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Comparative analysis of barnacle tropomyosin: Divergence from decapod tropomyosins and role as a potential allergen

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Abstract

Tropomyosin, a myofibrillar protein of 35–38 kDa, represents a major and cross-reactive allergen in decapod crustaceans. This study was initiated to clarify whether decapod-allergic patients also recognize tropomyosins of barnacles, crustaceans phylogenetically remote from decapods, which are locally consumed as a delicacy. On SDS-PAGE, a 37 kDa protein was observed in all the heated extracts prepared from two species of decapods (American lobster *Homarus americanus* and black tiger prawn *Penaeus monodon*) and two species of barnacles (acorn barnacle *Balanus rostratus* and goose barnacle *Capitulum mitella*). In immunoblotting, the 37 kDa protein was found to react with monoclonal antibodies against American lobster tropomyosin and hence identified as tropomyosin. The patient sera reacted to tropomyosins from both decapods and barnacles and the reactivity was abolished by preincubation with American lobster tropomyosin, demonstrating that barnacle tropomyosins are allergens cross-reactive with decapod tropomyosins. However, the amino acid sequence of acorn barnacle tropomyosin, deduced by cDNA cloning experiments, shares higher sequence identity with abalone tropomyosins than with decapod tropomyosins. In accordance with this, the phylogenetic tree made for tropomyosins from various animals showed that the acorn barnacle tropomyosin is evolutionally classified not into the decapod tropomyosin family but into the molluscan tropomyosin family.

Keywords: Allergen; Acorn barnacle; cDNA cloning; Cross-reactivity; Crustacea; Decapod; Goose barnacle; Tropomyosin

1. Introduction

Crustaceans are well known as a common cause of food allergy mediated by IgE antibodies. Since edible crustaceans mostly belong to the order Decapoda (including shrimps, prawns, crayfishes, lobsters, hermit crabs and crabs) of the class Malacostraca, previous studies on crustacean allergens have been performed only with decapods. As a result, the major allergen has been identified at the molecular level as tropomyosin, a myofibrillar protein of 35–38 kDa, common with the following decapods: Indian white shrimp *Penaeus indicus* (Shanti et al., 1993), brown shrimp *P. aztecus* (Daul et al., 1994), sand shrimp *Metapenaeus ensis* (Leung et al., 1994), American lobster *Homarus americanus* (Leung et al., 1998a), spiny lobster *Panulinus stimpsoni* (Leung et al., 1998a) and red crab *Charybdis feriatus* (Leung et al., 1998b).

Besides decapods, barnacles, members of the subclass Cirripedia of the class Thecostraca, are also edible crustaceans. They inhabit the rocky shores of warm seas and some species of them are consumed as a delicacy in coastal countries such as Japan and the Mediterranean countries. The subclass Cirripedia is further divided into two orders, Sessilia (acorn barnacles lacking a stalk) and Pedunculata (goose barnacles having a stalk). The acorn barnacle *Balanus rostratus* and the goose barnacle *Capitulum* (*Pollicipes*) *mitella* are the most consumed species in Japan and the goose barnacle *Pollicipes pollicipes* in the Mediterranean countries. Recently, barnacles were reported to cause IgE-mediated allergic reactions by two research groups (Moreno Escobosa et al., 2002; Marinho et al., 2006). A number of IgE-binding proteins were detected in the barnacle extract by immunoblotting experiments using sera from barnacle-allergic patients. Interestingly, one IgE-binding protein of about 37 kDa was suggested to be tropomyosin from the molecular mass. However, only one of the five barnacle-allergic patients was diagnosed to be also allergic to shrimp in one study (Marinho

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et al., 2006), while susceptibility to shrimp was unclear for the five barnacle-allergic patients in another study (Moreno Escobosa et al., 2002). It is therefore still unknown whether decapod-allergic patients recognize barnacle tropomyosin as well as decapod tropomyosin.

Tropomyosin is regarded as a pan-allergen that is implicated in IgE cross-reactivity among various invertebrates including crustaceans (decapods), mollusks, mites and cockroaches (Leung et al., 1996; Reese et al., 1999; Santos et al., 1999; Chuo et al., 2000; Ayuso et al., 2002a,b; Lehrer et al., 2003). In view of this, it is possible that barnacle tropomyosin is also cross-reactive with decapod tropomyosin, having a potential to cause adverse reactions in decapod-allergic patients. The present study was initiated to confirm this possibility by immunoblotting and immunoblotting inhibition, using two species of barnacles (acorn barnacle *B. rostratus* and goose barnacle *C. mitella*) locally consumed in Japan. Subsequently, the primary structure of acorn barnacle tropomyosin was elucidated by cDNA cloning to obtain a molecular basis for the confirmed cross-reactivity between tropomyosins from acorn barnacle and decapods.

2. Materials and methods

2.1. Crustacean samples

Live specimens of American lobster and acorn barnacle and fresh specimens of black tiger prawn *Penaeus monodon* were purchased at the Tokyo Central Wholesale Market. Specimens of goose barnacle were caught along the coasts of Noto Peninsula, Ishikawa Prefecture, and transported alive to our laboratory. Abdominal muscles collected from American lobster and black tiger prawn and muscles from acorn barnacle and goose barnacle were all stored at −20 °C until used. For cloning experiments, part of the muscle samples from acorn barnacle and goose barnacle was immediately frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Preparation of heated extract

Each muscle sample was homogenized with four volumes of 10 mM phosphate buffer (pH 7.0) containing 0.6 M KCl. Since this study was focused on tropomyosin, a thermostable protein, the homogenate was heated in a boiling water bath for 10 min to make thermolabile proteins insoluble. After centrifugation at 18,000 *g* for 20 min, the supernatant obtained was used as heated extract. Protein concentrations of the heated extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.3. Purification of American lobster tropomyosin

An acetone powder of myofibrillar proteins was prepared from the deep abdominal muscle of American lobster as reported by Greaser and Gergely (1971) and extracted with 25 mM Tris–HCl buffer (pH 8.0) containing 1 M KCl, 0.1 mM $CaCl_2$ and 1 mM dithiothreitol. The extract was subjected successively to salting-out with ammonium sulfate (50–60% saturation) and isoelectrical precipitation (pH 4.6) by the method of Cummins and Perry (1973). To remove small amounts of contaminated proteins, the tropomyosin-rich fraction thus obtained was further applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 1 mL/min with a linear gradient of acetonitrile (38.5–49.0% in 50 min) in 0.1% trifluoroacetic acid. The homogeneity of the final preparation was supported by SDS-PAGE. American lobster has previously been reported to contain three types of tropomyosin (fast, slow-twitch and slow-tonic) (Mykles et al., 1998; Medler et al., 2004). Since the deep abdominal muscle of American lobster is composed of fast muscle fibers (Mykles et al., 1998), our purified preparation was judged to be fast type tropomyosin.

2.4. Human sera

Sera were obtained from ten crustacean-allergic patients with clinical histories of immediate hypersensitive reactions after ingestion of crustaceans but without experiences of eating barnacles. The patients were all diagnosed to be allergic to decapod crustaceans based on the determined CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) classes of 2–5 against shrimp or both shrimp and crab. Written informed consent was obtained from each patient. In this study, sera from four healthy volunteers without adverse reactions after ingestion of any foods were pooled and used as a control. All sera were stored at −20 °C.

2.5. SDS-PAGE

SDS-PAGE was performed with a PhastSystem (GE-Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's instructions. Each sample was dissolved in 62.5 mM phosphate buffer (pH 7.5) containing 2.5% SDS and 5% dithiothreitol, heated at 100 °C for 10 min and subjected to electrophoresis on a PhastGel Gradient 8–25 gel (GE-Healthcare Bio-Sciences). Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA) were run as a reference, along with samples. After running, proteins were visualized by staining with Coomassie Brilliant Blue R-250.

2.6. Immunoblotting

Immunoblotting was carried out as reported previously (Motoyama et al., 2006). Briefly, the proteins separated by SDS-PAGE were electrotransferred from the gel to a nitrocellulose membrane, which was reacted successively with primary and secondary antibodies. To detect tropomyosin, either one of the two monoclonal antibodies (0.5 μg/mL of 2A7H6 and 1 μg/mL of 5G5E1) against American lobster tropomyosin (Lu et al., 2007) was used as a primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgM+IgG+IgA antibody (diluted 1:20,000; Cosmo Bio, Tokyo, Japan) as a secondary antibody. For the detection of IgE-binding proteins, patient serum (diluted 1:500) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1:5000; Kirkegaard and Perry Laboratories,

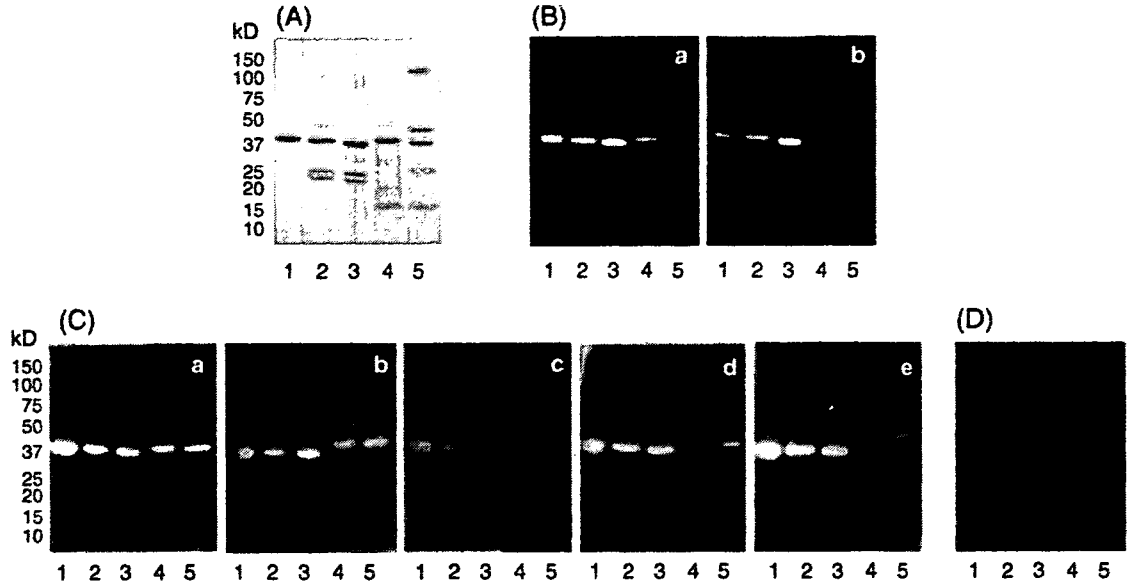

Fig. 1. Analysis of the heated extracts from decapods and barnacles by SDS-PAGE (A), immunoblotting using monoclonal antibodies against American lobster tropomyosin (B), immunoblotting using patient sera (C) and inhibition immunoblotting (D). Lanes: 1, tropomyosin purified from American lobster; 2, American lobster extract; 3, black tiger prawn extract; 4, acorn barnacle extract; 5, goose barnacle extract. (A) Heated extracts (1 μl each) with the following protein concentrations were subjected to SDS-PAGE: 3.6 mg/mL for American lobster, 6.6 mg/mL for black tiger prawn, 3.6 mg/mL for acorn barnacle and 4.4 mg/mL for goose barnacle. (B) Monoclonal antibodies: a, 2A7H6; b, 5G5E1. (C) Sera: a, pooled serum from ten patients; b, patient 1; c, patient 2; d, patient 3; e. patient 4. (D) Pooled serum (diluted 1:250) from ten patients was preincubated with an equal volume of inhibitor (American lobster tropomyosin) solution (20 μg/mL) and used as a primary antibody. The same results were also obtained with the sera from patients 1–4.

Gaithersburg, MD) were used as primary and secondary antibodies, respectively. Positive bands were visualized using an ECL Plus Western Blotting Detection System (GE-Healthcare Bio-Sciences) and an ECL Mini Camera (GE-Healthcare Bio-Sciences), according to the manufacturer's instructions. For IgE-immunoblotting inhibition, patient serum (diluted 1:250) was

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ATAGCCCTTTCGAGTTTGCGGCAGCAGGATTGTGGTTCTGACAGCTCTCGTCTGCTAAAGTCACTTGTAGCTTCGACATTCCTATTTAT    89

TTTATTTTATTTGTGTGTTAGGTGAGAATAAACTAAAAATCAGCCATGGATGCCATCAAGAAGAAGATGCAGGCAATGAAATTGGAAAAG   179
                                                M  D  A  I  K  K  K  M  Q  A  M  K  L  E  K    15

GAAAATGCCTTGGATAAAGCAGAACAGTTAGAACAAAAACTTAGGGATGTTGAGGAAACCAAAGCAAAGGCAGAGGAAGATCTTACTCTT   269
 E  N  A  L  D  K  A  E  Q  L  E  Q  K  L  R  D  V  E  E  T  K  A  K  A  E  E  D  L  T  L    45

CTCCAAAAGAAATACACAAACCTCGAGAATGAGTTCGACCAAGTCAACGAACAATACAATGAAGGTGTCACCAAGCTTGAGGTCTCCGAG   359
 L  Q  K  K  Y  T  N  L  E  N  E  F  D  Q  V  N  E  Q  Y  N  E  G  V  T  K  L  E  V  S  E    75

AAACGTGTAACAGAGGCAGAAGATGAAATCAAGGGtTACACTAGGcGTATCCAACTTTTAGAAGATGACCTTGAACGTACCCCAGTCAAG   449
 K  R  V  T  E  A  E  D  E  I  K  G  Y  T  R  R  I  Q  L  L  E  D  D  L  E  R  T  P  V  K   105

TTGGACGAAGCCACCTTTAAGTTGGAAGACGCAACCAAAACAGCAGATGAGAGTGAAAGAGGACGCAAGGTACTCGAGAGCAGAAGCATT   539
 L  D  E  A  T  P  K  L  E  D  A  T  K  T  A  D  E  S  E  R  G  R  K  V  L  E  S  R  S  I   135

GCTGATGATGATAGAATTGATGCATTAGAAAAACAAGTGAAAGATGCTAAATATGTTGCTGAGGAAGCCGACCGTAAATACGATGAGGCT   629
 A  D  D  D  R  I  D  A  L  E  K  Q  V  K  D  A  K  Y  V  A  E  E  A  D  R  K  Y  D  E  A   165

GCCCGTAAACTTGCTATCACTGAAGTGGATCTTGAACGCTCCGAGACCCGATTGGAGGCTGCTGAAGCCAAAATTACCGAACTCAGTGAG   719
 A  R  K  L  A  I  T  E  V  D  L  E  R  S  E  T  R  L  E  A  A  E  A  K  I  T  E  L  S  E   195

GAGCTGGCTGTGGTTGGTAATAACTGTAAGGCCCTGCAGAACGCCGTAGACCAGGCATCTCAGAGAGAAGACAGTTACGAGGAGACTATC   809
 E  L  A  V  V  G  N  N  C  K  A  L  Q  N  A  V  D  Q  A  S  Q  R  E  D  S  Y  E  E  T  I   225

CGTGACTTGACCCAGAGACTCAAGGACGCCGAGAATCGTGCGGCGGAGGCTGAACGCGTAGTCAACAAGCTGCAGAAGGAAGTTGACAGA   899
 R  D  L  T  Q  R  L  K  D  A  E  N  R  A  A  E  A  E  R  V  V  N  K  L  Q  K  E  V  D  R   255

TTAGAAGATGAATTACTTGCTGAAAAGGAGAAGTACAAGGCGATTAGTGACGAATTGGATCAAACATTTGCTGAGTTAGCTGGCATGTGA   989
 L  E  D  E  L  L  A  E  K  E  K  Y  K  A  I  S  D  E  L  D  Q  T  F  A  E  L  A  G  M  *   284

GACATCAACTTTTGTCAGACAACAAGAAAAATACACTTCATTTACATTTCTACAAAGCCCTGTTTCGCTGTTATTGATGAT          1070
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Fig. 2. Nucleotide sequence of cDNA encoding for acorn barnacle tropomyosin. The deduced amino acid sequence is denoted below the nucleotide sequence. Nucleotide and amino acid numbers are shown at the right. The region corresponding to the primer used in 3′RACE is indicated by a straight line and those corresponding to the primers used in 5′RACE by dotted lines. An asterisk represents the stop codon (TGA).

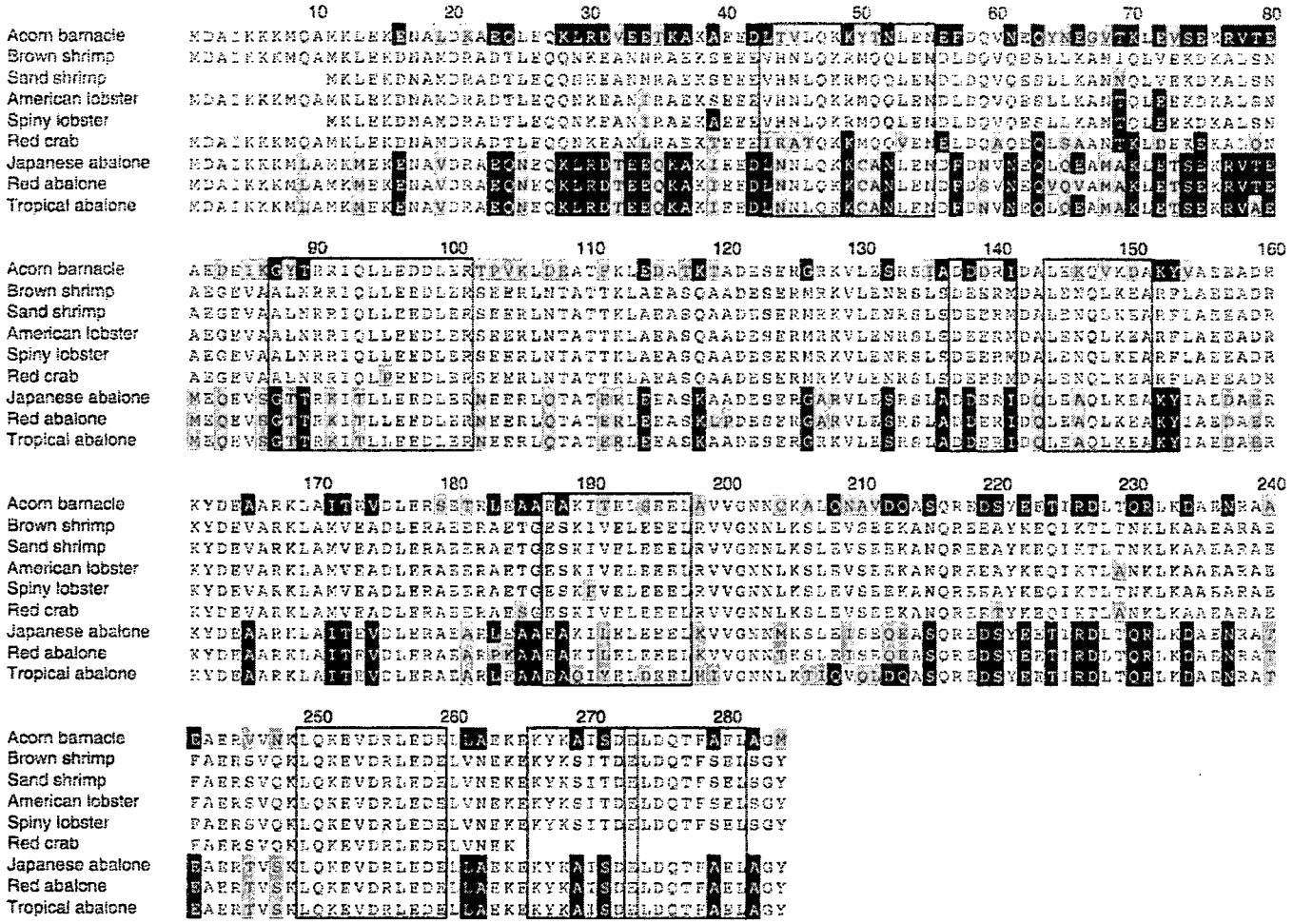

Fig. 3. Alignment of the amino acid sequence of acorn barnacle tropomyosin with those of decapod and abalone tropomyosins. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases): acorn barnacle (*Balanus rostratus*), AB269786; brown shrimp (*Penaeus aztecus*), DQ151457; sand shrimp (*Metapenaeus ensis*), U08008; American lobster (*Homarus americanus*) (fast type tropomyosin), AF034954; spiny lobster (*Panulinus stimpsoni*), AF030063; red crab (*Charybdis feriatus*), AF061783; Japanese abalone (*Haliotis diversicolor*), AF216518; red abalone (*Haliotis rufescens*), X75218; tropical abalone (*Haliotis asinina*), AY320360. The residues differing from brown shrimp tropomyosin are shaded. Of the different residues from brown shrimp tropomyosin, common residues between acorn barnacle tropomyosin and other tropomyosins are shown in white on the black background. The IgE-binding epitopes proposed for brown shrimp tropomyosin are boxed.

incubated with an equal volume of American lobster tropomyosin solution (20 μg/mL) at 37 °C for 1 h and used as a primary antibody.

2.7. Cloning experiments

Total RNA was extracted from muscle sample (2 g) of either acorn barnacle or goose barnacle with the TRIzol reagent (Invitrogen, Carlsbad, CA). For 3′ rapid amplification of cDNA ends (RACE), first strand cDNA was synthesized from 5 μg of total RNA using the 3′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) as recommended by the manufacturer and used as a template. A forward primer 5′-ATCAAGAAGAA-GATGCAGGCG-3′ (corresponding to ^{4}IKKKMQA^{10}), which was designed from the conserved sequence of the known cDNAs coding for tropomyosins from brown shrimp (DDBJ/EMBL/GenBank accession number: DQ151457), American lobster (AF034954 for fast tropomyosin, AF034953 for slow-twitch tropomyosin and AY521627 for slow-tonic tropomyosin) and red crab (AF061783), was used in PCR in combination with the abridged universal amplification primer (AUAP). Amplification was performed using HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, 55 °C for 20 s and 70 °C for 1 min; and 72 °C for 7 min. After electrophoresis on a 1.2% agarose gel and extraction with a QIAquick-Gel Extraction Kit (Qiagen, Tokyo, Japan), the PCR product was determined for its nucleotide sequence using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and a PRISM 310 genetic analyzer (Applied Biosystems). 5′RACE was carried out using the 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) following the manufacturer's instructions. First strand cDNA was synthesized from 5 μg of total RNA using the gene-specific primer 5′-GCGTCCTTGAGTCTCTGGGT-3′ (corresponding to ^{229}TQRLKDA^{235}). The first 5′RACE reaction using the gene-specific primer 5′-GCGTTCTGCAGGGCCTTACA-3′ (corresponding to ^{204}CKALQNA^{210}) and the abridged anchor

primer and the second 5'RACE reaction using the gene-specific primer 5'-TAGCAAGTTTACGGGCAGCC-3' (corresponding to ^{164}EAARKLAI^{171}) and the AUAP were completed under the same conditions as adopted for 3'RACE. Nucleotide sequencing of the second PCR product was also performed as in the case of 3'RACE.

2.8. Phylogenetic analysis

Multiple alignments of tropomyosins from various animals were performed with the Clustal W program (Higgins et al., 1994). Based on the results of multiple alignments, a phylogenetic tree was constructed using a MEGA 3.1 software (Kumar et al., 2004) by the neighbor-joining method (Saitou and Nei, 1987). The robustness of topology nodes was tested by the bootstrap method with 1000 replications.

3. Results

3.1. Allergens of barnacles

On SDS-PAGE, all the heated extracts from two species of decapods (American lobster and black tiger prawn) and two species of barnacles (acorn barnacle and goose barnacle) afforded a prominent protein band of about 37 kDa at almost the same position as American lobster tropomyosin (Fig. 1A). Irrespective of the species, the 37 kDa protein reacted with the monoclonal antibody 2A7H6 against American lobster tropomyosin (Fig. 1B), which recognizes both crustacean and molluscan tropomyosins (Lu et al., 2007), allowing us to conclude that the 37 kDa protein is tropomyosin of each decapod and barnacle. Very interestingly, another monoclonal antibody 5G5E1 specific only to crustacean tropomyosin (Lu et al., 2007) reacted to the 37 kDa protein in two species of decapods but showed no substantial reactivity to that in two species of barnacles (Fig. 1B). This result suggested significant differences in amino acid sequence between decapod and barnacle tropomyosins. Indeed, the elucidated amino acid sequence of acorn barnacle tropomyosin shares no high identity with decapod tropomyosins, as described below in more detail. In IgE-immunoblotting, no bands appeared when the control serum was used (data not shown), while the pooled serum from ten decapod-allergic patients and four individual patient sera were all reactive not only to tropomyosins from two species of decapods but also to those from two species of barnacles (Fig. 1C). As obviously recognized in the case of sera from patients 3 and 4, the tropomyosin bands obtained with two species of barnacles were considerably weak compared to those obtained with two species of decapods. This can be realized by assuming that the barnacle tropomyosins with significantly different amino acid sequences have a lower IgE-binding potency than the decapod tropomyosins. Besides tropomyosin, some IgE-reactive proteins, although much less potent in IgE-binding ability than tropomyosin, were also detected in the heated extracts from decapods and barnacles; for example, a 75 kDa protein in black tiger prawn reacted with the pooled

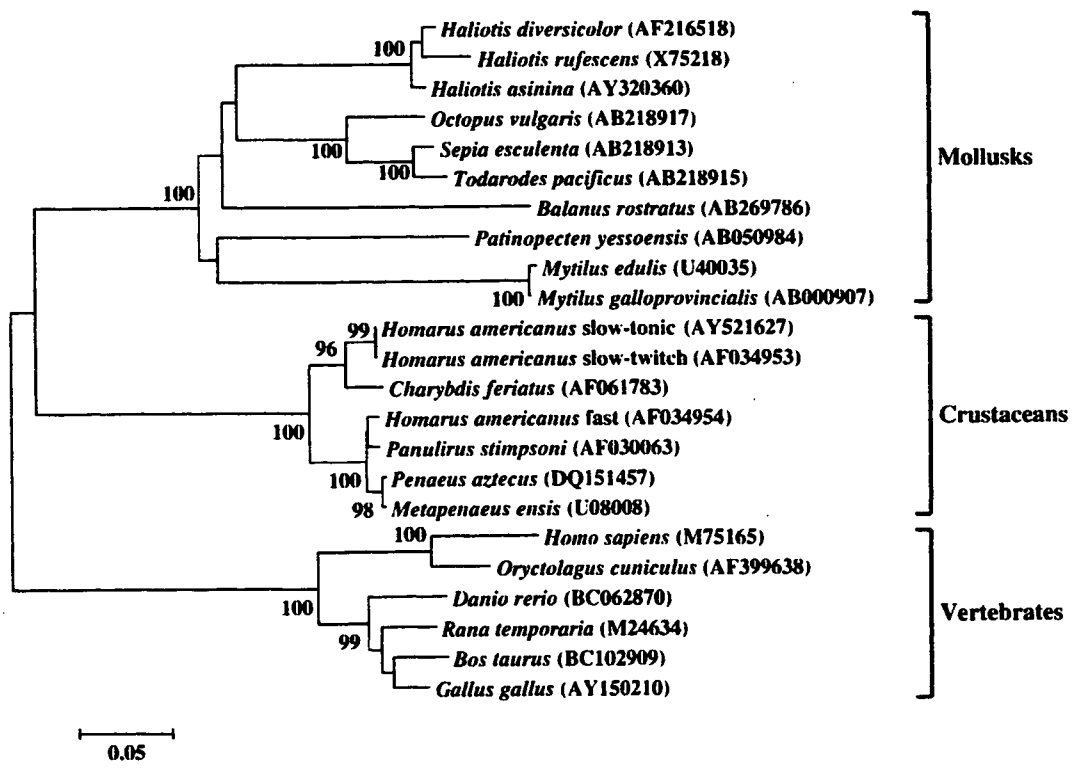

Fig. 4. Molecular phylogenetic tree for tropomyosins from various animals. The tree was constructed by the neighbor-joining method. Bootstrap values >70% are reported on nodes of the tree. The length of branches indicates the genetic distance between the tropomyosins. Accession numbers (DDBJ/EMBL/GenBank nucleotide sequence databases) are shown in parentheses after scientific names.

patient serum and a 30 kDa protein in acorn barnacle with the patient 4 serum. On the whole, however, tropomyosin was almost the sole IgE reactive protein, regardless of the crustaceans and patients. In IgE-immunoblotting inhibition, the patient sera preincubated with American lobster tropomyosin lost their reactivity to tropomyosins from both decapods and barnacles (Fig. 1D), demonstrating the cross-reactivity between decapod and barnacle tropomyosins.

3.2. Primary structure of acorn barnacle tropomyosin

In the case of goose barnacle tropomyosin, no amplified products were obtained by 3′RACE. On the other hand, a cDNA fragment encoding acorn barnacle tropomyosin was successfully amplified by 3′RACE, although the specific primer used was different by one nucleotide from the finally elucidated nucleotide sequence of the acorn barnacle tropomyosin. Based on the nucleotide sequence of the cDNA fragment, the remaining 5′ region sequence was analyzed by 5′RACE. The nucleotide sequence of the full-length cDNA (1070 bp) encoding acorn barnacle tropomyosin was thus established as shown in Fig. 2. This nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank databases under the accession number of AB269786.

An open reading frame of the acorn barnacle tropomyosin cDNA is composed of 852 bp coding for 284 amino acid residues from the putative initiating methionine to the putative last methionine. The deduced amino acid sequence of acorn barnacle tropomyosin is shown in Fig. 3, together with those from five species of decapod crustaceans and three species of abalones. Tropomyosins of decapod crustaceans share an extremely high sequence identity (more than 90% identity) with one another. Although acorn barnacle is a member of the Crustacea, its tropomyosin has only 58–61% sequence identities with decapod tropomyosins. Interestingly, a homology search by the BLAST program (Altschul et al., 1997) revealed that the highest sequence identity (76–78% identity) with acorn barnacle tropomyosin is displayed by abalone tropomyosins. In order to predict the evolutional position of the acorn barnacle tropomyosin, a molecular phylogenetic tree for tropomyosins was constructed. As shown in Fig. 4, crustacean (decapod), molluscan and vertebrate tropomyosins independently form a specific cluster and the acorn barnacle tropomyosin is classified into the molluscan tropomyosin family.

4. Discussion

Tropomyosin has been established to be a major and cross-reactive allergen in decapod crustaceans (Reese et al., 1999; Lehrer et al., 2003). Our immunoblotting data demonstrated that serum IgE of decapod-allergic patients reacts not only to tropomyosins from two species of decapods (American lobster and black tiger prawn) but also to those from two species of barnacles (acorn barnacle *B. rostratus* and goose barnacle *C. mitella*) locally consumed as a delicacy in Japan. IgE cross-reactivity between decapod and barnacle tropomyosins was also verified by immunoblotting inhibition using American lobster tropomyosin as an inhibitor. Based on these results, we recommend that decapod-allergic patients should avoid consumption of barnacles as well as decapods unless they are clinically diagnosed to be insensitive to barnacles.

Barnacles are not so widely consumed and hence barnacle allergies may be rare. It is, however, worth mentioning that barnacles are crustaceans but significantly distinct in appearance from decapods such as shrimps and crabs. Moreover, they are sessile crustaceans differing from decapods. Therefore, barnacles are not always correctly recognized as members of crustaceans and often misunderstood as members of sessile bivalves or snails by consumers and food servers. In other words, patients allergic to decapods but not to mollusks may mistakenly eat barnacles, resulting in the manifestation of allergic reactions. To prevent accidental incidents of fool allergy by consumption of barnacles, it is particularly important to let the public including decapod-allergic patients fully understand that barnacles are members of the Crustacea, having a potential to cause allergic reactions similar to decapods.

The significance of this study is the finding that the amino acid sequence of the acorn barnacle tropomyosin, elucidated by a cDNA cloning technique, is more homologous to those of abalone tropomyosins than to those of decapod tropomyosins. In accordance with this, the phylogenetic tree made for tropomyosins from various animals showed that the acorn barnacle tropomyosin is evolutionally classified not into the decapod tropomyosin family but into the molluscan tropomyosin family. From the viewpoint of molecular evolution, future study is needed to examine whether the sequence features observed with the acorn barnacle tropomyosin are a common feature of barnacle tropomyosins. In addition, it should be noted that such crustaceans as mantis shrimp (belonging to the order Stomatopoda of the class Malacostraca) and Antarctic krill (belonging to the order Mysidacea of the class Malacostraca) are also edible and may be allergenic. It is, therefore, interesting to elucidate the amino acid sequences of tropomyosins from these crustaceans not only for a molecular understanding on the possible cross-reactivity with decapod tropomyosins but also from the viewpoint of comparative biochemistry.

Although the sequence homology between acorn barnacle and decapod tropomyosins is not so high, the cross-reactivity between them is apparent from the immunoblotting inhibition data. This cross-reactivity can be partly realized by the fact that one (region 249–259) of the eight IgE-binding epitope regions (43–55, 88–101, 137–141, 144–151, 187–197, 249–259, 266–273 and 273–281; refer to Fig. 3) proposed for brown shrimp tropomyosin (Pen a 1) (Ayuso et al., 2002a,b) is completely conserved in the acorn barnacle tropomyosin. However, there are more than two alterations in the other seven regions between acorn barnacle and decapod tropomyosins. It is unclear whether these seven regions are also IgE-binding epitopes of the acorn barnacle tropomyosin. In order to understand in more detail the cross-reactivity between acorn barnacle and decapod tropomyosins, future study using synthetic peptides is needed on the IgE-binding epitopes of acorn barnacle tropomyosin. Such study will also provide helpful information on the IgE-binding epitopes of

abalone tropomyosins that are highly homologous to the acorn barnacle tropomyosin.

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Note

Specific Detection of Soybean Residues in Processed Foods by the Polymerase Chain Reaction

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A sensitive qualitative detection method for soybeans in foods by using the polymerase chain reaction (PCR) was developed. For specific detection of soybeans with high specificity, the primer pair of Gym 81/Gym 82 was designed on the gene encoding the Glycine max repetitive sequence. The trace amount of soybeans in commercial food products could be qualitatively detected by this method.

Key words: food allergy; soybean; *Glycine max* L.; detection method; polymerase chain reaction (PCR)

Food allergies have constituted an important health problem in recent years in industrialized countries, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy.[1–3] To prevent possible life-threatening reactions, the only effective treatment is to strictly avoid the consumption of these allergenic foods. Therefore, sufficient information about potentially allergenic ingredients in a food product is necessary.[4,5] In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to label eight kinds of food which contain ingredients known to be allergens, including soybeans.[6,7] In Japan, the Ministry of Health, Labor and Welfare has enforced a labeling system for allergenic food material since April 2002.[3] With this system, labeling of five food products (egg, milk, wheat, buckwheat, and peanuts) is mandatory, and is recommended for twenty other food materials such as soybeans and shrimp. Soybean labeling is particularly important, because of the almost unlimited uses of soybean, and the number of patients with an allergy to soybeans has been increasing.[8–11] In the present study, we developed a detection method for soybeans with high specificity and sensitivity using PCR. We show that the established method could be applicable for processed food products, and a trace amount of soybeans contained in a commercial food product can be detected by the proposed method.

Nine varieties of soybean (*Glycine max* L.), including seven varieties of domestic soybean (Toyokomachi, Toyomusume, Ryuhou, Tachinagaha, Enrei, Fukuyutaka and Murayutaka) and two varieties of U.S. soybean (Vinton and Navy), were collected in our laboratory. Legume relatives such as two varieties of the common bean (*Phaseolus vulgaris* cul. Tebou and *Phaseolus vulgaris* cul. Toramame), azuki bean, cowpea, broad bean, pea, lentil, lupine, chickpea and peanut, and grain samples of wheat, rye, barley, oats, corn, rice, buckwheat and rapeseed were also purchased by our laboratory. Eight kinds of nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan and walnut) were kindly provided by Morinaga Co., Ltd. Soybean-derived food materials including seasonings, lecithin, fiber and protein were collected by our laboratory, and ten kinds of commercial food products with soybeans in the list of ingredients were purchased from a local market in Japan.

The seed materials of the soybeans, other crops, food materials and commercial food products were ground with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and then the homogenous powders were used as the test samples. Wheat was used as the matrix to prepare the model mixed samples. Wheat grains were ground to a fine powder with a grinder and 0, 0.001, 0.005, 0.01, 0.1 and 100% of soybean flour (Nikka Fats & Oils, Tokyo, Japan) was then mixed according to the procedure described in a previous study with a slight modification.[12]

Genomic DNA was extracted from each plant material including soybean flour by using a silica-gel mem-

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Table 1. List of Designed Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	CP 03-5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	Chloroplast DNA, sense	Plants	123 bp
	CP 03-3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	Chloroplast DNA, antisense		
B:	Gym 81	5′-TCA GCA GAT TCA AAT CTC CCA GTG A-3′	Interspersed repetitive element 1, sense	Soybean	118 bp
	Gym 82	5′-CAT CTC AAG AAG CAG AGG AAA GGA C-3′	Interspersed repetitive element 1, antisense		

A: To confirm the validity of DNA extracted from plants for the polymerase chain reaction.
B: For specific detection of soybean.

brane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany) according to the procedure described in a previous study with some modification.[13] Genomic DNA was extracted from each soybean-derived food material and commercial food product by using an anion exchange-type kit (Genomic-tip 20/G; Qiagen, Hilden, Germany) according to the procedure described in a previous study.[14] The extracted DNA was diluted with the appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl, and stored at −20 °C until needed. When the concentration of the extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for the subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column by Operon Biotechnologies (Tokyo, Japan), and then diluted with the appropriate volume of DW to a final concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), all topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to a PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-220 DNA Engine DYAD; Bio-Rad Laboratories, CA, USA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out according to previous studies.[12,13]

To specifically detect soybeans by using PCR, we investigated the soybean-specific genes such as the Glycine max gene for Bd 30 K, the Kunitz trypsin inhibitor, beta-1,3-glucanase, cytosolic glutamine synthetase, the GB-D-II gene for proteinase inhibitor, vspA gene, the Bowman-Birk protease inhibitor, repetitive sequence (soybean interspersed repetitive element 1, SIRE-1) and satellite STR120-A.1. Based on these soybean-specific gene sequences, we designed the respective primer pair and examined PCR amplification by using the designed primer pairs and the mixed samples described later.

Consequently, the Glycine max repetitive sequence (SIRE-1) should be considered to be the most sensitive and specific for amplifying the gene sequence. Therefore, we chose a gene encoding SIRE-1 as a soybean-specific gene and designed the primer pair Gym 81/Gym 82 by referring to GeneBank Accession No. L06326. In addition, the primer pair, CP 03-5′/CP 03-3′, for the universal detection of DNA derived from plants was used to verify the extracted DNAs.[14] The primer pair, CP 03-5′/CP 03-3′, generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

As shown in Fig. 1A, a fragment (118 bp) amplified by using the primer pair Gym 81/Gym 82 was specifically detected from the soybean genomic DNA. In contrast, no amplified fragment was detected when the DNA was extracted from other legumes such as lupine and peanut, as shown in Fig. 1B. In addition, 16 kinds of other plant species (wheat, rye, barley, oats, corn, rice, buckwheat, rapeseeds, almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan and walnut) as the template DNA are shown in Fig. 1C and D. These data suggest that the soybean-genomic DNA can be specifically detected by using the Gym 81/Gym 82 primer pair.

To assess the sensitivity of the proposed PCR method, we tested the mixed wheat flour samples containing 0, 0.001%, 0.005%, 0.01%, 0.1% and 100% of the soybean flour powder. Fifty ng of the genomic DNA extracted from the mixed samples was amplified in the PCR reaction. As expected, the target sequence for the soybeans was clearly detected in the 0.001% to 100% mixed samples. This result suggested that the contaminated soybean flour in wheat flour can be at least detected at the level of 0.001% in a product without processing. To investigate the applicability of the soybean DNA detection method for commercial food products and food materials, we purchased 18 food items and tested them for the presence of soybean DNA by using the

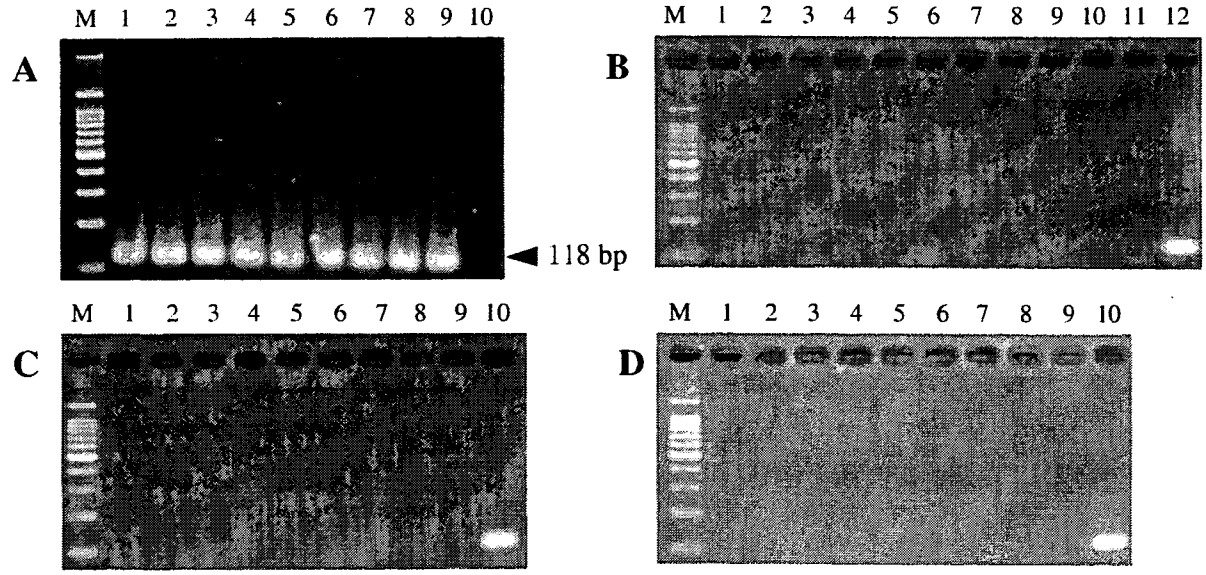

Fig. 1. Specificity of the Polymerase Chain Reaction Method Using the Primer Pair Gym 81/Gym 82 to Identify the DNA Sequence of the Gene Encoding Soybean Interspersed Repetitive Element 1.

A, Agarose gel electrophoresis of PCR products amplified from nine varieties of soybean genomic DNA [lane 1, Toyokomachi; lane 2, Toyomusume; lane 3, Ryuhou; lane 4, Tachinagaha; lane 5, Enrei; lane 6, Fukuyutaka; lane 7, Murayutaka; lane 8, Vinton; lane 9, Navy; lane 10, no template control; M, 100-bp ladder size standard]. B, Agarose gel electrophoresis of PCR products amplified from legume-relative genomic DNA [lane 1, Toramame; lane 2, Tebo; lane 3, azuki bean; lane 4, cowpea; lane 5, broad bean; lane 6, pea; lane 7, lentil; lane 8, lupine; lane 9, chickpea; lane 10, peanut; lane 11, no template control; lane 12, soybean; M, 100-bp ladder size standard]. C, Agarose gel electrophoresis of PCR products amplified from grain and rapeseed genomic DNA [lane 1, wheat; lane 2, rye; lane 3, barley; lane 4, oats; lane 5, corn; lane 6, rice; lane 7, buckwheat; lane 8, rapeseeds; lane 9, no template control; lane 10, soybean; M, 100-bp ladder size standard]. D, Agarose gel electrophoresis of PCR products amplified from nut genomic DNA [lane 1, almond; lane 2, cashew; lane 3, macadamia; lane 4, pistachio; lane 5, hazelnut; lane 6, Brazil nut; lane 7, pecan; lane 8, walnut; lane 9, no template control; lane 10, soybean; M, 100-bp ladder size standard].

proposed method. A sufficient amount (20 ng/μl) of genomic DNA for PCR was obtained from almost all the food products by using the purification method already described, except for the fried tofu and seasoning A. All the food product samples had soybean labeled on the list of ingredients. As shown in Table 2, the universal primer pair CP 03-5′/CP 03-3′ could generate a specific amplified fragment from all of the samples except for the fried tofu and seasoning A. In 16 food products except the fried tofu and seasoning A, soybean DNA was clearly detected with the PCR method by using the Gym 81/Gym 82 primer pair. Based on these results, we postulate that the absence of amplified PCR products from the fried tofu and seasoning A was probably due to the limited amount of genomic DNA in them. These results suggest that the proposed PCR method using the Gym 81/Gym 82 primer pair would be applicable for identifying soybean in processed food products, except for those that are highly processed.

In summary, a rapid PCR detection method was developed for the specific detection of soybeans. The methods reported in this study are simple, sensitive, and reliable for identifying a trace amount of soybean in processed foods. To minimize the risk of false negative or positive results, we believe that the PCR method can be used to complement the protein-based detection method.

Table 2. Investigation of Commercial Food Products (1–10) and Food Materials (11–18)

	Food item	CP 03-5′/CP 03-3′	Gym 81/Gym 82
1	Fish sausage	+	+
2	Loin roll	+	+
3	Bread	+	+
4	Soymilk drink	+	+
5	Biscuit	+	+
6	Roasted soybean tea	+	+
7	Potato chips	+	+
8	Fried tofu	−	−
9	Retort rice porridge	+	+
10	Retort sauce	+	+
11	Soy sauce	+	+
12	Miso	+	+
13	Seasoning A*	−	−
14	Seasoning B*	+	+
15	Soybean lecithin	+	+
16	Bean curd refuse powder	+	+
17	Soybean fiber	+	+
18	Soy protein	+	+

+, positive; −, negative

*Seasonings containing hydrolyzed soy protein.

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Note

Detection of Walnut Residues in Processed Foods by Polymerase Chain Reaction

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A sensitive qualitative detection method for walnut (*Juglans regia*) using polymerase chain reaction (PCR) was developed. For detection of walnuts with high specificity, the primer pair WAL-F/WAL-R was designed based on walnut matK genes. Trace amounts of walnuts in commercial food products can be qualitatively detected using this method.

Key words: allergen; walnut; pecan nut; PCR; restriction enzyme

Botanical food allergies, including allergy to walnuts (*Juglans regia*), represent a crucial and growing health problem in industrialized countries.[1–4] In Japan, it has become clear from epidemiological investigations that the number of patients with walnut allergy has increased.[5,6] The Ministry of Health of the Japanese government has enforced a labeling system for allergenic food materials since April 2002.[5] In this system, labeling of five food products (egg, milk, wheat, buckwheat, and peanuts) is mandatory, while it is recommended for 20 other food materials, including walnuts. Therefore, the development of a rapid detection method for hidden walnut residues in foods is desirable. Recently, a specific detection method for walnuts using real-time PCR equipment was reported by European researchers.[7] While the method is rapid, it also requires expensive real-time PCR instruments and specific probes. PCR methods for detecting wheat, peanuts, soybeans, and kiwifruit have already been established using conventional PCR and electrophoresis.[8–12] In the present study, we developed a detection method for walnuts with sensitivity and specificity using conventional PCR. We show that the method established can be applied to processed food products, and that even trace amounts of walnut contained in a commercial food product can easily be detected by it.

Walnuts and samples of kiwifruit, apple, yam, banana, and soybean were also purchased by our laboratory. Eight kinds of nuts (almond, cashew, macadamia, pistachio, hazelnut, Brazil nut, pecan nut, and walnut) were kindly provided by Morinaga Institute of Biological Science, Inc. (Yokohama, Japan).

Genomic DNA was extracted from each plant material, including walnuts, using a silica-gel membrane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany) according to the procedure described in a previous study, with some modifications.[13] Genomic DNA was extracted from each walnut-derived food material and commercial food product using an anion exchange-type kit (Genomic-tip 20/G; Qiagen), according to the procedure described in a previous study.[10] The extracted DNA was diluted with the appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl, and stored at −20 °C until needed. When the concentration of extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for the subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column by Operon Biotechnologies (Tokyo), diluted with the appropriate volume of DW to a final

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Table 1. List of Designed Primers for Detection of Walnut

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	CP 03-5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	Chloroplast DNA /sense	Plants	123 bp
	CP 03-3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	Chloroplast DNA /antisense		
B:	WAL-F	5′-GAT CTA TAT TGT TGG AAA ATG TAG C-3′	Chloroplast maturase (matK) gene/sense	Walnut	120 bp
	WAL-R	5′-GGT TAG AAT CAT TAG TGG AAA TCA G-3′	Chloroplast maturase (matK) gene/antisense		

A: For confirmation of validity of the DNA extracted from plants for polymerase chain reaction.
B: For specific detection of walnut.

concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to a PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-220 DNA Engine DYAD; Bio-Rad Laboratories, Hercules, CA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 64 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. When the CP 03-5′/CP 03-3′ primer pair was used, the annealing temperature was changed to 60 °C. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out following previous studies.[13] For the design of primer pair WAL-F/WAL-R, Vector NTI Advance version 10 (Invitrogen Japan, Tokyo) was used.

To detect walnuts specifically using PCR, we investigated genes specific to walnuts. We designed primer pairs for various walnut-specific gene sequences, and examined PCR amplification using the designed primer pairs on walnuts and the other nut and food samples. We found that the walnut matK gene was the most specific for amplifying the gene sequence. Hence, we chose the gene encoding walnut matK as a walnut-specific gene, and designed primer pair WAL-F/WAL-R by referring to GeneBank accession no. AF118027. In addition, the primer pair CP 03-5′/CP 03-3′, for the universal detection of DNA derived from plants, was used to verify the extracted DNAs.[10] The primer pair CP 03-5′/CP 03-3′ generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

As shown in Fig. 1, a fragment (120 bp) amplified using primer pair WAL-F/WAL-R was specifically detected in walnut genomic DNA. In contrast, no amplified fragment was detected when the DNAs extracted from other foods, such as kiwifruit and soybean, were used, as shown in Fig. 1B. In addition, eight other nut species (almond, cashew, macadamia, pistachio, hazelnut, brazil nut, pecan nut, and walnut) were used as template DNA (Fig. 1C and D). The results suggest that only the genomic DNAs from walnuts and pecan nuts are detected using the WAL-F/WAL-R primer pair. In addition, it was confirmed that the detection limit of this PCR for both walnut and pecan nut was 0.5 pg (10 ppm equivalency) when we tested salmon genome DNA samples containing walnut DNA or pecan nut DNA (data not shown). However, according to the current Japanese article standard classification, pecan nuts are not classified as part of the walnut family, and hence, it is necessary to distinguish walnuts and pecan nuts. The matK gene, which is the target gene of this PCR, is known to exist in many edible kinds of walnut, but the gene sequence is unknown for *Juglandaceae*, *Carya illinoensis*, which is a food pecan nut. Hence, for the first time, we identified the gene sequence of matK for pecan nuts using a TA cloning technique. For insertion into the pCR®II-TOPO® vector (TOPO TA cloning® kit, Invitrogen Japan), a partial pecan nut matK gene was obtained by PCR amplification from pecan nut genome DNA with a sense primer (walnut-378F, 5′-GGA TTT CTA ACC ATC TTG TTA TCC T-3′) and an antisense primer (walnut-1295R, 5′-TCC AGA AGA TGT TAA TCG TAA ATG A-3′), under the reaction conditions described above. The 1.0-kb PCR product was cloned into pCR®II-TOPO® vector by standard procedures, and its sequence was analyzed by Hokkaido System Science (Sapporo, Japan). The pecan nut matK gene of the 148 bp fragment, including a domain amplified using the walnut PCR method, is shown in Fig. 2A.

By sequence analysis, we found that the products amplified from walnut and pecan nuts both involve the restriction enzyme site AclI, and we further determined that the products amplified from the walnut matK gene but not from the pecan nut matK gene involve a restriction enzyme site, BfaI (Fig. 2A). Therefore, to determine whether BfaI enzyme digestion discriminates between walnuts and pecan nuts, we examined digestion by the restriction enzyme with an amplification product

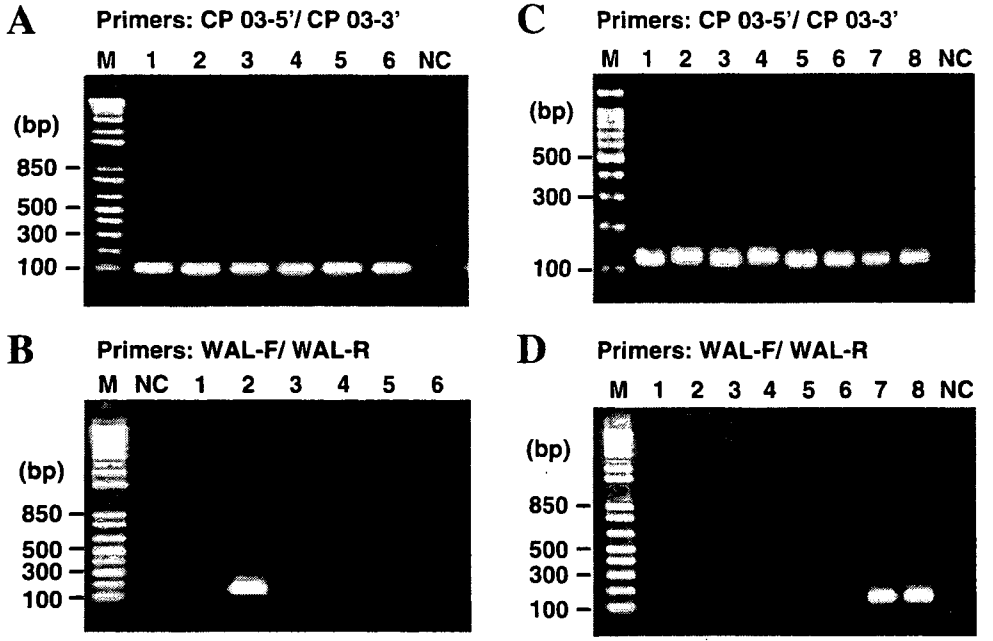

Fig. 1. Specificity of the PCR Method Using Walnut Primers (WAL-F/WAL-R).

A and B, Amplification of DNA of various plants species with primers for plants (A) or walnuts (B). Genome DNAs were obtained from kiwifruit (lane 1), walnut (lane 2), apple (lane 3), yam (lane 4), banana (lane 5), and soybean (lane 6). NC is the no-template control. Amplicons were electrophoresed in a 1.5% agarose gel. M, 100-bp ladder size standard. C and D, Amplification of DNA of various nut species with primers for plants (C) or walnuts (D). Genome DNAs were obtained from almond (lane 1), cashew (lane 2), macadamia (lane 3), pistachio (lane 4), hazelnut (lane 5), Brazil nut (lane 6), pecan nut (lane 7), and walnut (lane 8). NC is the no-template control. Amplicons were electrophoresed in a 2.5% agarose gel (plant) or a 1.5% agarose gel (walnut). M, 100-bp ladder size standard.

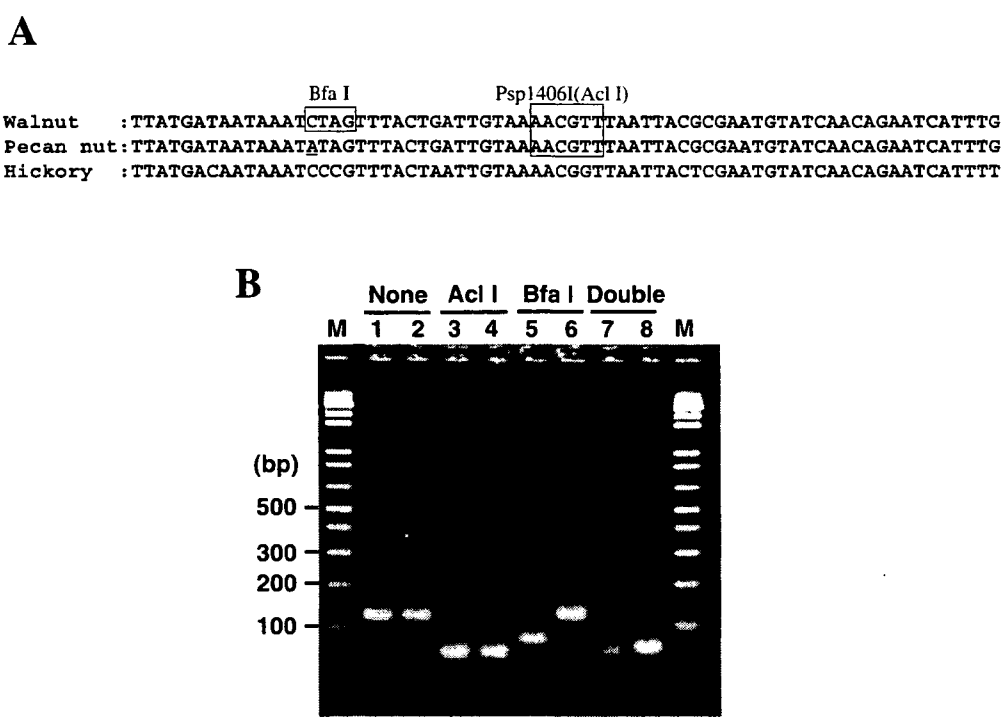

Fig. 2. Comparison of matK Genes of Walnut, Pecan Nut and Hickory (A) and Electrophoresis Analysis in 2.5% Agarose Gels of the Products Obtained with Endonuclease (B).

Primers WAL-F and WAL-R are underlined. GenBank accession nos.: walnut, AF118036, AF118027; hickory, AF118039. B, Odd numbers, walnut; even numbers, pecan nut; lanes 1 and 2, no endonuclease control; lanes 3 and 4, digestion with Acl 1; lanes 5 and 6, digestion with Bfa 1; lanes 7 and 8, digestion with Acl 1 and Bfa 1; M, 100-bp ladder size standard.

of walnut DNA and one of pecan nut DNA. As shown in Fig. 2B, we confirmed that the PCR products of the walnut matK gene were exactly digested with the restriction enzyme Bfa1 and were divided into two fragments while the PCR products of the pecan nut matK gene were not. These results suggest that Bfa1 enzyme digestion can discriminate between the PCR products of walnuts and pecan nuts.

To investigate the applicability of the walnut DNA detection method to commercial food products and food materials, we purchased 10 processed food samples and tested them for the presence of walnut DNA using our method. In two food product samples of mixed nuts and a bean-jam bun which included walnuts on the lists of ingredients, walnut DNA was clearly detected by the PCR method with the WAL-F/WAL-R primer pair. In the other eight processed food samples, such as snacks and cookies which did not include walnuts on the list of ingredients, no walnut DNA was detected using the PCR method with the WAL-F/WAL-R primer pair.

These results suggest that the proposed PCR method using the WAL-F/WAL-R primer pair is applicable in identifying walnuts in processed food products.

In conclusion, we developed a detection method for walnuts and pecan nuts using conventional PCR without expensive real-time PCR instruments. In addition, we discovered how restriction enzyme digestion of the PCR products can discriminate between walnuts and pecan nuts. To minimize the risk of false negative or positive results in the inspection for walnut contamination, we believe that the PCR method can be used to complement protein-based detection methods such as ELISA.

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Note

Specific Detection of Wheat Residues in Processed Foods by Polymerase Chain Reaction

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A sensitive qualitative detection method for wheat in foods using polymerase chain reaction (PCR) was developed. Trace amounts of wheat in commercial food products could be qualitatively detected by this method. The sensitivity of the proposed PCR method appears to be similar to that of ELISA. The present method should be very useful for detecting wheat residues in processed foods.

Key words: food allergy; common wheat; *Triticum aestivum* L.; detection method; polymerase chain reaction (PCR)

Food allergies have become an important health problem in recent years in industrialized countries, and it is estimated that approximately 8% of children and 2% of adults have some type of food allergy.[1–3] In particular, allergies to wheat have long been of importance, since wheat is consumed as a staple food in many countries.[4–7] To prevent possible life-threatening reactions, hypoallergenic foods might be useful,[8] but the most effective treatment is strictly to avoid the consumption of these allergenic foods. Therefore, accurate information about potentially allergenic ingredients in a food product is critical.[9,10] In 1999, the Joint FAO/WHO Codex Alimentary Commission agreed to label eight kinds of food that contain ingredients known to be allergens, including wheat.[11,12] In Japan, the Ministry of Health, Labor and Welfare has enforced a labeling system for allergenic food material since April, 2002.[3] With this system, labeling of five food ingredients (egg, milk, wheat, buckwheat, and peanuts) is mandatory.

In the present study, we developed a detection method for wheat with high specificity and greater sensitivity than previously published methods.[13,14] We show that the proposed method can be applied to processed food products, and that trace amounts of wheat contained in commercial food products can be detected by it.

Grain samples of common commercial wheat (*Triticum aestivum* L.) belonging to four classes, including Canada western red spring wheat, Australian standard wheat, Western white wheat, and domestic wheat (Norin 61), and Canadian amber durum wheat (*Triticum durum* L.) were collected in our laboratory. Grain samples of rye, barley, oats, rice, maize, soybean, foxtail millet, rapeseed, and buckwheat were also purchased. Nine different commercial food products with wheat in their lists of ingredients were purchased from a local market in Saitama.

The grain materials of wheat, other crops, and commercial food products were ground with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and then the homogenous powders were used as test samples for PCR. Maize was used as the matrix to prepare model mixed samples. Maize grains were ground to a fine powder with a grinder, and 0, 0.0001, 0.001, 0.005, 0.01, 0.1, 1, and 100% of wheat flour (Australian standard wheat) was then admixed according to the procedure described in a previous study, with a slight modification.[15]

Genomic DNA was extracted from each plant material, including model mixed samples, using a silica-gel membrane-type kit (DNeasy Plant Mini; Qiagen, Hilden, Germany). A 200-mg sample of ground sample was incubated for 15 min at 65 °C with the addition of 1.5 ml of buffer AP1 and 10 µl of RNase A, and then 400 µl of buffer AP2 was added to the mixture, which was cooled on ice for 5 min. Then the following steps were carried out according to previously described methods:[16] Genomic DNA was extracted from commercial food products using an anion exchange-type kit (Genomic-tip

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Table 1. List of Designed Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	CP 03-5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	Chloroplast DNA, sense	Plants	123 bp
	CP 03-3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	Chloroplast DNA, antisense		
B:	Wtr 01-5′	5′-CAT CAC AAT CAA CTT ATG GTG G-3′	Triticum aestivum triticin precursor, sense	Wheat	141 bp
	Wtr 10-3′	5′-TTT GGG AGT TGA GAC GGG TTA-3′	Triticum aestivum triticin precursor, antisense		

A. To confirm the validity of DNA extracted from plants for the polymerase chain reaction.
B. For specific detection of wheat.

20/G; Qiagen, Hilden, Germany) according to the procedure described in a previous study.[17] The extracted DNA was diluted with an appropriate volume of distilled water (DW) to a final concentration of 20 ng/μl and stored at −20 °C until needed. When the concentration of the extracted DNA was less than 20 ng/μl, an undiluted DNA extract was used for subsequent PCR analysis. The primers were synthesized and purified in a reversed-phase column (Operon Biotechnologies, Tokyo), diluted with an appropriate volume of DW to a final concentration of 50 μmol/l, and stored at −20 °C until needed.

The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 50 ng of genomic DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of the 5′ and 3′ primers, and 0.625 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), all topped up with DW. When the concentration of the extracted DNA was less than 20 ng/μl, 2.5 μl of an undiluted DNA extract was added to the PCR reaction tube. The reaction was buffered with PCR buffer II (Applied Biosystems), and amplified in a thermal cycler (PTC-220 DNA Engine DYAD; Bio-Rad Laboratories, Hercules, CA). The PCR step-cycle condition was as follows: pre-incubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min. After PCR amplification, agarose gel electrophoresis of the PCR product was carried out following previous studies.[15,18]

The amount of wheat protein in the commercial food products was measured using a sandwich ELISA kit for wheat (Morinaga, Yokohama, Japan) according to the procedure described in a previous study.[17] To detect wheat specifically by PCR, we investigated wheat-specific genes such as the triticum aestivum triticin precursor gene and the triticum aestivum glutathone S-transferase gene. Based on these wheat-specific gene sequences, we designed the respective primer pairs and examined PCR amplification using them on the mixed samples.

Consequently, the triticum aestivum triticin precursor gene proved to be more sensitive and specific than the glutathone S-transferase gene and others for amplifying the gene sequence. Triticum aestivum triticin is the storage protein and is synthesized specifically during seed development.[19] Hence, we chose the gene encoding the triticum aestivum triticin precursor as the wheat-specific gene and designed the primer pair Wtr 01-5′/Wtr 10-3′ on the coding region of that gene by referring to GeneBank accession no. S62630. In addition, the primer pair CP 03-5′/CP 03-3′, for universal detection of DNA derived from plants, was used to verify the extracted DNAs.[17] This primer pair generated a 123-bp amplified fragment. The sequences of the designed oligonucleotides in this study are listed in Table 1.

One to 32 μg genomic DNA from each 200 mg of grain samples was obtained by the purification method described above. As shown in Fig. 1A, a fragment (141 bp) amplified using the primer pair Wtr 01-5′/Wtr 10-3′ was specifically detected from common wheat and durum wheat genomic DNA. By contrast, no amplified fragment was detected when DNA was extracted from nine other plant species (rye, barley, oats, rice, maize, soybean, foxtail millet, rapeseed, and buckwheat) as the template DNA. Nucleotide sequence analysis of the PCR product obtained from common wheat and durum wheat confirmed that the intended sequence of the triticum aestivum triticin precursor gene had been amplified. These data suggest that wheat-genomic DNA can be specifically detected using the Wtr 01-5′/Wtr 10-3′ primer pair.

To assess the sensitivity of the proposed PCR method, we tested the mixed maize flour samples containing 0, 0.0001, 0.001, 0.005, 0.01, 0.1, 1, and 100% of wheat flour powder. Fifty ng of the genomic DNA extracted from the mixed samples was amplified in the PCR reaction. As expected, the target sequence for the wheat was clearly detected in the 0.005% to 100% mixed samples (Fig. 1B). This result suggests that wheat flour contamination of as low as 0.005% can be detected in an unprocessed food. To investigate the applicability of the wheat DNA detection method to commercial food products, we purchased nine food items with wheat in their lists of ingredients and tested them for the presence of wheat DNA using the proposed method. A sufficient amount (more than 20 ng/μl) of genomic DNA for PCR was obtained from all the food products, except for retort soup and sauce, using the described purification method. As shown in Fig. 1C, the universal primer pair CP 03-5′/CP 03-3′ generated a specific amplified

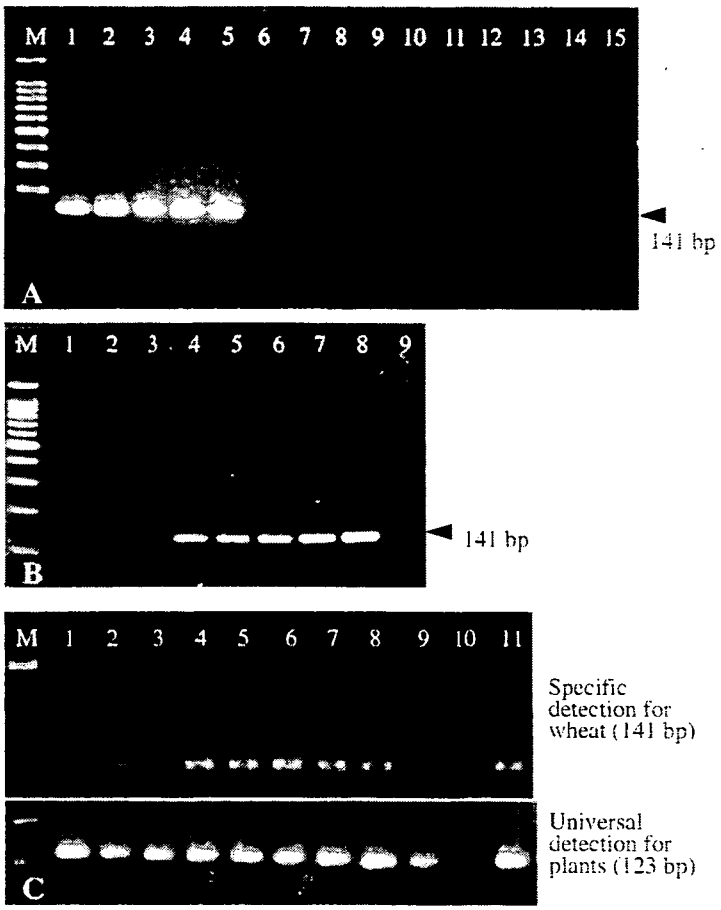

Fig. 1. Specific Detection of Wheat by PCR.

A. Specificity of the PCR method for wheat. The arrowhead indicates the expected PCR product. Lane 1, Canada western red spring wheat; lane 2, Australian standard wheat; lane 3, white wheat; lane 4, Japanese domestic wheat (Norin 61); lane 5, durum wheat; lane 6, rye; lane 7, barley; lane 8, oats; lane 9, rice; lane 10, maize; lane 11, soybean; lane 12, foxtail millet; lane 13, rapeseed; lane 14, buckwheat; lane 15, no template control; M, 100-bp ladder size standard. B. Sensitivity of the PCR method for wheat. The arrowhead indicates the expected PCR product. The genomic DNAs extracted from eight mixing levels of wheat flour in maize flour were used as the template DNA. Lane 1, 0%; lane 2, 0.0001%; lane 3, 0.001%; lane 4, 0.005%; lane 5, 0.01%; lane 6, 0.1%; lane 7, 1%; lane 8, 100%; lane 9, no template control; M, 100-bp ladder size standard. C. Investigation of commercial food products. Lane 1, fish meat paste; lane 2, pork sausage; lane 3, snacks; lane 4, chocolate; lane 5, cookies; lane 6, cracker; lane 7, instant Chinese noodle; lane 8, retort soup; lane 9, retort sauce; lane 10, no template control; lane 11, wheat; M, 100-bp ladder size standard.

fragment from all of the samples. In the nine food products, except for the retort sauce, wheat DNA was clearly detected by the PCR method using the Wtr 01-5′/Wtr 10-3′ primer pair (Fig. 1C and Table 2). Based on these results, we postulate that the absence of amplified PCR products from the retort sauce was probably due to the limited amount of wheat-derived genomic DNA in it. The wheat protein in retort sauce was also not detected by the ELISA method, and that result appears to be consistent with that for the present PCR method (Table 2). These results suggest that the proposed PCR method using the Wtr 01-3′/Wtr 10-5′ primer pair is applicable in identifying wheat in processed food products, except for those that are highly processed, and that the sensitivity of the PCR method appears to be similar to that of the ELISA method.

In summary, a rapid PCR detection method was

Table 2. Analyses of Wheat Protein and Wheat DNA in Commercial Food Products

No.	Food item	Protein-ELISA method	PCR method
		Protein conc. (ppm)	Result
1	Fish meat paste	0.4	Positive
2	Pork sausage	10.4	Positive
3	Snacks	1.0	Positive
4	Chocolate	7.2	Positive
5	Cookies	>12.8	Positive
6	Cracker	>12.8	Positive
7	Instant Chinese noodle	>12.8	Positive
8	Retort soup	8.5	Positive
9	Retort sauce	nd	Negative

nd, not detecteed; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction

developed for specific detection of wheat. The methods reported in this study are simple, sensitive, and reliable in identifying trace amounts of wheat in processed foods. To minimize the risk of false negative or positive results, we believe that the PCR method can be used to complement the protein-based detection method.[20]

Acknowledgments

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