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Genomic DNA Sequence of Mackerel Parvalbumin and a PCR Test for Rapid Detection of Allergenic Mackerel Ingredients in Food

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Abstract Mackerel (*Scomber japonicus*) often causes severe allergic reactions in sensitive people. Food containing undeclared mackerel may pose a risk to such people. The major allergenic protein in fish such as mackerel, codfish, and Alaska pollack has been found to be parvalbumin. In this study, we developed a polymerase chain reaction (PCR) method to detect mackerel DNA using primers corresponding to the parvalbumin gene. We cloned and sequenced 1.5 kb of parvalbumin gene by PCR using mackerel genomic DNA as a template. Nucleotide sequence analysis of genomic parvalbumin gene, composed of 4 exons and 3 introns, allowed the selection of two pairs of oligonucleotide primers specific for mackerel. These primers successfully enabled PCR amplification of specific regions of genomic parvalbumin DNA from mackerel, but no amplification from 8 other fish samples, surimi, and 6 boiled fish pastes. The sensitivity of this method was sufficient to detect 5 ng of purified mackerel DNA mixed with 50 ng of surimi DNA. This rapid and specific method for the detection of allergenic mackerel would be beneficial in reducing food allergy caused by the ingestion of hidden allergen in processed food.

Keywords: mackerel, *Scomber japonicus*, parvalbumin, rapid detection, PCR

Introduction

Fish is an important nutrient source in the human diet, however compounds within fish products are amongst the most common causes of food allergy. Fish allergy is an IgE-mediated disease with clinical manifestations ranging from urticaria and angioedema to anaphylaxis in hypersensitive individuals (1, 2). The first fish allergen identified was parvalbumin of the cod *Gadus callarias* (3). Parvalbumins are calcium-binding sarcoplasmic proteins with a molecular mass of about 12 kDa, which are found at high levels in the white muscles of lower vertebrates and at lower levels in the skeletal muscle of higher vertebrates (4, 5). Parvalbumins are the major allergen in various species of fish such as carp *Cyprinus carpio*, salmon *Salmo salar*, and mackerel *Scomber japonicus* (6-8).

Three species of mackerels (Japanese mackerel *S. japonicus*, Pacific mackerel *Scomber australasicus*, and Atlantic mackerel *Scomber scombrus*) are representative of the genus Scombridae (9, 10). Among them, *S. japonicus*, which inhabits the coasts of Korea and Japan, is consumed mainly in Korea (11). Mackerel is considered to be responsible for the majority of fish allergy in Korea and Japan (12, 13). Foods containing even a trace of *Scomber* mackerel may cause serious allergenic symptoms in fish-allergic patients (14). Although allergenic compounds must be identified on the labels of processed foods, unintentional contamination, or mislabeling of commercial products with these compounds may happen during food processing procedures. Therefore the development of an accurate, rapid, and highly sensitive detection method for the identification of hidden allergens in processed foods is necessary for the food industry.

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Various methods for the detection of potential allergens in food products have been reported (15). Generally, in order to identify the presence of allergenic ingredients in food, specific proteins or DNA fragments are detected as target materials. Protein-based methods usually involve immunochemical detection methods such as enzyme-linked immunosorbent assay (ELISA), while DNA detection methods are based on the amplification of a specific DNA fragment by polymerase chain reaction (PCR) (15-17).

In this study, parvalbumin genomic DNA from the mackerel *S. japonicus* was cloned and sequenced. We developed a PCR method based on the nucleotide sequence of parvalbumin genomic DNA for the rapid detection of mackerel *S. japonicus* in processed food.

Materials and Methods

Fish samples and DNA purification Specimens of nine marine fish species were purchased from fish suppliers in Seoul (Table 1). All samples were cut into fillets and immediately stored in laboratory freezer at -80°C until use. Six boiled fish pastes were purchased from a grocery store in Seoul. Genomic DNA was purified from small muscle fragments (about 20 mg) by using a Power Prep™ DNA Extraction kit (Kogenebiotech, Seoul, Korea). The DNA concentration was determined using a spectrophotometer (A_{260}).

Cloning of mackerel parvalbumin genomic DNA All primers used in this study are shown in Table 2. The parvalbumin cDNA sequence (accession number: AB 091470) of *S. japonicus* was obtained from the NCBI's GenBank database. Genomic mackerel DNA was amplified with the parvalbumin cDNA-specific forward primer SJ9 (5'-CCCTACAAAGCAAAAACATC-3') and reverse primer SJ487 (5'-GCATAGGAGGAAAGGTCTC T-3') by PCR. The amplified 1.5 kb DNA fragment containing part of the parvalbumin gene was inserted into

Table 1. List of marine fish species used in this study

Species	Common name
Scomber japonicus	Mackerel
Gadus macrocephalus	Pacific cod
Theragra chalcogramma	Alaska pollack
Hoplobrotula armata	Armored weasel-fish
Stephanolepis cirrhiter	Thread-sail filefish
Scomberomorus niphonicus	Japanese Spanish mackerel
Cololabis saira	Pacific saury
Clupea pallasii	Pacific herring
Engraulis japonicus	Japanese anchovy

the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformed into the *Escherichia coli* host Mach1^{TM}-T1^{R}. The plasmid was purified from a transformant and grown in LB broth containing 0.01% ampicillin. The 5.4 kb recombinant plasmid pCR2.1-TOPO containing the parvalbumin gene was isolated and confirmed by restriction enzyme analysis and DNA sequencing. The resulting plasmid was named pSJ15.

Table 2. Primers used in this study

Primer	Sequence
SJ9	5'-CCCTACAAAGCAAAAACATC-3'
SJ487	5'-GCATAGGAGGAAAGGTCTCT-3'
SJ106	5'-GTAGTTTCGACCACAAAAAGTT-3'
SJG107f	5'-AGCTATTCTGTATCGCTTCG-3'
SGG297r	5'-GGTGTGAGTCTTACTTCAGC-3'
SJG441r	5'-ACTGCTGTATAGGTGATAGG-3'

PCR conditions PCR reactions were conducted in 25 μL volumes containing an appropriate amount of template DNA, 300 nM of each primer, 2.5 mM of dNTPs, 5 μL of 5× reaction buffer, 0.5 U of Taq polymerase and deionized water. The PCR reactions were run on an PCR Express thermocycler (Hybaid, Waltham, MA, USA), using the following program: 1 cycle of 3 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C and finally 2 min at 72°C. Each reaction was conducted in triplicate. Five μL of PCR product was loaded onto a 2% agarose gel containing ethidium bromide and visualized under UV illumination.

ATGGCCTTTGCAAGTGTACTGAAAGATGCTGAGGTCACTGCAGCCCTGGATGGGTG

CAAAGgtgactcatcatgagacaatcacagctttaatctaatctaatctcagctat

tctgtatcgcttcgttaacaacccattatcattttcag**CCGCTGGTAGTTTCGACC**

ACAAAAAGTTCTTCAAGGCATGCGGTCTGTCCGGCAAGTCCACCGATGAAGTCAAG

AAGGCTTTCGCCATCATTGACCAGGACAAGAGCGGCTTCATTGAGGAGGAGGAGCT

GAAgtaagactcacacctgcgactgcgcacccagcaccagatggtatcctcttctg

tgctcaacaacagaccatgtagtcttttatgatgaaattatttggcacagaggtta

ataaaaaaatacattattgcatcttctgtcctatcacctatacagcagtaatattg

gtagtgtgattaaatatttgaaatgcctgattctacttaatttatcatattttaaa

tcttcaaatagattatgtatgcgctttaaaacaataggcatgcctctagatataac

gtcagcatcagtggataaagtgatagggttgtgggatcatgttgcatatgaagtta

tgaagtgtgtctcagccgatggggagctgtggaccgtgaactaaccacctctgttc

gcgcacag**GCTGTTCCTGCAGAACTTCAAGGCAGGTGCACGCGCACTCTCCGACGC**

CGAGACCAAGGCTTTCCTCAAGGCCGGTGACAGCGACGGTGATGGCAAGATCGGTA

TTGATGgtaagcactttgagatgcaggggcattgtttaactgtgtttggtgaaatt

gatgccctcagctgttggttttcctctgtctctccctcatcatag**AGTTTGCAGCC**

ATGATTAAGGGATAA

Fig. 1. The complete nucleotide sequence of parvalbumin genomic DNA of *Scomber japonicus*. The nucleotide sequence of exons and introns are shown in capital and lowercase, respectively. The translational start (ATG) and stop (TAA) codons are underlined.

Results and Discussion

Nucleotide sequence of mackerel parvalbumin genomic DNA A number of cDNA sequences of the parvalbumin gene in fish including mackerel, codfish (19), and salmon (20) have been reported, but the genomic sequence of the parvalbumin gene has not yet been determined. We found that PCR assays based on the mackerel parvalbumin cDNA sequence were unable to successfully differentiate mackerel from other fishes including cod, herring, and pollack (data not shown). Thus, we attempted to clone the parvalbumin gene from mackerel *S. japonicus* genomic DNA in order to design mackerel-specific primers based on the nucleotide sequence of parvalbumin genomic DNA. A 1.5 kb DNA fragment was obtained by PCR with the SJ9/SJ487 primer set using *S. japonicus* genomic DNA as a template. Sequencing results showed that the mackerel parvalbumin genomic DNA was composed of 911 base pairs (GenBank accession number: EF016113). Comparison of genomic and cDNA sequences revealed that the mackerel parvalbumin gene consisted of 4 exons and 3 introns of various lengths (Fig. 1). The nucleotide sequences of all 4 exons of the parvalbumin gene were identical to the parvalbumin cDNA sequence of *S. japonicus*. The complete amino acid sequence deduced from the parvalbumin gene contained 109 residues with an average molecular mass of 11,545 Da.

Specificity of PCR assay The PCR method has many advantages over the ELISA method for the detection of allergenic proteins. The target DNA is more stable than the protein during food processing and is efficiently extracted with harsh extraction steps (15). PCR amplification of the 5S rDNA sequence of *Scomber* mackerel was recently developed for the detection of allergenic mackerel ingredients in seafood (18). This PCR assay successfully amplified the flanking nontranscribed spacer region of the 5S rDNA from *Scomber* mackerel, but not from 12 other fish species tested. In this study, we developed a PCR assay directly targeting genomic parvalbumin DNA, a major allergenic protein in mackerel, to detect mackerel ingredients in food. We designed the forward primer SJG107f and two reverse primers SJG297r and SJG441r from the intron sequence of parvalbumin genomic DNA. The ability of PCR to correctly identify mackerel from other fish in food products was assessed. Combining the SJ106 forward primer originating from the cDNA sequence with the primers above, we performed PCR with 100 ng of purified DNA from mackerel and 8 other fish species including Pacific cod, Alaska pollack, armored weasel-fish, thread-sail filefish, Japanese Spanish mackerel, Pacific saury, Pacific herring, and Japanese anchovy. As shown in Fig. 2, primer sets SJG107f/SJG297r, SJ106/SJG441r, and SJG107f/SJG441r generated 190 bp, 284 bp, and 334 bp amplicons, respectively, from mackerel DNA only. This result shows that these three selected primer pairs are specific to mackerel. The specificity of these three primer sets was utilized to detect the presence of allergenic mackerel ingredients in seafood. However, no mackerel-specific DNA fragments were amplified in the PCR assay from DNA extracted from six boiled fish pastes (Fig. 3). These results suggest that these selected primer sets are useful for detecting mackerel ingredients in processed foods.

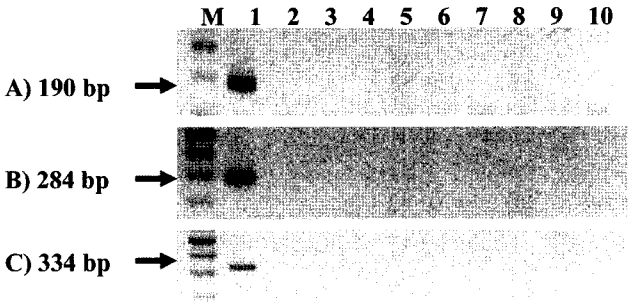

Fig. 2. PCR amplification of genomic DNA from various marine fish with the specific primer pairs SJG107f-SGG297r (A), SJ106-SJG441r (B), and SJG107f- SJG441r (C). PCR products were analyzed by electrophoresis through a 1.5% agarose gel. Lane M, 100-bp ladder; lane 1, mackerel; lane 2, Pacific cod; lane 3, surimi; lane 4, Alaska pollack; lane 5, thread-sail filefish; lane 6, armored weasel fish; lane 7, Japanese Spanish mackerel; lane 8, Pacific saury; lane 9, Pacific herring; lane 10, Japanese anchovy.

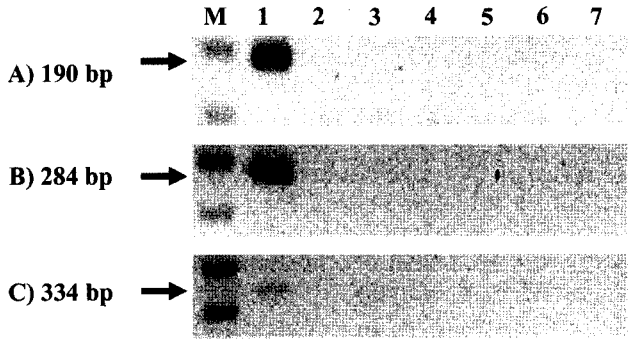

Fig. 3. PCR amplification of DNA extracts from various boiled fish pastes with the specific primer pairs SJG107f-SGG297r (A), SJ106-SJG441r (B), and SJG107f- SJG441r (C). Lane M, 100-bp ladder; lane 1, positive control (mackerel); lane 2 to 7, boiled fish pastes A to F.

Limits of PCR detection The detection of allergens in food products can be very difficult, as they are often present only in trace amounts or are masked by the food matrix (15). Therefore, the development of accurate, reliable, and highly sensitive detection methods is of high importance for the food industry. To determine the detection limit of the PCR assay, PCR was performed on increasing amounts of purified mackerel DNA (1.25, 2.5, 5, and 10 ng per reaction). Although ordinary PCR results are qualitative, the SJG107f-SGG297r, and SJ106-SJG441r primer sets were able to detect as little as 5 and 10 ng, respectively (Fig. 4). This is a level of sensitivity similar to that reported for the detection of mackerel genomic DNA (10 ng), targeting the 5S rRNA gene (18). The influence of non-specific background DNA on the detection limit was also examined. Purified DNA of surimi, a minced fish meat made from white-fleshed fish such as pollack (21), was mixed with mackerel DNA before amplification. The concentration of non-target surimi DNA was held constant

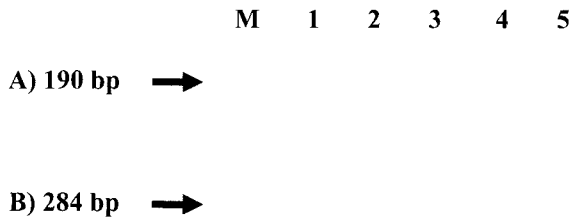

Fig. 4. Defining the limits of PCR detection using the specific primer pairs SJ106-SJG441r (A) and SJG107f-SJG441r (B). Fifty ng of purified surimi DNA was mixed with purified mackerel genomic DNA (1.25 to 10 ng) to introduce non-specific background DNA. Lane M, 100-bp ladder; lane 1, negative control (surimi); lane 2, 1.25 ng; lane 3, 2.5 ng; lane 4, 5 ng; lane 5, 10 ng.

at 50 ng while increasing the amount of target DNA (1.25, 2.5, 5, and 10 ng). There were no significant differences in the performance of the PCR assay regardless of the presence of non-specific DNA in the reaction mixture (data not shown).

In summary, we determined the nucleotide sequence of parvalbumin genomic DNA from mackerel *S. japonicus*. Based on this new sequence data we have developed a mackerel-specific PCR method which allows the identification of mackerel among other fish. Our method is able to detect as little as 5 ng of mackerel genomic DNA per assay. The mackerel-specific PCR assay provides a valuable tool for the rapid detection of hidden mackerel ingredients in processed food.

Acknowledgments

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