FOOD COMPOSITION AND ADDITIVES

Detection of Parvalbumin, a Common Fish Allergen Gene in Food, by Real-Time Polymerase Chain Reaction

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Fish, as one of the most common causes of IgE-mediated food hypersensitivity, has recently received increasing attention from the food industry and legislative and regulatory agencies. A real-time polymerase chain reaction assay based on TaqMan-MGB probe technology was developed for the detection of parvalbumin, a major fish allergen gene. The assay had a sensitivity up to 5 pg purified fish DNA and had no cross-reaction with other species, such as cattle, sheep, swine, chicken, shrimp, lobster, crab, squid, clam, rice, soybean, maize, and potato. The coefficient of variation for both intra- and interexperimental variability demonstrated high reproducibility and accuracy. The assay proved to be a potential tool for the detection and label management of fish allergens in food.

Fish is one of the most common causes of IgE-mediated food hypersensitivity. For sensitized individuals, contact and ingestion of fish is very dangerous, and can elicit a variety of clinical symptoms, ranging from mild to severe and even death (1, 2). Complete and strict avoidance of the offending food is the only way to prevent these allergic reactions. Many countries, such as the United States, Japan, Canada, Switzerland, Great Britain, and the European Union have implemented food allergen labeling regulations to protect food-allergic consumers. Even with the regulations, mistakes in food labeling and cross-contamination occur from time to time, though not always intentionally (3). Therefore, the development of a rapid, sensitive, and specific method for the detection of fish allergens in food is in demand.

In recent years, researchers have carried out in-depth studies on fish allergens that trigger allergic reactions and have identified parvalbumins as the major allergy-eliciting proteins that are responsible for over 95% of atopic responses. They are small [about 12 kilodaltons (kDa), composed of 108–109 amino acid residues] calcium-binding proteins with a remarkable resistance to thermal, chemical, and enzymatic degradation (4, 5). Parvalbumins are mainly expressed in fish muscle, and are much lower in the dark muscle than in the white muscle (6). Studies have also shown that parvalbumins are cross-reactive fish allergens, containing most of the fish-specific IgE epitopes (7–9). This study reports a real-time polymerase chain reaction (PCR)-based assay using the parvalbumin-specific primers and probe that enable the detection of fish allergens in food.

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Experimental

Samples

Specimens of 30 fish species were included in our study, which comprised 30 genera in 22 families (Table 1). Fish allergen-free samples, such as soybean, rice, maize, beef, chicken, pork, mutton, squid, oyster, clams, scallops, shrimp, crab, and lobster, were used for the analysis of assay specificity. Most of the fish specimens were samples from the trading companies for exit-entry inspection and quarantine; others and reference materials were purchased in local supermarkets in Qingdao, China.

DNA Extraction

For DNA preparation of plant samples, the commercial kit (Jiemen Biotech Co., Shanghai, China) was used according to the manufacturer's instruction. For animal samples, the DNA extraction protocol was modified to extend the Proteinase K digestion at 56°C. Briefly, 10 g sample was weighed out and finely ground with a grinder. Subsequently, 100 mg of fine particulates was lysed not only with lysis buffer but also with Proteinase K until no tissue was visible. Phenol–chloroform extraction was then performed. After centrifugation, the upper aqueous phase was transferred into a new tube, and buffer that was provided with the kit was added to precipitate DNA. The resulting DNA pellet was dissolved and applied to the silica column. The column was washed twice with washing buffer, and the DNA was eluted with elution buffer. The purity and concentration of DNA were determined by measuring the absorption at 260 and 280 nm. After UV quantification, the concentration of each DNA sample was adjusted to 10 ng/μL.

Table 1. Names and categories of fish samples studied

Common name	Scientific name	Category
Blue whiting	Micromesistius poutassou	Gadiformes, Gadidae, Micromesistius
Pacific cod	Gadus macrocephalus	Gadiformes, Gadidae, Gadus
Alaska Pollock	Theragra chalcogramma	Gadiformes, Gadidae, Theragra
Haddock	Melanogrammus aeglefinus	Gadiformes, Gadidae, Melanogrammus
Saithe	Pollachius virens	Gadiformes, Gadidae, Pollachius
Southernhake	Muraenolepis microps	Gadiformes, Muraenolepididae, Muraenolepis
Pacific hake	Merluccius productus	Gadiformes, Melanonidae, Merluccius
Hoki	Hymenocephalus striatissimus	Gadiformes, Macrouridae, Hymenocephalus
Grass carp	Ctenopharyngodon idellus	Cypriniforms, Cyprinidae, Ctenopharyngodon
Crucian	Carassius auratus	Cypriniforms, Cyprinidae, Carassius
Carp	Cyprinus carpio	Cypriniforms, Cyprinidae, Cyprinus
Bighead carp	Aristichthys nobilis	Cypriniforms, Cyprinidae, Aristichthys
Small yellow croaker	Pseudosciaena polyactis	Perciformes, Sciaenidae, Larimichthys
Red fish	Sciaenops ocellatus	Perciformes, Sciaenidae, Sciaenops
Belt fish	Trichiurus haumela	Perciformes, Trichiuridae, Trichiurus
Surfperch	Ditrema temmincki	Perciformes, Embiotocidae, Ditrema
Red seabream	Pagrosomus major	Perciformes, Sparidae, Pagrosomus
Silvery pomfret	Pampus argenteus	Perciformes, Stromateidae, Pampus
Stamp	Trachurus japonicus	Perciformes, Carangidae, Trachurus
Sea perch	Lateolabrax japonicus	Perciformes, Serranidae, Lateolabrax
Mackerel	Scomber scombrus	Perciformes, Scombridae, Scomber
Yellowfin tuna	Thunnus albacares	Perciformes, Thunnidae, Thunnus
Golden threadfin bream	Nemipterus virgatus	Perciformes, Nemipteridae, Nemipterus
Olive flounder	Paralichthys olivaceus	Pleuronectiformes, Paralichthyidae, Paralichthys
Atlantic halibut	Hippoglossus hippoglossus	Pleuronectiformes, Pleuronectidae, Hippoglossus
Dory	Zeus faber	Zeiformes, Zeidae, Zeus
Greenling	Hexagrammos otakii	Scorpaeniformes, Hexagrammidae, Hexagrammos
Alfonsino	Beryx splendens	Beryciformes, Berycidae, Beryx
Big pacific-salmon	Oncorhynchus keta	Salmoniformes, Salmonidae, Oncorhynchus
Catfish	Parasilurus asotus	Siluriformes, Siluridae, Silurus

Primers Design

Based on the sequence alignment of the published nucleotide sequences of parvalbumin genes (GenBank Accession Nos. AM497928, AM497927, AB091470, AB211364, AB211365, AB211366, AJ292211, AJ292212, DD137485, and DQ374441), a fish-specific and universal TaqMan probe and primer pair were designed with Primer Express software (Applied Biosystems, Foster City, CA). The forward primer was 5′-CAGGACAAGAGTGGCTTCAT-3′; the reverse primer was 5′GAAGTTCTGCAGGAACAGCTT-3′. The probe was 5′-AGGAGGAYGAGCT-3′ (Y was T or G), labeled with a reporter dye (FAM) at the 5′ end, and a quencher dye and a minor groove binder (MGB) at the 3′ end. These primers and probe were synthesized by ShineGene Bio-Technologies Inc. (Shanghai, China). The primers were purified by polyacrylamide gel electrophoresis and the probe was purified with high-performance liquid chromatography.

PCR Amplification

The optimized PCR amplification system had a total volume of 25 μL:1 × Master Mix, containing HotStarTaq DNA polymerase, $MgCL_2$, dNTP (Tiangen Biotech Co. Ltd, Beijing, China), 400 nmol/L each primer, 400 nmol/L probe, and 5 μL template DNA. Amplification was performed in an ABI PRISM 7900 (Applied Biosystems), and the PCR program was as follows: predenaturation at 94°C for 2 min; 45 cycles of amplification (94°C for 20 s, 55°C for 20 s, and 68°C for 40 s). Each sample was amplified in triplicate.

Table 2. Real-time PCR threshold cycle of fish samples studied

Sample	Mean Ct[a]
Blue whiting	24.45 ± 0.08
Pacific cod	24.36 ± 0.04
Alaska Pollock	29.71 ± 0.12
Haddock	23.25 ± 0.03
Saithe	24.67 ± 0.13
Southern hake	26.25 ± 0.09
Pacific hake	29.47 ± 0.13
Hoki	29.29 ± 0.11
Grass carp	21.24 ± 0.04
Crucian	22.04 ± 0.08
Carp	21.02 ± 0.09
Bighead carp	20.79 ± 0.06
Small yellow croaker	22.56 ± 0.11
Red fish	29.17 ± 0.06
Belt fish	24.78 ± 0.1
Surfperch	20.06 ± 0.04
Red seabream	22.82 ± 0.05
Silvery pomfret	27.37 ± 0.09
Stamp	21.85 ± 0.02
Sea perch	22.71 ± 0.05
Mackerel	27.01 ± 0.12
Golden threadfin bream	ND[b]
Olive flounder	30.26 ± 0.07
Atlantic halibut	30.16 ± 0.17
Dory	23.74 ± 0.08
Greenling	24.99 ± 0.07
Alfonsino	25.67 ± 0.04
Big pacific-salmon	28.03 ± 0.07
Catfish	27.15 ± 0.08
Yellowfin tuna	ND

[a] 50 ng fish DNA per reaction; Ct, the number of PCR cycles to reach the fluorescence threshold.

[b] ND = Not detected.

Table 3. Real-time PCR results of food products studied

Food sample	Fish declaration	PCR result
Fish steak	+	+
Fish ball 1	+	+
Fish ball 2	+	+
Caviar	+	+
Sausage 1	+	+
Imitation crab	+	+
Imitation shrimp	+	+
Can	+	+
Shrimp ball	M[a]	+
Sausage 2	M	–

[a] M = May contain traces of fish.

Results and Discussion

Applicability of the Assay

To determine the efficiency of the primers and probe, 30 different fish species were collected. Apart from golden threadfin bream and yellowfin tuna, 28/30 fish species samples yielded positive amplification (Table 2). Interestingly, the dissociation curve of golden threadfin bream showed a single and characteristic melt peak in real-time PCR when SYBR Green I was used. It is reported that the TaqMan-MGB-based assay is much more specific than the SYBR Green I-based assay, which can detect a single base difference. Thus, some samples may test negative by the probe-based real-time PCR but positive by the double-stranded DNA-binding dye-based PCR assay, because of base difference in nucleotide sequence between forward and reverse primer. As for yellowfin tuna, our amplification result was consistent with the previous study (10) in which the researchers found that tuna did not contain the parvalbumin protein. An allergen of about 46 kDa in yellowfin tuna, which does not belong to the parvalbumin group, could explain tuna's weak allergenic capacity.

Ten food products were purchased from local supermarkets and tested with parvalbumin-specific primers and probe to further validate the applicability of the assay. Of the 10 products, 9 were found to harbor fish allergens, including one labeled with "may contain traces of fish" (Table 3, Figure 1).

Repeatability and Reproducibility of Assay

In order to evaluate the repeatability and reproducibility of the method, 5 samples at a concentration of 10 ng/μL were randomly chosen as PCR templates, and amplified in triplicate in an experiment and performed 3 times. The results of the TaqMan assay showed that the coefficient of variation values for both intraexperimental and interexperimental ranged from 0.84 to 2.11% and 0.16 to 0.43%, respectively (Table 4). These results suggest that the method has good repeatability and reproducibility.

Sensitivity of the Assay

To determine the applicability of the TaqMan assay, we studied 30 fish species samples. As shown in Table 2, almost all the samples yielded positive amplification. Nevertheless, some samples had various threshold cycles, and even a difference of 10 cycles, though the same amounts of DNA had

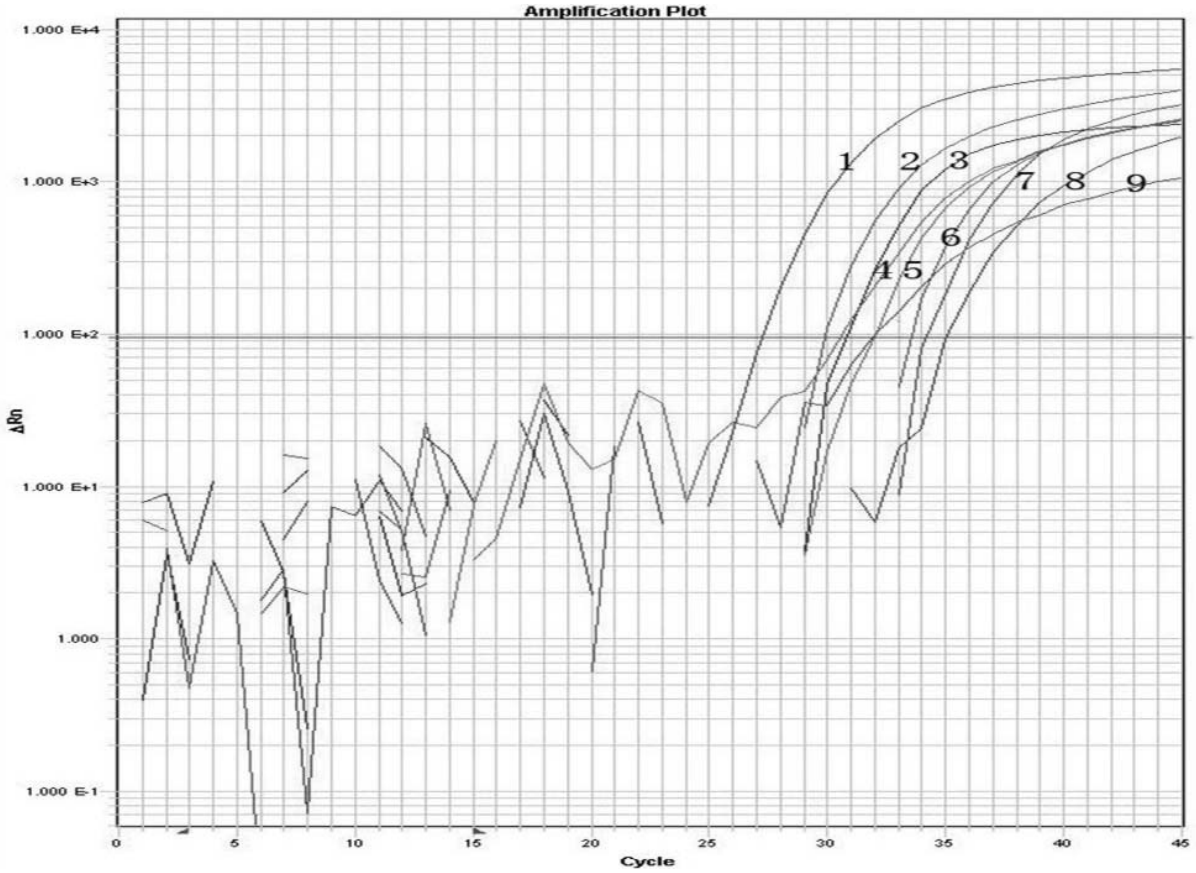

Figure 1. Real-time PCR amplification plot of food products: Lane 1, fish ball 2; Lane 2, can; Lane 3, caviar; Lane 4, fish ball 1; Lane 5: imitation crab; Lane 6: fish steak; Lane 7: imitation shrimp; Lane 8: shrimp ball; Lane 9: sausage 1.

been used in the PCR amplification. Surfperch gave the smallest mean threshold cycle (Ct) values (20.06), and olive flounder gave the largest mean Ct values (30.26), indicating that the copy number of gene-coded parvalbumin varied in different fish species. To determine the sensitivity of this method, we serially diluted DNA solution of surfperch and olive flounder with soybean DNA solution, respectively. The results showed that the concentration of 10 pg for surfperch and 5 pg for olive flounder were the lowest DNA concentrations that yielded reproducible positive results. The linearity was observed when DNA was amplified at concentrations between 10 pg and 100 ng, and 5 pg and 10 ng, respectively (Figures 2 and 3).

Specificity of Assay

Specificity is always of primary concern. To determine the specificity of the assay, DNA was extracted from beef, mutton, pork, chicken, shrimp, lobster, crab, squid, clam, rice,

Table 4. Coefficient of variation values for both intra- and interexperimental of some samples

	Interexperimental							
	Experiment 1		Experiment 2		Experiment 3		Intraexperimental	
Sample	Mean Ct	CV, %	Mean Ct	CV, %	Mean Ct	CV, %	Mean Ct	CV, %
Alaska Pollock	29.71 ± 0.12	0.4	30.52 ± 0.06	0.2	30.65 ± 0.1	0.33	30.29 ± 0.51	1.65
Dory	23.74 ± 0.08	0.34	23.83 ± 0.04	0.17	24.13 ± 0.07	0.29	23.9 ± 0.2	0.84
Greenling	24.99 ± 0.07	0.28	25.87 ± 0.06	0.23	25.56 ± 0.11	0.43	25.47 ± 0.45	1.77
Alfonsino	25.67 ± 0.04	0.16	25.44 ± 0.09	0.35	26.15 ± 0.07	0.27	25.75 ± 0.36	1.4
Striped barb	27.37 ± 0.09	0.33	28.15 ± 0.07	0.25	28.53 ± 0.05	0.18	28.02 ± 0.59	2.11

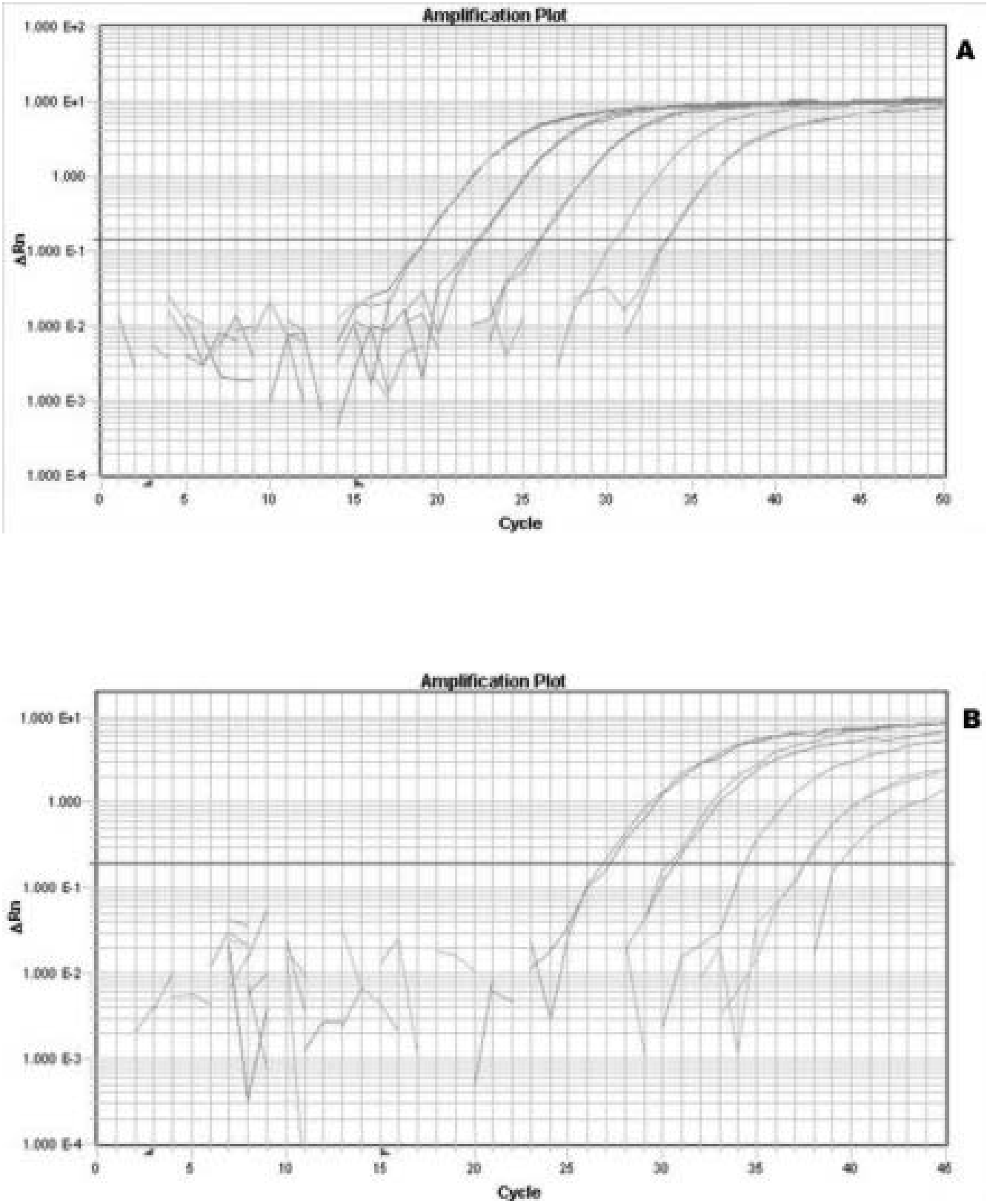

Figure 2. Real-time PCR amplification plot of serial dilution of fish DNA. Amplification plot generated by 5 concentrations of (A) surfperch DNA (100, 10, and 1 ng; and 100 and 10 pg); (B) olive flounder DNA (10 and 1 ng; and 100, 10, and 5 pg).

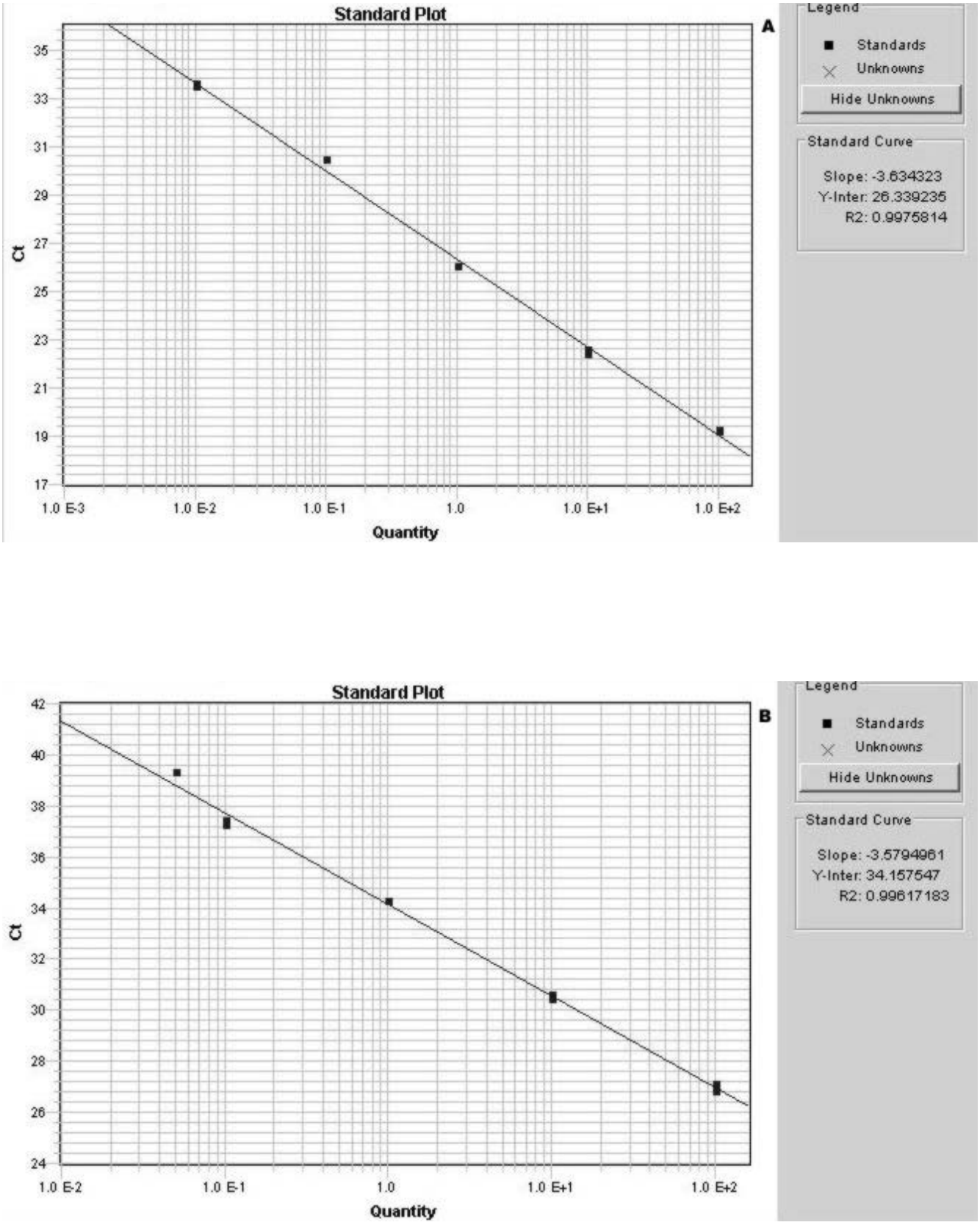

Figure 3. Standard plot of fish samples: (A) Surfperch, (B) olive flounder.

soybean, maize, and potato, and then amplified with parvalbumin-specific primers and the TaqMan-MGB probe. As expected, no typical amplification curves were observed, which demonstrated that primers and probe had no cross-reactions with these species.

The problem of allergens in food has received increasing attention. The routine methods for the detection of food allergens involve enzyme-linked immunosorbent assay and PCR. The former is a protein-based method and the latter is a method operating on the DNA level. Because of changes of protein structure in food processing, and the possibility that the special food matrix will affect the performance of the protein-based assay, the PCR assays are more desirable for processed products. PCR test kits for fish allergens are now commercially available. However, almost all of them focus on the identification of fish ingredient by targeting the cytochrome b or 5S RNA gene. In this study, we chose parvalbumin as the marker of real-time PCR. The detection of parvalbumin gene showed a higher precision in indicating the presence of fish allergens to some extent. The amplification product was only 56 base pairs, which made it a favorable method for processed foods, taking into account the degradation of DNA in processing (11).

Studies have shown that allergic reactions to food are highly individual; in other words, there is enormous variation in the sensitivity and severity levels among allergen-sensitive individuals. For some hypersensitive patients, even trace amounts can elicit life-threatening allergic reactions. It is difficult to determine safe doses of allergens (i.e., thresholds), which has been a challenge to researchers. In our study, the same number of fish samples was confirmed to be different in copy numbers of the parvalbumin gene. Among 30 fish species included, the copy number of olive flounder was the lowest. However, even trace olive flounder (5 pg) can be detected with the developed method. This suggests that it is feasible to use the parvalbumin gene segment as a detection marker of fish allergen, and the developed real-time PCR method is a potential tool for the detection and label management of fish allergen in food.

In contrast to the rich variety of fish, our data are still spare. Further research is needed to confirm the applicability of this method for additional fish species and determine the relationship between the copy numbers of parvalbumin and allergenicity.

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