13.7	2.7	159
sturgeon	0.5	0.8	4.0−14.6	3.9	110
haddock	0.3	0.6	2.7−19.4	2.4	179

Table 2. LODC, LOD, CV, and Recovery Rates of Cod Parvalbumin Spiked to Various Fish Gelatins

	LODC (mg/kg)	LOD (mg/kg)	CV (%)	recovery rates between 60 and 400 mg/kg (%)
fish gelatin 1	7.0	14.4	1.5−5.2	88−130
fish gelatin 2	13.8	21.8	0.8−8.0	93−140
hydrolyzed fish gelatin	11.0	20.0	2.5−9.3	79−130

Table 3. Parvalbumin Contents in Two Different Isinglass Samples Derived by Various Calibration Materials

parvalbumin used for calibration	isinglass derived from sturgeons (mg/kg)	isinglass derived from non-sturgeon fishes (mg/kg)
cod		82.6 ± 8.4
hake		113.0 ± 10.5
tilapia		14.5 ± 1.3
pollock		46.7 ± 7.1
sturgeon	414.7 ± 30.6	
haddock		42.5 ± 2.6
mean value		59.9 ± 6.0

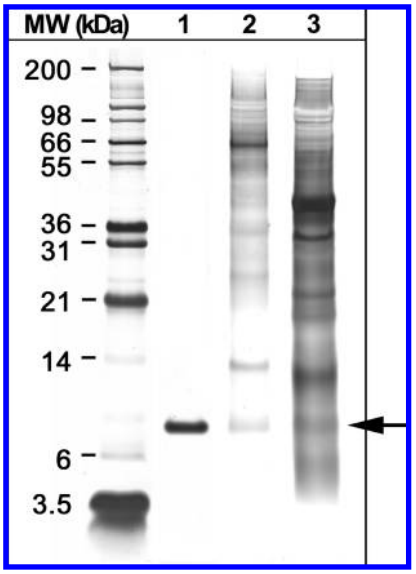

Figure 5. SDS-PAGE with silver staining of the two positive tested isinglass samples. The arrow indicates parvalbumin detected with silver staining. Lanes: MW, molecular weight ladder; 1, cod parvalbumin; 2, isinglass from sturgeon; 3, isinglass from non-sturgeon fishes.

No reactions were found in vivo by skin prick tests (SPTs) using two different fish gelatins in a total of seven allergic individuals (*21*, *24*), whereas Hansen et al. observed positive skin reactions with cod fish gelatin in 3 of 30 patients allergic to fish. However, positive SPTs are not necessarily proof of allergic reactions, and no patient with a positive skin reaction revealed any clinically relevant allergic reactions after a cumulative ingestion of 14.6 g of cod fish gelatin (*23*).

In conclusion, no parvalbumin was detected in various fish gelatins by the competitive ELISA and, also, no evidence exists that fish gelatin can cause allergic reactions after oral administration.

Contrary to fish gelatins, definite amounts of the parvalbumins were detected in the isinglass samples. Many fish species possess a fast-twitch muscle tissue in the swim bladder that allows them to produce species-specific sounds. These muscles contain high amounts of parvalbumin and could be the cause of the high parvalbumin amounts found in this study (*25*, *26*). These amounts were found in a broad range from 0 to 414.7 ± 30.6 mg/kg in four commercial products. This variation could be due to the various production procedures applied by the different manufacturers (*27*, *28*). The production process includes several steps, such as washing and cleaning, conditioning in hot water, removal of muscle layers and blood vessels, and treatment with hydrogen peroxide, but does not have a high level of standardization. Thus, residual levels of parvalbumins may vary between various manufacturers.

Elsewhere, parvalbumin was detected in various isinglass preparations in levels up to 35 mg/kg with a sandwich ELISA specific to cod (*27*). However, it remains unknown whether this sandwich ELISA presented sufficient cross-reactivity to parvalbumins from other fish species and, thus, whether parvalbumin amounts were underestimated in that study. Due to the findings of this earlier study, the industry has adopted a code of good manufacturing practice (GMP) to reduce the parvalbumin content in isinglass. This code includes additional washing stages, sieving steps, and the exclusion of fish species with high parvalbumin levels in the swim bladder. Investigation of nine isinglass samples treated according to this GMP revealed amounts of parvalbumin of < 0.7 mg/kg with the same cod-specific sandwich ELISA (*27*). Nonetheless, considerably higher parvalbumin amounts were found in this study by a competitive indirect ELISA sensitive to various fish parvalbumins. These findings suggest that the isinglass production and the GMP should be monitored with respect to the level of parvalbumins to ensure consumer safety. Therefore, the competitive indirect ELISA developed in this study seems to present a good and reliable tool.

As stated in the Introduction, isinglass is commonly used in the treatment of some beverages. After treatment, the insoluble and precipitated isinglass material is removed from the beverage along with the adsorbed substances. Recommendations for the dosage of isinglass are in the range of 0.005−0.05 g/L (*27*, *29*). Anyhow, no conclusion could be made yet about the amounts of parvalbumin that could possibly migrate from the isinglass material into the beverage and remain in the final product. The extractability from isinglass material in the various beverage matrices has not been established, and some ingredients that are naturally present in the affected beverages, particularly polypenolic compounds, are known for their protein-precipitating properties. Additional applied processing, such as filtration or stabilization, is also known to contribute to the removal of proteins as demonstrated earlier (*8*, *15*). Therefore, it would be highly speculative to give any conclusions now concerning the hazards of isinglass used for the treatment of beverages for consumers allergic to fish. Furthermore, a serious lack of clinical data concerning parvalbumin amounts that could be considered to be clinically safe [usually defined as "no observed adverse effect level" (NOAEL)] must be noted. A few studies have indicated that allergenic amounts of fish muscle are about a few milligrams, but the data about parvalbumin contents in the used materials are nonexistent (*7*). Unfortunately, the content of parvalbumin in fish muscle is highly variable among different fish species and between different muscle positions as demonstrated in this and in another study (*20*). Thus, clinical studies should be well elaborated and carefully arranged to assess data about the NOAEL of the dominant fish allergen parvalbumin in humans. According to the results in the present study, the treatment of beverages with 0.05 g/L isinglass may cause parvalbumin amounts, in the worst case, of up to 0.021 mg/L. This is significantly lower than the threshold doses reported for fish and other food allergens (*7*). Adverse reactions from the consumption of beverages treated with isinglass are currently unknown. This could be due to an

absence of allergic reactions or the fact that these reactions are under-reported because fish allergens are usually not expected in beverages by consumers or physicians. No clinical reactions were observed by Kirschner et al. using SPTs with three different isinglass preparations and in a DBPCFC with 200−300 mL of wine treated with up to 2.5 mL/L of various 2% isinglass preparations (= 0.05 g/L)(*24*). No adverse reactions were visible in a DBPCFC performed by Rolland et al. using 200 mL of various isinglass-fined wines and 10 humans allergic to fish (*30*).

Many different nations, such as the member states of the European Community, Australia, New Zealand, the United States, and Japan, have introduced special rules for labeling fish gelatin and isinglass because they are derived from an allergenic foodstuff. Exceptions from labeling have been granted by the European Community for the use of fish gelatin and isinglass as fining agents in wine and beer production (Directive 2007/68/EC). According to the results published in this study, fish gelatin and fish skin used as a raw material were found to contain no detectable amounts of parvalbumin. No clinical reactivity was found for fish gelatin in two studies (*23*, *24*). No evidence exists indicating that the fish collagen protein itself can trigger allergic reactions (*8*, *9*). Thus, no reason is given to consider the products as allergenic. On the contrary, isinglass was found to contain high amounts of parvalbumin, but it is used in small amounts in the treatment of beverages. Therefore, it appears currently unclear whether beverages that were treated with isinglass could present a health risk to humans.

In conclusion, a sensitive and reliable competitive ELISA was developed and shown to be able to detect parvalbumins from all investigated fish species within a narrow range of 0.1−0.5 mg/L. Parvalbumin was undetectable in fish gelatins and a fish skin used as raw material in fish gelatin production. Isinglass, with a usage of 0.005−0.05 g/L in the beverage industry, was found to contain parvalbumin amounts of up to 414.7 ± 30.6 mg/kg. Consequently, fish gelatins appear to be of no risk for consumers allergic to fish. However, both the introduction of parvalbumin to beverages by the treatment with isinglass and the potential allergenicity of these parvalbumin amounts need to be clarified.

ABBREVIATIONS USED

B_0, blank value; CV, coefficient of variation; DBPCFC, double-blinded placebo-controlled food challenge; ELISA, enzyme-linked immunosorbent assay; GMP, code of good manufacturing practice; LODC, limit of decision; LOD, limit of detection; NOAEL, no observed adverse effect level; OD, optical density; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; SPT, skin prick test; Tris, tris(hydroxymethyl)-aminomethane; Tween 20, polyethylene-sorbitan monolaurate.

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