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Quantification of Parvalbumin in Commercially Important Mediterranean Seafood Species using Real Time PCR

Dimitra HOUHOULA, Polytimi DIMITRIOU, Gena MENGJEZI, Vasiliki KYRANA and Vladimiros LOUGOVOIS

Department of Food Technology, Faculty of Food Technology and Nutrition, Technological Educational Institute of Athens, Egaleo, Athens, Greece

Abstract

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The parvalbumin allergen gene was quantified in various types of seafood using RT PCR. Freshly harvested specimens from 25 species of finfish, molluscs, and crustacean shellfish, commonly consumed in the Mediterranean region, were included in the investigation. DNA was extracted using the commercial NucleoSpin Food kit. The amplification of the parvalbumin gene was performed by RT PCR. Sixteen out of the 25 species examined yielded positive amplification. Positive samples, including several species of fish (Atlantic mackerel, horse mackerel, sheepshead, red mullet, sandsmelt, pandora, saddled sea bream, gilthead sea bream, red sea bream, European sea bass, blue whiting, anchovy, sardine) and cephalopods (cuttlefish, musky octopus), exhibited largely variable thresholds differing by as much as 12 cycles. Even though equal amounts of DNA were used in PCR amplification the copy number of gene-encoded parvalbumin varied between the fish species. The assay proved to be a potential tool for the detection and label management of fish allergens in food. The studies have shown that allergic reactions to food are highly individual. For some hypersensitive patients, even trace amounts can cause life-threatening allergic reactions. The results of the present study indicate that several seafood species, commonly consumed in the Mediterranean region, may pose a threat for hypersensitive individuals.

Keywords: protein quantification; Real Time PCR; seafood allergy

Seafood is among the eight major groups of allergens that are responsible for more than 90% of all food allergies. Fish contain a wide variety of proteins, but only few of them are known to cause allergic reactions (Lehrer *et al*. 1996). Parvalbumin, a low molecular weight (12 kDa), heat-stable, calcium-binding sarcoplasmic protein, containing 108–109 amino acid residues, represents the major fish allergen. More than 95% of fish-allergic patients have been found to have specific immunoglobulin E (IgE) to this protein and many of the IgE-binding epitope on this allergen are present in various fish species (de Martino *et al*. 1990; Bugajska-Schretter *et al*. 1998). High amino acid sequence homologies and antibody cross-reactivities have been demonstrated for parvalbumins in Japanese eel and bigeye tuna (Shiomi *et al*. 1999) mackerel, salmon and horse mackerel (Hamada *et al*. 2003). Parvalbumin is extremely abundant in fish muscle, where it plays an important role in relaxation. White muscles generally contain more parvalbumin than dark muscles, which makes the latter much less allergenic. Kobayashi et al. (2006) showed that horse mackerel white muscle contained 5–6 times more parvalbumin than the dark muscle. They reported that parvalbumin content varied markedly among fish, with species such as Japanese amberjack (*Seriola quinqueradiata*) containing very low amounts of the allergen. The first reported allergenic fish parvalbumin was from Baltic cod (*Gadus callarias*) designated as "allergen M" (later named Gad c 1) and was identified by Elsayed and Bennich (1975). Methodologies for the detection of fish allergens already exist, which are based on diverse technologies and can be designed for different purposes. Several papers have reported on the development of ELISAs and PCRs to detect parvalbumin in fish products (Hilger *et*

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al. 2004; Gajewski & Hsieh 2009; Griesmeier *et al*. 2010; Hildebrandt & Garber 2010; Li *et al*. 2011). Recent validation studies by Faeste and Plassen (2008) developed a sandwich ELISA for the quantification of fish in foods using polyclonal anti-cod parvalbumin antibody as the capture and detector antibody. The assay had a detection limit of 0.01 mg parvalbumin/kg food, equivalent to 5 mg fish/kg food. Choi and Hong (2007) published a PCR method using primers that specifically targeted the gene of mackerel parvalbumin. However, the use of this method was limited to the detection of allergenic residues derived from mackerel but not from other fish species. The aim of the present study was to implement a sensitive RT PCR for the detection and quantification of the parvalbumin gene in seafood species commonly consumed in the Mediterranean region, with a view to providing data that would protect sensitised individuals from the severe consequences of fish-induced allergy.

MATERIAL AND METHODS

Food collection. During the study period (March 2013 to June 2013), freshly harvested specimens from a total of 25 species (Table 1) of finfish, molluscs, and crustacean shellfish commonly consumed in the Mediterranean region were collected from super markets. Fish allergen free samples such as beef, chicken, and pork, were used for the assay specificity, as negative controls.

Genomic DNA extraction and quantification. The NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) and the Bio Scientific (Austin, USA) were used for the extraction of the 25 species and for the 3 fish allergen free samples, comparing the extraction yields and cleanup of the genomic DNA. Composite samples of three fish from each species were extracted in duplicate. All extraction methods were used according to the manufacturer's instructions, with some modifications. The NucleoSpin

Table 1. Real-Time PCR threshold cycle and quantification of parvalbumin gene in fish specimens studied

No.	Common name	Scientific name	Mean Ct	DNA yield (ng/100 mg fish)
1	Anchovy	*Engraulis encrasicolus*	28.12 ± 0.05	0.083
2	Chub mackerel	*Scomber japonicus*	27.05 ± 0.04	0.13
3	Horse mackerel	*Trachurus trachurus*	31.25 ± 0.06	0.0013
4	White sea bream	*Diplodus sargus*	31.12 ± 0.05	0.0013
5	Red mullet 1	*Mullus sumuletus*	29.12 ± 0.05	0.013
6	Picarel	*Spicara smaris*	32.12 ± 0.05	0.001
7	Common pandora	*Pagellus erythrinus*	26.08 ± 0.08	0.6
8	Saddled bream	*Oblada melanura*	32.12 ± 0.09	0.001
9	Gilthead sea bream	*Sparus aurata*	23.15 ± 0.04	>13
10	Red sea bream	*Pagrus major*	27.12 ± 0.05	0.13
11	European sea bass	*Dicentrarchus labrax*	29.12 ± 0.05	0,013
12	Cuttlefish 1	*Sepia officinalis*	30.09 ± 0.04	0.0053
13	Sardine	*Sardina pilchardus*	26.05 ± 0.04	0.6
14	Musky Octopus	*Eledone moschata*	31.12 ± 0.08	0.0013
15	Red mullet 2	*Mullus sumuletus*	27.05 ± 0.07	0.13
16	Cuttlefish 2	*Sepia officinalis*	31.09 ± 0.04	0.0013
17	Pink shrimp 1	*Penaeus* sp.	not detected	not detected
18	Atlantic mackerel	*Scomber scombrus*	not detected	not detected
19	Octopus	*Octopus vulgaris*	not detected	not detected
20	Warty venus	*Venus verrucosa*	not detected	not detected
21	Squid	*Loligo vulgaris*	not detected	not detected
22	Pink shrimp 2	*Penaeus* sp.	not detected	not detected
23	Norway lobster	*Nephrops norvegicus*	not detected	not detected
24	Brown venus	*Callista chione*	not detected	not detected
25	Mediterranean mussel	*Mytilus galloprovincialis*	not detected	not detected

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Food kit was finally selected to extract all of the samples. About 100 mg of each sample were used for the extraction. The purity and concentration of DNA were determined spectrophotometrically. All samples were stated neat and were diluted 1/10 in dH_2O.

RT-PCR assay. The protocol was an in-house established Real-time PCR (RT-PCR) assay using the protocol of Sun *et al.* (2009) with some modification. The reactions were performed in a 25-µl final volume, containing 8 µl of the eluted DNA, 12.5 µl of Master Mix (KAPA Probe Fast qPCR; Kapa Biosystems, London, UK), and 0.4 µM of each primer, a forward (5'-CAGGACAAGAGTGGCTTCAT-3') and a reverse (5'-GAAGTTCTGCAGGAACAGCTT-3'). The probe 0.4 µM was 5'-AGGAGGAYGAGCT-3' (Y was T or G) labelled with a reporter dye (FAM) at the 5' end, and a quencher dye a minor groove binder (MGB) at the 3' end. The amplification conditions consisted of a 2 min initial denaturation step at 95°C, followed by 40 cycles of 20 s denaturation at 95ºC, 20 s annealing at 55°C, and 72°C for 30 seconds.

Creation of standard curves for Real-Time PCR analysis. Step One plus™ Real Time PCR System (Applied Biosystems, Grand Island, USA) was used for the PCR assay. For RT fluorescence, the measurements were compiled in every cycle. All reactions included negative controls containing the amplification master mix and dH_2O that had been used for reagent preparation.

For positive controls and DNA quantification, a standard curve was designed using known concentrations (KC) of DNA extracted from gilthead sea bream (*Sparus aurata*) (KC_1 = 13 ng/100 mg, KC_2 = 1.3 ng/100 mg, KC_3 = 0.13 ng/100 mg, KC_4 = 13 pg/100 mg, KC_5 = 1.3 pg/100 mg, and KC_6 = 130 fg/100 mg). A cycle threshold value (Ct) was defined as the cycle of the RT-PCR at which a significant fluorescence increase was detected in comparison to the negative control and the blanks, then increase being associated with an exponential growth of PCR product during the log-linear phase. This increase was calculated using the equation: $DRn = Rn^+ - Rn^-$, where: Rn^+ – fluorescence of the sample of interest containing all components at any given time after the onset of the reaction, Rn^- – fluorescence of the same sample detected in the baseline value at the beginning of the reaction. DRn, which is the difference between Rn^+ and Rn^-, serves as an indicator of the magnitude of the signal generated by the PCR and, when plotted against the cycle numbers, produces the amplification curves and determines the Ct.

The slope of the amplification curve during the log-linear phase was used to calculate the amplification efficiency (Eff), using the formula: $Eff = 10^{(-1/slope)} - 1$.

RT-PCR runs were acceptable only when the negative control had an undetectable Ct, the KC_2 and KC_3 had Ct between 25 and 27, and the efficiency of the PCR was 90–100%.

All samples were tested neat and were diluted 1/10 in dH_2O for the detection of inhibition. The inhibition was defined as a positive PCR result with a diluted specimen, while a negative PCR result was obtained with the specimen tested undiluted.

RESULTS AND DISCUSSION

DNA extraction and quantification. Optimisation of the genomic DNA extraction from protein rich foods such as seafood was the first issue of this work. The DNA extraction methods critically affect PCR sensitivity. The optimisation of these methods can improve the yield of DNA for the detection and quantification of parvalbumin gene. In order to achieve this, two different extraction methods were tested (data not shown) and the NucleoSpin Food kit was finally selected. About 130 ng of genomic DNA starting from 100 mg of fish was obtained with the NucleoSpin Food kit.

Dynamic range, analytical sensitivity and specificity. RT PCR protocols did not produce any results using the extracted DNA from fish allergen free samples such as beef, chicken and pork. Based on the DNA quantification performed, reproducible analytical sensitivities of RT PCR were 130 fg/100 mg of fish.

Sensitivity and specificity. Overall, 25 species of finfish, molluscs, and crustacean shellfish commonly consumed in the Mediterranean region were collected from super markets. From the 25 products, a total of 16 (64%) examined yielded positive amplification. Positive samples, including fish (Atlantic mackerel, horse mackerel, sheepshead, red mullet, sandsmelt, pandora, saddled sea bream, gilthead sea bream, red sea bream, European sea bass, blue whiting, anchovy, sardine) and cephalopods (cuttlefish, musky octopus) exhibited largely variable thresholds, differing by as much as 9 cycles, even though equal amounts of DNA were used in PCR amplification. Gilthead sea bream (*Sparus aurata*), sardine (*Sardina pilhardus*), and Common Pandora (*Pagellus erythrinus*) gave a lower mean Ct values range from 23.00 to 26.00. Picarel (*Spicara smaris*) and saddled sea bream (*Oblada melanura*) gave the highest mean Ct value (32.12), indicating that the

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copy number of gene-coded parvalbumin varied with different fish species. It is worth noting that red mullet specimens originating from the Aegean or the Atlantic coast of Morocco produced values differing by a factor of 10, indicating that factors other than the species may also have an impact. When diluted specimens were examined, 10 inhibition cases were detected. More specifically, in 10 samples a negative PCR result was obtained when they were tested undiluted, whereas they were defined positive when diluted 1/10 in dH_2O.

Reproducibility of the assay. In order to evaluate the reproducibility of the method, 5 samples at a concentration of 1.3 ng/µl were randomly chosen as PCR templates and the experiment was repeated 3 times. The results of the TaqMan assay showed that the coefficient of variation values for both intra-experimental and inter-experimental data ranged from 0.46% to 0.80% and 0.23% to 0.71%, respectively (Table 1). These results suggest that the method presents good reproducibility.

DNA quantification. Analytical DNA quantification of all positive specimens, using RT-PCR protocol is shown in Table 2. RT-PCR technology has been extensively evaluated in food allergen quantification. More specifically, it has been used for direct detection of allergen substances in food, using technologies like SYBR Green, and hydrolysis TaqMan probes. RT-PCRs are rapidly reporting as a result of faster turnaround times. In addition, no post-PCR processing is necessary, and both the amplification and detection are performed in a single closed tube, thus minimising the risk of carry over or cross-contamination. Quantification is another potential advantage of RT-PCR protocols, which nevertheless needs to be further evaluated, in order to reach definite conclusions regarding the improvement of the detection of potential allergens traces. Sharp and Lopata (2014) reported that fish often contain both α- and β-parvalbumins. Most fish express two or more different β-parvalbumin isoforms. These β isoforms can differ significantly in amino acid composition and/or sequence. The differences in β-parvalbumin isoforms within a single species can result in a fish allergic patient reacting to one isoform more than another, which adds to the complexity of diagnosing fish allergy and detecting allergenic parvalbumin. Sun *et al.* (2009) developed a real-time PCR method using a probe and primers that specifically detect the parvalbumin genes in 28 out of 30 fish species, with the exception of golden threadfin bream and yellowfin tuna. The sensitivity of the assay was reported to be as low as 5 pg of purified fish DNA. Their assay did not amplify DNA from 13 non-fish species. As reported by the authors, more research is required to verify the applicability of the method for further fish species and to correlate the DNA copy numbers with the actual amount of allergenic fish residues present in foods. Faeste and Plassen (2008) developed a quantitative sandwich ELISA for the determination of fish in foods. Specific antibody used in this assay was produced through immunisation of a rabbit with purified cod parvalbumin. The obtained polyclonal rabbit anti-cod parvalbumin antibody was used as a capture and detection (after previous conjugation with biotin) antibody to construct a sandwich ELISA. The assay was used for the quantification of 32 fish species in different food matrices with a limit of detection of 5 mg fish/kg food. The assay showed high specificity to fish no cross-reactivity to meat, shellfish or food additives. However, out of all fish tested species, twelve provided recovery rates lower than 1%. Also Shibahara *et al.* (2013) developed a sandwich ELISA that showed 22.6–99.0% reactivity (based on the reactivity to Pacific mackerel parvalbumin) to parvalbumins from various species of fish. The limits of detection and quantitation were estimated to be lower than 1%. A similar observation was made by Chen *et al.* (2006) with variable immunoreactivity

Table 2. Intra- and inter-experimental test results of 5 randomly chosen samples

Sample	Inter-experimental						Intra-experimental	
	experiment 1		experiment 2		experiment 3			
	mean Ct	*CV*%	mean Ct	*CV*%	mean Ct	*CV*%	mean Ct	*CV*%
1	25.72 ± 0.15	0.58	25.45 ± 0.18	0.71	25.68 ± 0.10	0.39	25.89 ± 0.12	0.46
2	24.74 ± 0.10	0.40	24.50 ± 0.08	0.33	25.10 ± 0.12	0.48	24.98 ± 0.20	0.80
3	25.99 ± 0.08	0.30	24.55 ± 0.15	0.61	25.38 ± 0.18	0.71	25.58 ± 0.16	0.63
4	25.57 ± 0.06	0.23	25.95 ± 0.20	0.71	25.52 ± 0.15	0.59	25.99 ± 0.18	0.69
5	25.01 ± 0.14	0.56	25.45 ± 0.18	0.71	25.58 ± 0.20	0.78	25.75 ± 0.15	0.58

Ct – cycle threshold value; *CV*% – coeffient of variance

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of the commercially available mouse monoclonal antifrog parvalbumin antibody against the raw extracts from several fish species. Similar results were observed in our study. The reduction of the allergenic reactivity of parvalbumin in different species of fish ranged from 10 to 5000 fold as a result of different isoforms of parvalbumin.

CONCLUSIONS

For some hypersensitive patients, even trace amounts of allergens can cause life-threatening allergic reactions. The RT PCR assay proved to be a potential tool for the detection and label management of fish allergens in food. The food industry has a responsibility to produce food that is safe for all consumers including food allergic people. Studies have shown that allergic reactions to food are highly individual. Improved allergen traceability through the food chain may aid the consumers with allergen avoidance. For some hypersensitive patients, even trace amounts can bring about life-threatening allergic reactions.

References

Bugajska-Schretter A., Elfman L., Fuchs T., Kapiotis S., Rumpold H., Valenta R., Spitzauer S. (1998): Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion. Journal of Allergy and Clinical Immunology, 101: 67–74.

Chen L., Hefle S.L, Taylor S.L., Swoboda I., Goodman R.E. (2006): Detecting fish parvalbumin with commercial mouse monoclonal anti-frog parvalbumin IgG. Journal of Agricultural and Food Chemistry, 54: 5577–5582.

Choi K.Y., Hong K.W. (2007): Genomic DNA sequence of mackerel parvalbumin and a PCR test for rapid detection of allergenic mackerel ingredients in food. Food Science and Biotechnology, 16: 67–70.

de Martino M., Novembre E., Galli L., de Marco A., Botarelli P., Marano E., Vierucci A. (1990): Allergy to different fish species in cod-allergic children: *in vivo* and *in vitro* studies. Journal of Allergy and Clinical Immunology, 86: 909–914.

Elsayed S., Bennich H. (1975): The primary structure of allergen M from cod. Scandinavian Journal of Immunology, 4: 203–208.

Faeste C.K., Plassen C. (2008): Quantitative sandwich ELISA for the determination of fish in foods. Journal of Immunology Methods, 329: 45–55.

Gajewski K.G., Hsieh Y.H. (2009): Monoclonal antibody specific to a major fish allergen: parvalbumin. Journal of Food Protection, 72: 818–825.

Griesmeier U., Vázquez-Cortés S., Bublin M., Radauer C., Ma Y., Briza P., Fernández-Rivas M., Breiteneder Griesmeier H. (2010): Expression levels of parvalbumins determine allergenicity of fish species. Allergy, 65: 191–198.

Hamada Y., Tanaka H., Ischitzaki S. (2003): Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergens of mackerels. Food and Chemical Toxicology, 41: 1149–1156.

Hildebrandt S., Garber E.A.E. (2010): Effects of processing on detection and quantification of the parvalbumin gene in Atlantic salmon (*Salmo salar*). Food Chemistry, 119: 75–80.

Hilger C., Thill L., Grigioni F., Lehners C., Falagiani P., Ferrara A., Romano C., Stevens W., Hentges F. (2004): IgE antibodies of fish allergic patients cross-react with frog parvalbumin. Allergy, 59: 653–660.

Kobayashi A. Tanaka H., Hamada Y., Ishizaki S., Nagashima Y., Shiomi K. (2006): Comparison of allergenicity and allergens between fish white and dark muscles. Allergy, 61: 357–363.

Lehrer S.B., Horner W.E., Reese G. (1996): Why are some proteins allergenic? Implications for biotechnology. Critical Review in Food Science and Nutrition, 36: 553–564.

Li Z., Zhang Y., Pawar R., Wang G., Lin H. (2011): Development of an optimized protein chip for the detection of fish parvalbumin allergen. Current Analytical Chemistry, 7: 349–356.

Sharp M.F., Lopata A.L. (2014): Fish allergy: in review. Clinical Reviews in Allergy & Immunology, 46: 258–271.

Shibahara Y., Uesaka Y., Wang J., Yamada S., Shiomi K. (2013): A sensitive enzyme-linked immunosorbent assay for the determination of fish protein in processed foods. Food Chemistry, 136: 675–681.

Shiomi K., Hamada Y., Sekiguchi K., Shimakura K., Nagashima Y. (1999): Two classes of allergens parvalbumins and higher molecular weight substances, in Japanese eel and bigeye tuna. Fish Science, 65: 943–948.

Sun M., Liang C., Gao H., Lin C., Deng M. (2009): Detection of parvalbumin, a common fish allergen gene in food, by real-time polymerase chain reaction. Journal of AOAC International, 92: 234–240.

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Corresponding author:

Dimitra Panagiotis Houhoula, PhD, Technological Educational Institute of Athens, Faculty of Food Technology and Nutrition, Department of Food Technology, Agiou Spyridonos Str., Egaleo, 12210, Athens, Greece; E-mail: dhouhoula@teiath.gr