A method for the detection of shrimp/prawn and crab DNAs to identify allergens in dried seaweed products

(Received August 4, 2014)
(accepted November 28, 2014)

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Abstract

Crustacean protein (tropomyosin) has frequently been detected in processed foods containing seaweed. In Japanese regulations for the labeling of allergenic food ingredients, the PCR method for detecting extracted shrimp/prawn and crab DNA is stipulated to discriminate shrimp/prawn and crab in processed foods. It has, however, been difficult to extract shrimp/prawn and crab DNA in processed foods including seaweed. We modified the DNA extraction protocol of the DNeasy mericon Food kit, and compared the yield and purity of DNA extracted from dried seaweed powder containing 1, 5, 10, 100, or 10,000 µg/g of freeze-dried edible shrimp/prawn or crab using various commercially available DNA extraction kits. The improved DNA extraction method provided sufficient yield and purity of extracted DNA suitable for the detection of specific DNA using the PCR method. To directly evaluate the applicability of the DNA extraction method, we employed PCR amplification with primers (PyrbL01–5′/PyrbL01–3′) designed for the detection of the Pyropia yezoensis rbcL gene. The primer pair could generate amplicons from several commercial nori food products and dried seaweed powder containing shrimp/prawn or crab. The limit of detection for shrimp/prawn or crab DNA extracted by the improved DNA extraction method is 1 µg per g dried seaweed powder. In conclusion, we showed that the improved method is simple, rapid and highly sensitive, and can be used to detect shrimp/prawn and crab DNA in dried seaweed food products.

Keywords: allergen, crustacean, DNA extraction method, dried seaweed product, PCR

I Introduction

The Japanese Ministry of Health, Labour, and Welfare (MHLW) stipulated the allergen labeling system by amending the Food Sanitation Law in April 2001. In particular, the labeling of egg, milk, wheat, buckwheat, and peanut ingredients in any commercial processed food became mandatory in April 2002 in response to individuals with food allergies. Therefore, the MHLW has prescribed official Japanese methods for determining allergens to validate the labeling of food products.

The labeling of shrimp/prawn and crab became mandatory in June 2008, and the enzyme-linked immunosorbent assay (ELISA) methods for quantitative screening and PCR for qualitative confirmation were announced as the official methods for the detection of shrimp/prawn and crab 1). Two commercially available ELISA kits for screening were validated according to international validation protocols 2) and were used to validate the labeling for shrimp/prawn and crab 3). However, because of the high amino acid sequence homology between shrimp/prawn and crab 4), these ELISA kits failed to distinguish between shrimp/prawn and crab tropomyosin. Furthermore, these ELISA kits can detect tropomyosin derived from other crustaceans and insects not encompassed by the food labeling regulation. In addition, PCR is commonly used to identify either shrimp/prawn or crab contamination and to exclude false positives.

Crustacean protein was frequently detected in a recent survey of processed food products primarily containing

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seaweed (seaweed products)\(^5\). Since many crustaceans such as the skeleton shrimp (Pseudoprotella phasma) inhabit the same river and sea environments where seaweed is harvested, the contamination of seaweed products with crustacean protein is thought to occur as a result of bycatch. A definitive confirmation test for crustacean protein contamination of seaweed products using PCR has been impeded by the difficulties of extracting DNA. Although several reports show the successful detection of crustacean contamination in young sardines (shirasu), dried young sardines (chiriminenjyako) and fish paste, confirmation of crustacean contamination in seaweed products has been problematic\(^6\)-\(^8\).

Seaweed is a generic name for algae used as a food ingredient, and includes red algae, green algae, and cyanobacteria (blue-green algae). Seaweed is widely consumed as a raw and processed food, and contains proteins, carbohydrates, minerals, dietary fiber, and vitamins\(^9\). Therefore, seaweed products are long-standing popular foods in coastal countries such as Japan\(^10\). However, the intake of seaweed products might induce an allergic response in some patients with an allergy to crustacean because of bycatch contamination. Thus, to protect the health of consumers and guarantee their right to information, the detection of crustacean in seaweed is necessary to prevent responses in those allergic to crustacean.

In this study, model seaweed processed food samples containing 1, 5, 10, 100, or 10,000 µg/g of shrimp/prawn or crab were examined using an improved DNA extraction method, and the limits of detection (LODs) for shrimp/prawn and crab DNA were determined using qualitative PCR. Subsequently, the applicability of the DNA extraction method was confirmed in commercial dried sheets of seaweed (nori food products).

II Materials and Methods

1. Samples

The nori food products were purchased at a supermarket in Chiba, Japan and ground using a mill mixer (IMF-700G; Iwatani, Co., Ltd, Tokyo, Japan) to prepare powdered nori model samples containing shrimp/prawn or crab. Qualitative analysis using the FA test Immunochromato-crustacean “Nissui” (LOD, 1.0 µg/g; Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan) confirmed the absence of crustacean protein in the nori powder. Frozen black tiger prawn (Penaeus monodon) and frozen boiled hair crab (Erimacrus isenbeckii) were purchased at a supermarket in Chiba, Japan for use as standard materials. After shell removal, freeze-drying and grinding, powdered black tiger prawn and hair crab were prepared from edible body parts (muscle) as standard shrimp/prawn or crab materials. To investigate the sensitivity of the established method, samples were powdered using a mill mixer and prepared to contain 1, 5, 10, 100, or 10,000 µg/g of freeze-dried shrimp/prawn or crab per g dried nori powder.

2. ELISA

To detect crustacean tropomyosin, the commercially available ELISA kit FA test for EIA-crustacean (N kit; Nissui Pharmaceutical) and Crustacean (M kit; Maruha Nichiro Holdings Inc., Ibaraki, Japan) were used according to the manufacturer’s instructions and the official method notified by Consumer Affairs Agency, Government of Japan\(^11\).

3. Pretreatment of samples

The moisture content of nori food products is generally < 10%\(^12\), and absorption of the DNA extraction buffer into samples often inhibits DNA extraction from foods such as dried seaweed products. Thus, nori food products tend to completely absorb water under all DNA extraction methods except for the cetyl trimethyl ammonium bromide (CTAB)-based method described in the following section 4. In these procedures, samples (1.0 g) were completely mixed with 10 mL of ultra-pure distilled water (Life Technologies, Carlsbad, CA, USA) by inversion in 50 mL centrifuge tubes. Mixtures were then centrifuged at 3,000 × g for 10 min and supernatants were carefully removed. Finally, precipitated samples were used as water-absorbed samples for subsequent DNA extraction.

4. Extraction of DNA

DNA was extracted using a DNeasy Plant mini kit (DPM method; Qiagen, Hilden, Germany), Genomic-tip 20 G kit (GT method; Qiagen), the DNeasy mericon Food kit (DMF method; Qiagen), and the CTAB-based method. The DPM method was performed according to the manufacturer’s instructions with slight modifications. Briefly, water-absorbed samples were mixed with 10 mL of AP1 buffer at 65°C and 10 µL of RNase A (17,500 U; Qiagen) and then incubated at 65°C for 15 min. Subsequently, 3,250 µL of AP2 buffer was added, and the mixtures were incubated at room temperature for 10 min. The solutions were centrifuged at 5,000 × g for 5 min and supernatants were obtained and centrifuged again at 10,000 × g for 5 min. The resulting supernatants were then transferred to a QIAshredder Spin Column and centrifuged at 10,000 × g for 2 min. The eluates (3 mL) were mixed with 4.5 mL of AP3/ Ethanol buffer and transferred to DNeasy Mini Spin Columns. The columns were washed twice with 500 µL of AW/Ethanol buffer by centrifugation, and DNAs were finally eluted with 100 µL of distilled water.

The GT method was performed according to the manufacturer’s instructions with slight modifications. Briefly, 20 mL of G2 buffer and 200 µL of α-amylase (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) were added to pretreated samples, mixed completely by inverting in 50 mL centrifuge.
tubes, and incubated at 37°C for 1 h. Subsequently, 100 μL of Proteinase K (> 600 mAU/mL; Qiagen) and 20 μL of RNase A were added, and the mixtures were incubated at 50°C for 2 h. After centrifugation, supernatants were transferred to 15 mL centrifuge tubes and then loaded on a Genomic-tip equilibrated with 1.0 mL of QBT buffer. The column was washed three times with a total of 6 mL of QC buffer, and the bound DNA was twice eluted with a total of 2 mL of QF buffer at 65°C. DNA was precipitated by the addition of 2 μL of Ethaminate (Wako Pure Chemical Ind., Ltd., Osaka, Japan) and 1.4 mL of isopropanol followed by centrifugation and 70% ethanol wash, and the DNA was finally dissolved in 100 μL of distilled water at 65°C.

The DMF method was performed according to the manufacturer's instructions with slight modifications. Briefly, water-absorbed samples were mixed with 10 mL of Food Lysis buffer and 25 μL of Proteinase K and incubated at 60°C for 30 min. The mixtures were then cooled on ice to room temperature. After centrifugation at 2,500 × g for 5 min, 700 μL aliquots of supernatant were mixed with 500 μL of chloroform in 1.5 mL microtubes and vigorously vortexed. The mixtures were then centrifuged, and 450 μL of the supernatant (aqueous phase) and 1.8 mL of PB buffer were mixed in 15 mL centrifuge tubes. The mixtures were then transferred to QIAquick Spin Columns, and the columns were washed with 500 μL of AW2 buffer. The columns were centrifuged at 17,900 × g for 1 min until all residual AW2 buffer was drained; after the addition of 100 μL of EB buffer, the QIAquick Spin Columns were kept at room temperature for 1 min. Finally, the DNA was eluted into 1.5 mL microtubes by centrifugation. The modified DNA extraction method was designated as the DMF-mSEP method.

The CTAB-based method was performed according to the procedures described in the Japanese official standard methods.

5. Spectrophotometric evaluation of extracted DNA

The concentration and quality of extracted DNA were evaluated by measuring ultraviolet (UV) absorption using a Bio-Spec mini spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). DNA purity was estimated according to ratios of absorbance at 260 nm and 230 nm (A260/230), and 260 nm and 280 nm (A260/280); absorption at 320 nm was subtracted as background.

6. PCR analysis and agarose gel electrophoresis

For universal detection, the AN1–5′/AN2–5′/AN3–3′ primer pair for detecting common animals and CP03–5′/CP03–3′, Plant01–5′/Plant01–3′ or Placon5/Placon3 primer pairs (Promega KK, Tokyo, Japan) for common plant DNA were used. The universal detection for seaweed was performed with the PyrbcL01–5′ (GGTCCTGCAACTGGATTGAT)/PyrbcL01–3′ (AGGAATCAAGACGCGCTT) primer pair, which was designed using published sequences of the Pyropia yezoensis chloroplast ribulose-1, 5-bisphosphate carboxylase (rbcL) gene (GenBank: AB243204) using Primer3 free online software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/, last accessed March 2014). The specific detection of shrimp/prawn and crab was performed with ShH12–05′/ShH13–03′ and CrH16–05′/CrH11–03′ primer pairs, respectively, as previously described (5, 10). All PCR primer pairs except for Placon5/Placon3 were synthesized and purified by FASMAC Co., Ltd. (Kanagawa, Japan) using reverse-phase columns. Specific primers were purchased from FASMAC for the detection of Akiami paste and mantis shrimp. PCR was performed using PCR AmpliTaq Gold PCR Master Mix (AmpliTaq reagent; Life Technologies) and AmpliTaq plus (Ampdiect reagent; Shimadzu). PCR reactions with the plant primer pairs CP03–5′/CP03–3′ and Plant01–5′/Plant01–3′ were performed using AmpliTaq reagent in a total volume of 25 μL in 0.2 mL reaction tubes containing 50 ng of template DNA, 1 × PCR buffer II, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 0.2 μmol/L forward and reverse primers, and 0.625 units of Taq DNA polymerase. PCR conditions were as follows: 10 min of preheating at 95°C and 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by 7 min at 72°C. Another PCR reaction for plants was performed with Placon5/Placon3 according to the manufacturer's instructions. To detect animal and crab DNA, MgCl₂ concentrations in the PCR reactions were 3.0 mmol/L and 2.0 mmol/L, respectively, as described in the official method (5). To detect shrimp/prawn, the concentration of ShH12–05′/ShH13–03′ primers in PCR reactions was 0.3 μmol/L each. PCR conditions for animal and crab DNA amplifications were as follows: 10 min of preheating at 95°C and 40 cycles of 30 s at 95°C, 30 s at 50°C or 54°C for animal or crab, respectively, and 30 s at 72°C, followed by 7 min at 72°C. PCR conditions for shrimp/prawn DNA amplifications were as follows: 10 min of preheating at 95°C and 45 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, followed by 7 min at 72°C. PCR reactions for the detection of all targets were also performed using Ampdirect reagent in a total volume of 25 μL, in 0.2 mL reaction tubes containing 50 ng template DNA, 1 × Ampdirect plus, 0.4 μmol/L forward and reverse primers and 0.625 units of Taq DNA polymerase. PCR conditions were as for the AmpliTaq reagent, and all PCR reactions were performed using a Veriti thermal cycler (Life Technologies). After PCR amplification, the products (7.5 μL) were mixed with 6 × loading buffer (1.5 μL; Wako Pure Chemical Ind., Ltd.) and electrophoresed at a constant voltage (100 V) on 4% agarose gels in 0.5 × TBE buffer (44.5 mmol/L Tris, 44.5 mmol/L boric acid, and 0.1 mmol/L EDTA; pH 8.3) using a Mupid-exU (Advance Co.,
LTD., Tokyo, Japan). After electrophoresis, gels were stained in 0.5 × TBE buffer containing 0.5 μg/mL ethidium bromide for 20 min and destained in 0.5 × TBE buffer for 20 min. The gels were photographed using a FAS-III Model-TM20 (Toyobo Co., Ltd., Osaka, Japan).

III Results and Discussion

1. Design of PCR primers for P. yezeoensis DNA

Preliminary data showed poor DNA extraction efficiency of commercial nori food products using the three DNA extraction methods of the Japanese official standard methods, due to the high viscosity of supernatants during cell lysis and the absorption of DNA extraction buffers into samples. The GT method is frequently used for DNA extraction to confirm the accuracy of ingredient labels on foodstuffs, and provides sufficient quantities of pure DNA for PCR reactions. However, the transfer and elution through the column is time consuming, and the DNA extracted from commercial nori food products failed to amplify using any primer pairs for universal detection of plants (CP03–5′/CP03–3′, Plant01–5′/Plant01–3′, and Placon5/Placon3 primer pairs; data not shown).

To avoid false-negatives, the applicability of extracted DNA in PCR should be confirmed. Major seaweed species in nori food products include P. yezeoensis, Pyropia tenera, and Pyropia pseudolinearis; in Japan, P. yezeoensis f. narawaeensis is the most popularly consumed. Therefore, we designed the PCR primers Pyrbcl01–5′/Pyrbcl01–3′ (152 bp) from the P. yezeoensis rbcL gene. The primer pairs designed from the genome DNA sequence of P. yezeoensis were able to generate amplicons from several commercial nori food products (data not shown). Furthermore, extracted DNAs obtained from powdered nori model samples containing 1, 5, 10, 100, or 10,000 μg/g of freeze-dried shrimp/prawn or crab were successfully amplified (Fig. 1).

Fig. 1. Agarose gel electrophoresis of PCR products (rbcL) using the Pyrbcl01–5′/Pyrbcl01–3′ primer pair from samples containing 1, 5, 10, 100, or 10,000 μg/g of shrimp (A) or crab (B) in powdered nori products. Arrows and arrowheads indicate the expected PCR amplification products for P. yezeoensis chloroplast rbcL (152 bp) and the position of 200 bp in 20 bp ladder size standard, respectively. Lanes M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals).

Thus, PCR using the Pyrbcl01–5′/Pyrbcl01–3′ primer pair can be used to validate extracted DNA obtained from nori food products.

2. Protocol optimization of the DNeasy mericon Food (DMF) kit

The CTAB-based method is widely used to extract total DNA from food materials and can effectively remove substances that inhibit PCR, such as certain proteins and polysaccharides. However, this method has a number of disadvantages, including complex procedures and low DNA yields. Nori food products contain substantial amounts of characteristic polysaccharides such as alginate, fucoidan, carrageenan, and agarose. In particular, the sulfated polysaccharide porphyran is regarded as a strong PCR inhibitor in plants and warrants use of the CTAB-based method for seaweed products.

Therefore, we optimized the total DNA extraction procedure using a DNeasy mericon Food (DMF) kit, which is based on the CTAB-based method. The standard protocol (SP) and the small fragment protocol (SFP) are described in the manufacturer's instructions, and sample weights required for these protocols are 2.0 g and 200 mg, respectively. The SFP procedure of the DMF kit is designed for effective extraction of total DNA from processed foods. However, the DNA extracted from processed foods has typically been subjected to extensive thermal treatments, high pressure, irradiation, pH changes and drying, thus resulting in highly fragmented DNA. Consequently, the SFP procedure uses column-binding conditions that are optimized for maximal recovery of short DNA fragments (shorter than 100–200 base pairs).

We then compared the six procedures shown in Table 1. Since the presence of crustacean in nori food products is irregular, the use of small amounts of a given sample might be limiting. For this reason, under all conditions, total DNA was extracted from 1 g samples. Ten milliliters of Food Lysis Buffer were added to water-absorbed samples in SP, SFP, and mSFP (modified SFP). Twenty milliliters of Food Lysis Buffer were added to non-water-absorbed samples in SP2, SFP2, and mSFP2 (modified SFP2) procedures, and samples were thoroughly agitated and mixed to produce sufficient volumes of supernatant. The resulting yield and purity of extracted DNA are shown in Table 1.

Regardless of the water absorption pretreatment, the mSFP and mSFP2 procedures gave the best yield and purity of extracted DNA compared with the SP (SP2) and SFP (SFP2) procedures. Pretreatment increased the DNA yields of all procedures. Furthermore, we considered the SFP procedure to be a simple step. These data show that the pretreatment facilitates DNA extraction from dried foods such as nori food products. To further increase in the yield of extracted DNA,
the mixture volume of the supernatant and PB buffer were varied 450 µL and 1.8 mL, respectively. In the following experiment, the modified procedure of mSFP of the DMF kit (DMF-mSFP method) was used to prepare high quality and quantity total DNA from nori food products.

3. DNA extraction from powdered nori samples containing shrimp/prawn or crab

The yield and purity of DNA extracted from powdered nori model samples spiked with five different concentrations of shrimp/prawn or crab were evaluated using the DPM, GT, CTAB, and DMF-mSFP extraction methods. The DNA was twice extracted from each sample. The yield and purity of DNA extracted from the shrimp/prawn and crab model samples are shown in Tables 2 and 3, respectively.

The highest yield of DNA extracted from the shrimp/prawn model samples was achieved with the GT method. In contrast, the CTAB-based method produced the lowest yield.

<table>
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<th>Table 1. Conditions of DNA extraction and spectrophotometric analysis of extracted DNAs</th>
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<td>Protocol</td>
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<tr>
<td>SP</td>
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<td>mSFP</td>
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<td>SP2</td>
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<td>mSFP2</td>
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<td>SP2</td>
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<sup>a) </sup>DNA extractions were carried out by DMF method.

SP, SFP, mSFP and SFP2 were treated for water absorption of samples with DNase free water, whereas SP2, SFP2 and mSFP2 were not. SPS(SF2) and SFP(SF2) indicate standard protocol and small fragment protocol in DNeasy mericon Food Handbook, respectively. mSFP(mSFP2) varied in volume of supernatant as compared to SFP(SF2).

<table>
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<th>Table 2. Spectrophotometric analysis of DNAs extracted from model foods (nori containing shrimp)</th>
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<tr>
<td>Shrimp Conc. (µg/g)</td>
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<tr>
<td>SPSP2</td>
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<tr>
<td>10,000</td>
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<tr>
<td>100</td>
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<sup>b) </sup>Modified method using DNeasy Plant Mini kit (Qiagen, Hilden, Germany)
<sup>c) </sup>Modified method using Genomic-tip 20/G (Qiagen)
<sup>d) </sup>Modified method using mSFP2 protocol of DNeasy mericon Food kit (Qiagen) as shown in Table 1
<sup>e) </sup>The Japanese official method described in reference No.1

<table>
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<th>Table 3. Spectrophotometric analysis of DNAs extracted from model foods (nori containing crab)</th>
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<td>Crab Conc. (µg/g)</td>
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<td>2.6</td>
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<sup>b) </sup>Modified method using DNeasy Plant Mini kit (Qiagen, Hilden, Germany)
<sup>c) </sup>Modified method using Genomic-tip 20/G (Qiagen)
<sup>d) </sup>Modified method using mSFP2 protocol of DNeasy mericon Food kit (Qiagen) as shown in Table 1
<sup>e) </sup>The Japanese official method described in reference No.1

Abbreviations and symbols are as in Table 2.
due to the use of aliquots of lysates from starting samples. The DNA extraction using the DMF-mSFP method resulted in the second highest yield of DNA.

The contaminants were assessed according to absorption ratios of A260/280 and A260/230 respectively, which are widely used to evaluate DNA quality for PCR amplification. According to the official method, the A260/280 and A260/230 absorption ratios of recommended DNA quality for the following PCR detection are 1.2 – 2.5 and >2.0, respectively. The A260/280 absorption ratio of DNA extracted by all methods fell within the range 1.6 – 2.0; in contrast, only the A260/230 absorption ratio of DNA extracted by the DMF-mSFP method are in the range of recommended DNA quality. These results suggest that the DMF-mSFP method effectively remove certain polysaccharides (Table 2). The yield and purity of DNA extracted from the crab model samples showed similar tendencies to those of the shrimp/prawn model samples (Table 3).

Since seaweed generally contains large amounts of polysaccharides and is highly viscous, DNA extraction using the GT method, which involves gravity-flow chromatography, is time consuming; similarly, the CTAB-based method is time consuming as it involves multiple steps for removing certain proteins and polysaccharides. In contrast, the DPM and DMF-mSFP methods were performed using silica membrane-type spin columns, allowing the rapid extraction by simultaneous procedures from multiple samples. However, the DPM method gave low-purity DNA, judging from the A260/230 values. According to these examinations, the results suggest that the DMF-mSFP method is superior to the other methods for extracting total DNA from nori food products.

In the official method for allergenic substance testing, PCR for the detection of common animal or plant DNA is required to assess the validity of extracted DNA and to avoid false-negative results. To directly evaluate the applicability of DNA extracted by the DMF-mSFP method, PCR with three kinds of universal plant primer pairs (CP03-5’/CP03-3’, Plant01-5’/Plant01-3’ or Placon5/Placon3) for the detection of common plant DNA was performed using the DNA extracted from the shrimp/prawn or crab model samples and AmpliTaq reagent. The PCR generated no plant specific products (data not shown). In addition, the PCR with the universal animal primer pairs (AN1-5’/AN2-5’/AN-3’) for the detection of common animal DNA was performed using the DNA extracted from both model samples. Several amplification products (ranging from 40 bp to 600 bp) in the PCR test for shrimp/prawn model samples were obtained (data not shown). By contrast, no amplification products in the PCR test for model samples containing crab were obtained (data not shown). The similar PCR test using animal specific primer pairs and Ampdirect reagent also gave several non-specific products (data not shown).

The primer pair for detecting common animal DNA is designed from a highly conserved region of the 16S rRNA gene of mitochondrial DNA. However, this region carries various nucleotide insertions and deletions among animal species, and the specific amplification product generated using the universal animal primer pair should be 370–470 bp in length. Accordingly, we considered that many amplification products generated in the PCR test using the universal animal primer pair cannot be used to evaluate the validity of extracted DNA.

Therefore, we evaluated the validity of extracted DNA using PCR amplification with primers (PyrbclL01-5’/PyrbclL01-3’) designed for the detection of the P. yezoensis rbcL gene and Ampdirect reagent (Fig. 1). The region of the P. yezoensis rbcL gene was clearly amplified using DNA extracted from the shrimp/prawn or crab model samples.

These results demonstrated the superiority of the DMF-mSFP method for detecting shrimp/prawn and crab DNA in nori food products.

4. Sensitivity of shrimp/prawn and crab DNA detection

The LODs for the specific detection of shrimp/prawn and crab DNA were determined (Fig. 2). The LODs for the specific detection of shrimp/prawn and crab DNA were 5 pg/g and 100 pg/g, respectively. However, use of the Ampdirect reagent led to LODs of 1 pg/g in both specific detections. Although LODs for the specific detection of shrimp/prawn DNA using AmpliTaq reagent were similar to those using Ampdirect reagent, LODs of crab DNA between PCR using AmpliTaq reagent and Ampdirect reagent differed greatly (Fig. 2). We speculate that these differences might be attributed to the neutralization of PCR inhibitors, or the high amplification efficiency of Taq DNA polymerase in the Ampdirect reagent.

5. Specific detection of shrimp/prawn or crab DNA from commercial dried seaweed products

To investigate the applicability of detection methods for shrimp/prawn and crab DNA in commercial nori food products, we employed eight commercial nori food products that did not declare shrimp/prawn and crab contents on their label. Concentrations of crustacean tropomyosin for the nori food products were determined using two ELISA kits (Table 4).

As shown in Table 4, the crustacean protein concentrations in the nori food products were 3.0 – 8.8 μg/g and 1.3 – 8.0 μg/g using N and M kits, respectively. Furthermore, no sample contained over 10 μg/g crustacean protein concentration. The
Fig. 2. Sensitivity of the specific detection method for shrimp (A) or crab (B) in powder nori products using two different PCR reagents

Arrows and arrowheads indicate the expected PCR amplification products and the position in 20 bp ladder size standard, respectively. AmpliTaq reagent, AmpliTaqGold (Life Technologies). Ampdirect reagent, Ampdirect plus (Shimadzu). Lanes M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals). “+” and “-” mean the lanes in which the amplified band was detected and not, respectively.

Table 4. Detection of crustacean proteins and DNA (crab and shrimp) in dry seaweed products and spectrophotometric analysis of extracted DNA by DMF

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>ELISA (µg/g)</th>
<th>PCR</th>
<th>Extracted DNA</th>
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<tr>
<td></td>
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<td>Control (Seaweed), Shrimp, Crab</td>
<td>DNA (µg)</td>
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<tr>
<td>1</td>
<td>0.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>7.6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
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<td>5</td>
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<tr>
<td>8</td>
<td>0.3</td>
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a) FA test ElA-crustacean (Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan)
b) Crustacean kit (Maruha Nichiro Holdings, Inc., Ibaraki, Japan)
c) To evaluate the validity of DNA extracted from seaweed products for the PCR
d) For specific detection of shrimp using the ShH12-05'/ShH13-03' primer pair
e) For specific detection of crab using the CrH16-05'/CrH11-03' primer pair

nori food products were then subjected to DNA extraction followed by control (PyrbCL)-PCR, shrimp-PCR, and crab-PCR. The DNA was simply and rapidly extracted from all samples using the DMF-mSFP method, with yields of 13 - 95 µg and A260/280 and A260/230 absorption ratios of 2.00 – 2.21 and 2.78 – 4.89, respectively (Table 4). All extracted DNA was in the range of recommended DNA yield and quality described in the Japanese official method. PCR with the PyrbCL01-5'/PyrbCL01-3' primer pair was then performed to validate extracted DNA using the Ampdirect PCR reagent and specific amplification products were observed. These results suggested that PCR inhibitors in the extracted DNA did not influence the PCR reactions (Fig. 3).

In subsequent shrimp-PCR and crab-PCR, samples 1, 2, 5, and 6 were positive in both reactions. In a previous study, shrimp-PCR gave false-negative results with the Akiami paste shrimp (Acetes japonicus), whereas crab-PCR gave false-positive results with the mantis shrimp (Oratosquilla oratoria). Therefore, to avoid false results, the detection of Akiami paste and mantis shrimp were examined in samples 3, 4, 7, and 8, and samples 1, 2, 5, and 6, respectively, using specific Akiami paste and mantis shrimp primers. Consequently, no amplification products were observed in all examined samples (data not shown), suggesting that samples 1, 2, 5, and 6 are mixed with both shrimp/prawn and crab.

The official PCR methods for shrimp/prawn and crab taxonomically detect Pernaoidae, Sergestidae family of the suborder Dendrobranchiata and infraorder Caridea, Astaciidae, Achelata of the suborder Pleocyemata, and infraorder Brachyura and the Lithodidae family of the infraorder Anomura, respectively. The amplification regions of both PCR protocols were on the mitochondrial 16S rRNA gene, allowing the detection of all species of these groups. Using the developed DNA extraction method for the detection of shrimp/prawn and crab DNA in the commercial nori food products enabled the detection of DNA of shrimp/prawn and
Fig. 3. Investigation of commercial seaweed products
Control (seaweed) evaluation of the validity of DNA extracted from seaweed products using PCR amplification with the PyrbcL01-5' PyrbcL01-3' primer pair. Arrows indicate expected PCR amplification products. The lane numbers correspond to the sample numbers from Table 4. Lane M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals). crab, with a detection limit of 1 µg/g in both model samples. However, samples 3, 4, 7, and 8 were negative in both PCR tests, even though according to the ELISA results, crustacean protein was present at greater than 1 µg/g in those samples. These results suggest that samples 3, 4, 7, and 8 contain crustaceans of the Amphilopoda, Mysida, and Euphausiacea families, which are not subject to mandatory labeling for shrimp and crab.

The present DNA extraction methods are applicable to the identification of shrimp/prawn or crab DNA in commercial nori food products. Further studies are required to investigate discrepancies between the results of ELISA and PCR methods.

IV Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research "KAKENHI" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by grants from the Japanese Ministry of Health, Labor, and Welfare.

V References


特定原材料検査における乾燥海苔製品中のえび・かに DNA 検出法の検討

（2014年8月4日受付）
（2014年11月28日受理）

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キーワード: 特定原材料、海藻類、DNA抽出方法、乾燥海苔製品、ポリ変異連鎖反応

概要

甲殻類タンパク質（トロポミオン）は、海苔を含む加工食品で高頻度に検出されている。アレルギー物質の表示に関する日本規制では、抽出されたエビとカニの DNA を検出するための PCR 法が、加工食品中のエビとカニを区別するために規定されている。海苔を含有した加工食品では、エビとカニの DNA を抽出することが難しい。我々は、DNeasy mericon Food kitのDNA抽出プロトコールを改良し、凍結乾燥したエビとカニの可食部を1 ～ 10,000 μg/g 含む乾燥海苔粉末から抽出された DNA を用いて、いくつかの市販の DNA 抽出キットと収量・精製度の比較検討を行った。その結果、改良した DNA 抽出方法は、PCR 法を用いた特異 DNA の検出に適した十分な DNA の収量、精製度を示した。抽出 DNA の適用性を直接評価するために、スパシノリ rbcl 連代子の塩基配列からプライマーベアを作成した。このプライマーベアは、いくつかの市販の海苔製品や、エビまたはカニを含んだ乾燥海苔粉末から増幅産物を生成することが可能であった。改良した DNA 抽出方法により抽出されたエビとカニのDNAの検出下限値は、海苔乾燥粉末1 g あたり、それぞれ1 μg であった。また、市販の海苔製品においても良好な適用性を示した。改良した方法は、簡便、迅速、高感度であり、乾燥海苔製品において、エビとカニのDNAを検出するために利用可能であった。