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Analytical Methods

Detection of peanut (*Arachis hypogaea*) allergen by Real-time PCR method with internal amplification control

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ABSTRACT

Specific primer sets were designed based on the DNA sequence of Ara h 1, one of the major peanut (*Arachis hypogaea*) allergens, and a competitive internal amplification control (IAC) was designed by compound primer technology. By choosing 314 copies/PCR as the IAC dosage, a Real-time PCR method with IAC was established for detecting peanut allergen Ara h 1 DNA. The method showed high specificity with a detection limit of 0.005% peanut. A series of commercial food products with/without peanut components were tested. Among these products, the peanut allergen Ara h 1 DNA could be detected in 12 products labelled containing peanut ingredients, in two without a declaration of peanut and one labelled that was produced in a facility that produced peanut-containing foods. This indicates that the method is highly sensitive for the detection of peanut ingredients in foods.

1. Introduction

Hypersensitivity reaction to dietary substances from peanuts is a public health problem, even a trace amount of peanut can trigger severe allergic reactions. The symptoms of peanut allergies varied from mild urticaria to life-threatening anaphylactic shock. Indeed, several fatal cases caused by peanut allergies have already been reported (Bock, Muñoz-Furlong, & Sampson, 2001, 2007; Zhao-Yun, 2001). Generally, peanut allergies do not disappear with age (Sampson, 2004). There is currently no effective therapy for peanut allergies (Kun-Mei, Jia-Jie, Hai-Yan, Xiao-Yu, & Zhi-Gang, 2010). Avoiding potential peanut-containing foods is the only way for sufferers to avoid being harmed. Therefore, establishing a reliable method for the detection of peanut allergens is very important.

Real-time PCR technique is for DNA detection and quantitation, which monitors the accumulation of fluorescent signal produced during the amplification of a DNA template in real time. In recent years, it has been widely used in the detection of food allergens, such as celery (Fuchs, Cichna-Markl, & Hochegger, 2012), crustacean (Herrero, Vieites, & Espiñeira, 2012) and peanut (López-Calleja et al., 2013). In Real-time assay, the presence of impurities and other inhibitors that are present during DNA extraction may lead to the false-negative results, which mean originally positive samples were detected as negative. False-negative results may result in fatalities in peanut allergy sufferers who inadvertently consume contaminated products (Xiao-Hua & Xian-Ming, 2010). Adding an IAC sequence to the PCR reaction system is one of the ways to improve the reliability of PCR results. In this study, internal amplification control (IAC), an artificial DNA sequence was constructed and co-amplified simultaneously with target gene. PCR combined with IAC have been widely used in detecting foodborne pathogens, such as *Vibrio parahaemolyticus* (Xiao-Hua et al., 2010) and *Salmonella* (Xian-Long et al., 2011). However, this method has not been reported in food allergens detection. Given this, we established a Real-time PCR with IAC method, and applied for detecting peanut allergen Ara h 1 DNA in the commercial food products.

2. Materials and methods

2.1. Samples preparation and pre-treatment

All samples used in this study were purchased from local supermarket and pre-treated. Among them, Peanut (*Arachis hypogaea*), sorghum (*Sorghum bicolor*), corn (*Zea mays*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), buckwheat (*Fagopyrum esculentum*), oat (*Avena sativa*), rice (*Oryza sativa*), millet (*Setaria italica*), soybean (*Glycine max*), peas (*Pisum sativum*), sesame (*Sesamum indicum*), celery (*Apium graveolens*), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), pear (*Pyrus bretschneideri*), shrimp (*Palinuridae*), crab (*Liocarcinus marmoreus*), oyster (*Crassostrea gigas*), atlantic salmon (*Salmo salar*), 8 kinds of biscuits, 2 kinds of powders, 2 kinds of candies, and 2 kinds of nuts were grinded

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for DNA extraction and stored at 4 °C. Liquid samples, including cow's milk and 6 kinds of drinks were used for DNA extraction without pre-treatment.

2.2. DNA extraction

DNA from peanut (including peanut with wheat powder as matrix), sorghum, corn, barley, wheat, buckwheat, oat, rice, millet, soybean, peas, celery, tomato, apple, pear and were extracted by the modified CTAB (Cetyl Trimethyl Ammonium Bromide) method (Qin et al., 2009; Moller, Bahnweg, Sandermann, & Geiger, 1992). 0.2 g samples were grinded and incubated in 800 μL TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS), including 50 μg Proteinase K at 60 °C for 1 h. Salt concentration was adjusted to 1.4 M with 5 M NaCl, 1/10 volume 10% CTAB (cetyltrimethylammonium bromide) was added, incubating for 30 min at 65 °C, and centrifuged for 10 min. Supernatant was transferred to a new tube, equal volume phenol–chloroform–isoamyl alcohol (phenol: chloroform: isoamyl alcohol, 25:24:1, v/v) was added and centrifuged. Supernatant was transferred to a fresh tube, equal volume SEVAG (chloroform: isoamyl alcohol, 24:1, v/v) was added and centrifuged. Supernatant was transferred to a fresh tube, 3 μL RNase (10 mg/mL) was added and the sample was incubated at 37 °C for 30 min. 0.7 volume isopropanol was added to precipitate DNA, sample was placed at −20 °C for approx 60 min, and centrifuged. Supernatant was removed and pellets were washed twice with cold 70% ethanol, dried in ambient, and dissolved in about 50 μL ddH_2O. The DNA were stored at −20 °C. DNA was extracted from cow's milk using the method described previously (Qing-Jin, Zhi-Rui, Wen-Ju, & Qin, 2012). The Plant Genomic DNA Kit (TIANGEN, Beijing, China) was used to extract DNA from sesame exactly according to the manufacturer's instructions. The QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, MD, USA) was used to extract DNA from shrimp, crab, oyster and atlantic salmon exactly according to the manufacturer's instructions. The GMO food DNA Extraction Kit (TIANGEN, Beijing, China) was used to extract DNA from various commercial food products exactly according to the manufacturer's instructions. The DNA extractions were repeated 3 times and mixed for the following experiment. DNA concentrations (ng/μL) were determined spectrophotometrically using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, MA, USA).

2.3. Primers

Primer sets Ara h 1-F/R were designed using the Primer Premier 5.0 (Premier, CA) based on the Ara h 1 gene (AF432231) from peanut. Sequences of specific primer sets 18S rRNA-F/R based on the nuclear 18S rRNA gene were derived from previous research (Shu-Ya, Hua-Jie, Yi-Ning, Ni, & Fu-Wei, 2012). The primers FSL-F/R were designed based on the gene encoding *FSL R2-561* (NC017546) gene from *Listeria* monocytogenes. All primers were synthesized by Sangon Biotech (Shanghai, China). The sequences of all primers are listed as follows, Ara h 1-F, CTGGAAACCTTG AACTCGT, Ara h 1-R, GTTGATGGCTACTGGATGA, 18S rRNA-F, TCTGCCCTATCAACTTTCGATGGTA, 18S rRNA-R, AATTTGCGCGCCT GCTGCCTTCCTT, FSL-F, TAGCCAACCGATGTTTCTGTATC, FSL-R, CAT CATTTAGCGTGACTTTCTTTCA.

2.4. Conventional PCR

The concentrations of the reagents in the final volume of 20 μL were: 2 μL 10× Buffer (Mg^{2+} plus) (Takara Biotechnology, Dalian, China), 3.2 μL dNTP (2.5 mM) (Beijing ComWin Biotech, Beijing, China), 0.2 μL rTaq (5 U/μL) (Takara Biotechnology, Dalian, China), 1 μL primers (each 3 μM), 2 μL DNA template, 11.6 μL double distilled water. The reaction was run on an iCycler® Thermal Cycler (Bio-Rad, USA) and the temperatures applied were as follow: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s (FSL-F/R), 54 °C for 30 s (18S rRNA-F/R), 59 °C for 30 s (Ara h 1-F/R and IAC-F/R) and 72 °C for 25 s; followed by one step at 72 °C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel in 1 × TAE and analysed with Tanon-3500 Gel Image System (Tanon Science and Technology, Shanghai, China).

2.5. Real-time PCR

The Real-time PCR reaction was performed in a final volume of 20 μL, containing 10 μL 2 × SYBR® Green Real-time PCR Master Mix (Toyobo, Japan), 1 μL primers (each 3 μM), 50 ng DNA template, IAC (optimised concentration), double distilled water up to 20 μL. Application was carried out in a CFX96 Real-time PCR System (Bio-Rad, USA), using the following PCR programme: initial denaturation at 95 °C for 30 s; 40 cycles consisting of denaturation at 95 °C for 10 s, annealing at 59 °C for 15 s, extension at 72 °C for 25 s, data acquisition of the signal took place once every 0.5 °C, continuous 5 s during annealing and elongation of each cycle from 55 °C to 95 °C.

2.6. IAC construction

The IAC primer sets IAC-F/R (IAC-F, CTGGAAACCTTGAACTC-GTTAGCCAAC, IAC-R, GTTGATGGCTACTGGATGACATCATTT) were formed by connecting part sequences of the primers FSL-F/R to 3′ end of Ara h 1-F/R primer pairs and synthesized by Sangon Biotech (Shanghai, China). While the IAC construction was carried out according to the compound primer technique (Siebert & Larrick, 1993), the scheme was showed in Fig. 1A. Firstly, a 351 bp amplicon was obtained when *Listeria* monocytogenes DNA was amplified based on FSL-F/R primer sets (Fig. 1B). Secondly, the 351 bp amplicon and IAC-F/R primer sets were used as template and primers to generate a 389 bp product (Fig. 1C). Thirdly, the obtained 389 bp fragment was ligated into the pMD18-T Simple Vector (Takara Biotechnology, Dalian, China), and transformed into *Escherichia coli* DH5α (TIANGEN, Beijing, China), then positive clones were selected by a PCR screening with the vector specific M13-F/R primers. Finally, IAC-plasmid purification was carried out using the TIANprep Mini Plasmid Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions and sequenced by Sangon Biotech (Shanghai, China). The copy numbers of IAC-plasmid could be calculated as follows: $N = (m/M) \times 6.02 \times 10^{23}$, where N equals to the copy numbers of IAC (copies), m equals to the mass of IAC (g), M equals to the molecular weight of IAC (1.92×10^6 g/mol).

3. Results

3.1. Amplification of specific primers based on 18S rRNA

Specific 18S rRNA-F/R primers were used to amplify various DNA samples from peanut and other samples. DNA extracted from all the plants and animal species yielded amplicons with the same length (approximately 137 bp), indicating that these DNA samples were all available for PCR amplification.

3.2. Sensitivity of the Real-time PCR method without IAC

The DNA from peanut powder serially diluted with wheat powder were extracted and amplified with primers Ara h 1-F/R. The result was shown in Fig. 2. 0.005% peanut or above was capable to be detected, while no signal was observed below 0.005%.