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Liquid-phase hybridization based PCR-ELISA for detection of genetically modified organisms in food

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Abstract

Polymerase chain reaction (PCR) screening for presence of transgenic components in food is becoming a routine method in modern food analysis. To develop a high throughput method for quantitation of the PCR products is needed for automatic industry analysis. Here we described an in situ liquid-phase hybridization (LPH) for PCR-enzyme linked immunoabsorbent assays (PCR-ELISA) that was widely used for quantitation of PCR products. In LPH-PCR-ELISA, the biotinylated PCR product was hybridized with digoxigenin-labeled probes in the PCR reaction mixture immediately after PCR cycles and the hybridizations was incorporated into the PCR program. Subsequent enzyme conversion of substrate gave distinct OD values when detecting samples with genetically modified organisms (GMOs) labels in the different concentrations. The described method enabled a fast, specific, and accurate detection of GMOs components in food products and thus can be developed to a full-automatic method for routine analysis of raw and processed food products in large sample number.

Keywords: Genetically modified organisms; PCR-enzyme linked immunoabsorbent assays; Liquid-phase hybridization

1. Introduction

Recent statistics data in the International Service for the Acquisition of Agri-biotech Applications (ISAAA) showed that cultivation of global transgenic crop had rapidly grown up to 58.7 million ha in the year of 2002. The top two transgenic crops were soybean (36.5 million ha) and maize (12.4 million ha). Although the demand for commercial use of genetically modified organisms (GMOs) was constantly increasing, it was highly controversial in development of GMOs due to its potential harms to human health and ecological environment of the world. Currently, most countries required labeling products containing GMOs above a certain percentage. The EU allowed up to 1% of adventitious presence of authorized GMOs in imported food (EC regulations 258/97/CE & 49/2000). China also issued "Regulations on Safety of Agricultural Genetically Modified Organisms" on 23 May 2001, emphasizing controls over GMOs and GMO-related products.

The need to monitor and verify the presence and the amount of GMOs in agricultural products has generated a demand for analytical methods. Lüthy (1999) reported that analytical technique divides into detecting the introduced DNA and the expressed protein in transgenic plants. The method of detecting expressed protein (such as enzyme linked immunoabsorbent assays (ELISA)) was simple, highly specific, and easy to quantitate, although the sensitivity was low and frequently it failed to detect the fully processed products (Anklam, Gadani, & Heinze, 2002). The methods of detecting target DNAs with polymerase chain reaction (PCR) was reliable and highly sensitive, although it was difficult to use the routing agarose gel analysis and southern blot confirmation of the PCR products for massive samples (Meyer, 1999). Here we described an improved liquid-phase hybridization (LPH) PCR-ELISA technique for specific detection of PCR products. In this method, the

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biotinylated PCR products were in situ hybridized with the digoxigenin-labeled probe in the PCR reaction mixtures, and then captured with Streptavidin coated tubes. The specific bound biotin- and digoxigenin-labeled DNA fragments were subsequently quantitate with ELISA analysis. The data showed here indicated that the LPH-PCR-ELISA was both reliable and highly sensitive. In addition, the LPH-PCR-ELISA described here was convenient and easy to be converted to a large scale, high-throughput automatic method.

2. Materials and methods

2.1. Materials

Import soybean and maize samples were gifts from the Xiamen Port of China; Roundup Ready soybean standard samples, Bt1 maize standard samples were purchased from USA SDI Company. The oligonucleotides were customer synthesized by the Shanghai Sangon Bioengineering Technological Service, Ltd.

2.2. DNA extraction and PCR analyses

Genomic DNAs were extracted with CTAB protocol as described (Wang & Fang, 2002) or with the Wizard® kit (Promega, USA) according to manufacture's procedures, and quantitated with a UV-spectrophotometer based on absorptions of OD_{260}. The DNA fragment containing the *cauliflower mosaic virus* 35S promoter was PCR amplified with forward primer 35S-F (5′ Biotin-GCT CCT ACA AAT GCC ATC A 3′) and reverse primer 35S-R (5′ GAT AGT GGG ATT GTG CGT CA 3′). The PCR fragment was hybridized with the digoxigenin-labeled probe 35S-P (5′ DIG-CAA CCA CGT CTT CAA AGC AA 3′). The DNA fragment containing the *Agrobacterium tumefaciens* nos terminator with forward primer nos-F (5′ Biotin-GAA TCC TGT TGC CGG TCT TG 3′) and reverse primer nos-R (5′ TTA TCC TAG TTT GCG CGC TA 3′), and hybridized with the digoxigenin-labeled probe nos-P (5′ DIG-TGC CGG TCT TGC GAT GAT TAT CAT A 3′). The control DNA fragment from the soybean lectin locus with forward primer lec-F (5′ Biotin-CCA GCT TCG CCG CTT CCT TC 3′) and reverse primer lec-R (5′ GAA GGC AAG CCC ATC TGC AAG CC 3′), and hybridized with the digoxigenin-labeled probe lec-P (5′ DIG-CTT CAC CTT CTA TGC CCC TGA CAC 3′); and the control DNA fragment from the maize invertase locus with forward primer inv-F (5′ Biotin-TGG CGG ACG ACG ACT TGT 3′) reverse primer inv-R (5′ AAA GTT TGG AGG CTG CCG T 3′), and hybridized with the digoxigenin-labeled probe inv-P (5′ DIG-CGA GCA GAC CGC CGT GTA CTT CTA CC 3′). All PCR reactions were carried out in 50 μl reaction mixtures (containing 1 × PCR Buffer, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer, 1 unit of Taq DNA Polymerase, and 2 μl DNA templates) for 35 cycles at 94 °C for 20 s, 54 °C for 40 s and 72 °C for 60 s with the specific primers as described in a GeneAmP 9600 PCR system (Perkins Elmer, USA), with a pre heating at 94 °C for 3 min and post PCR incubation at 72 °C for 3 min. The PCR products were then analyzed with electrophoresis on agarose gel (1%, w/v) and southern blotting on nylon membrane (0.45 μm) as described (Wang & Fang, 2002).

2.3. Coating microplates with streptavidin

The microplate wells were incubated with 100 μl binding buffer (5 mg/L streptavidin diluted in PBS) overnight at 4 °C and then 100 μl sealing buffer overnight at 4 °C as described (Li, Cheng, & Luo, 2001). Then the microplate wells can store for 6 months at 4 °C.

2.4. LPH-PCR-ELISA

For PCR-ELISA analyses, 50 μl hybridization buffer (5 × SSC, 1% SDS w/v, 50% methanamide v/v) including 40 μM digoxigenin-labeled probes was added to each sample immediate after the PCR reaction. The hybridization was carried out by further incubation the reaction mixture at 92 °C 5 min, 55 °C 5 min, and then room temperature for 30 min. The hybridization products (50 μl) were then transferred to the streptavidin-coated microplates, mixed with 50 μl hybridization buffer, and incubated at 50 °C for 30 min. After washed with 150 μl wash buffer I for 4 times, 100 μl AP solution diluted with 0.5% BSA (1:1000) was added to each well. The plate were incubated for 60 min at 50 °C, and then washed 4 times with 150 μl wash buffer II. Subsequently, 100 μl substrate buffer (0.1% pNPP w/v, 1 M diacetyl amide, 1 mM $MgCl_2$) was add to each well. After incubation at room temperature for 30 min at room temperature, the reaction was stopped by adding of 50 μl 1 M H_2SO_4. The plate was measured at 405 nm with a microplate reader (Bio-rad Model 450, USA).

2.5. Solid-phase hybridization (SPH) based PCR-ELISA

Solid-phase hybridization was performed as described elsewhere (Brunnert, Spener, & Börchers, 2001). Briefly, biotin-labeled PCR products are annealed chemically with NaOH and then hybridized with digoxigenin-labeled probes immobilized in tubes. After washing, specific bound biotin–digoxigenin-labeled fragments were accessed with ELISA and the absorption of OD_{405} was determined as in LPH-PCR-ELISA.

3. Results and discussion

3.1. Extraction genomic DNAs and PCR analyses GMOs components

The genomic DNA was extracted from soybean and maize sample with or without GMOs components by the CTAB protocol or the Wizard Kit. Generally, the DNA concentration was in the range of 80–150 ng/μl with an OD_{260}/OD_{208} ratio of 1.6–1.7 in samples purified with the CTAB protocol, and 15–40 ng/μl with the OD_{260}/OD_{208} ratio of 1.7–1.8 in samples purified with the Wizard Kit. Both the CTAB-purified and Wizard Kit-purified DNAs were tested in pilot experiments to determine whether the DNA samples were pure enough for the PCR analysis, the results showed no significant difference in the PCR products among these samples (data not shown), suggesting that the quality of DNA purified with the two methods was good enough for the analysis.

To determine whether the primers listed in Section 2 were suitable for detecting GMOs components, we first analyzed both soybean and maize samples with or without GMOs components by PCR with the CaMV 35S promoter and T-nos specific primers. The PCR products were analyzed with agarose electrophoresis and then verified with Southern blotting. The results clearly showed that the 195 bp fragment representing the CaMV 35S promoter and 180 bp fragment representing the T-nos terminator were only presence in the samples labeled with 0.15% and 0.3% GMOs either in agarose gel or the southern blotting (Fig. 1), which indicated that the two sets of primers were able to specifically amplified GMOs components in both soybean and maize samples.

3.2. PCR-ELISA analysis with liquid-phase hybridization

Immediately after PCR reaction, the digoxigenin-labeled probes were directly added to each PCR reaction mixtures for on-site hybridization as described in Section 2. The biotin- and digoxigenin-labeled DNA hybrid fragments were then captured with streptavidin-coated microplates. The specific bound biotin- and digoxigenin-labeled DNA hybrid fragments were accessed as described in Section 2. The results showed that negative control samples had a background OD_{405} absorption of 0.05 or less, either with primers specific for the CaMV 35S promoter or T-nos terminator in both soybean (Fig. 2A) and maize (Fig. 2B) samples. The cut-off value (about 0.20) was determined as the mean of negative control samples (0%) plus three times the standard deviation from triplicate assays. In contrast, OD_{405} values of samples containing GMOs at indicated percentage were greater than 0.2 in both soybean (Fig. 2A) and maize (Fig. 2B) samples either with the primers for CaMV 35s Promoter or for T-nos terminator, and exhibited a linear increase as the percentage of GMOs component increase. In addition, the OD_{405} values of both none-GMOs and GMOs samples were greater than 0.2 when analyzed with the primers specific for lectin or invertase reference genes (data not shown). All together, the data showed here indicated that the limit of detection (LOD) of the LPH-PCR-ELISA is 0.3% for Roundup Ready soybean and 0.15% for Bt1 maize regardless which GMOs marker was used.

To further validate the data derived from the LPH-PCR-ELISA analysis, we employed the solid-phase hybridization (SPH) based PCR-ELISA to access the presence of GMOs components in the same set of samples, and found no significant difference between the data generated from the LPH- and SPH-PCR-ELISA, although the OD_{405} background of SPH-PCR-ELISA is somewhat higher than that of LPH-PCR-ELISA (data not shown). Although the streptavidin-coated tubes have a long shelf life and a small variation, the SPH-PCR-ELISA overall is complicate, time consuming, and has high OD_{405} background (Li et al., 2001). In comparison, the improved LPH-PCR-ELISA is relatively simple and fast, which also has a low OD_{405} background. In addition, the LPH-PCR-ELISA is relatively easy to be adapted to a large-scale automatic procedure.

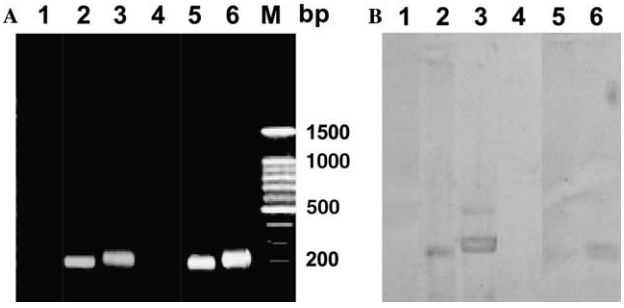

Fig. 1. Detection of GMOs components with PCR analysis. The DNA fragments representing the T-nos terminator and CaMV 35S promoter were PCR amplified from soybean (lanes 1–3) and maize (lanes 4–6) samples with the indicated primers described in Section 2, and analyzed with agarose gel electrophoresis (A) and Southern blotting (B). 1, soybean sample with 0% GMOs components; 2–3, soybean with 0.3% GMOs; 4, maize sample with 0% GMOs; 5–6, maize sample with 0.15% GMOs; M: 100 bp DNA ladder.

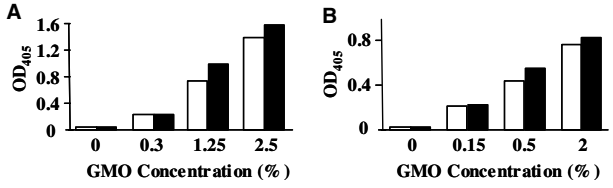

Fig. 2. Detection of GMOs components in samples with GMOs labels with LPH-PCR-ELISA. DNA samples from soybean (A) and maize (B) samples with the indicated GMOs components were analyzed with LPH-PCR-ELISA as described in Section 2. The data represented average of triplicated sample. Solid box, T-nos terminator; hollow box, CaMV 35S promoter.

3.3. Detection of GMOs components in unlabeled samples with LPH-PCR-ELISA

To detect whether unlabeled product samples contain GMOs components, we employed the LPH-PCR-ELISA to analyze 13 samples including 7 soybean samples and 6 maize samples. The results (Fig. 3) showed that the OD_{405} values of 7 samples (S1, S2, S6, S7, and M1, M3, M6) were greater than 0.20 and comparable to that of the positive control (S9 for soybean, M8 for maize), which was at least 2 times as high as that of the negative controls (S8 for soybean, M7 for maize). The other 6 samples (S3, S4, S5, and M2, M4, M5) had an OD_{405} values similar to that of the negative control and the blank control (BC), which was small than 0.20. In addition, the OD_{405} values of the lectin and invertase reference genes in soybean and maize samples were greater than 0.20. Therefore, we concluded that the soybean sample S1, S2, S6, S7 and maize sample M1, M3, M6 were GMOs positive; and that soybean sample S3, S4, S5 and maize sample M2, M4, M5 were GMOs negative. The results were further confirmed with agarose gel electrophoresis, which further indicated that the LPH-PCR-ELISA was a valid method for detection of GMOs in foods.

In PCR-based assays, the results are usually analyzed by electrophoresis and southern blotting. Gel electrophoresis method is rapid but hazardous, and southern blotting method makes the testing of multiple samples tedious and time-consuming.

The PCR-ELISA technology combines high efficiency and high sensitivity advantages of PCR with high specificity and high accuracy advantages of ELISA, and therefore has been widely used in medical diagnosis (Li et al., 2001), although it is relatively new in detection of GMOs in food. Up to date, PCR-ELISA procedures rely mainly on solid-phase hybridization for detection of the amplified products. In this report we described an alternative strategy that employed liquid-phase hybridization immediately post PCR cycles to incorporate digoxigenin into the PCR products for detection with ELISA. Normally, the liquid-phase hybridization generates more uniform hybrids and generates less error than the solid-phase hybridization. In LPH-PCR-ELISA, the amplified PCR products are annealed physically (heat denatured) on site and the subsequent hybridization procedure is also carried out in the same PCR instrument, which significantly simplifies procedure and time of the assay. In addition, both annealing and hybridization steps can be incorporated into the PCR program, which make it relatively easy for future adaptation to an automatic procedure for identification of GMOs components in multiple food samples. The LPH-PCR-ELISA has a more accurate digital readout than conventional agarose gel electrophoresis and can be used for quantitative access of GMOs components in food. The quality of DNA and the efficacies of DNA amplification and hybridization are among the major concerns in PCR-ELISA detection of GMOs in food (Brunnert et al., 2001). We had purified the DNA with the CTAB method and the Wizard Kit, and analyzed GMOs components in these DNA samples. We found both DNA samples were suitable for the LPH-PCR-ELISA detection of GMOs components. Yet, the CTAB method is more economical, high output. Although the DNA samples extracted with the Wizard Kit has high purity, it is more costly and the yield is relatively low. Therefore, we recommend to use CTAB purified DNA from multiple samples for the LPH PCR-ELISA detection of GMOs components in food.

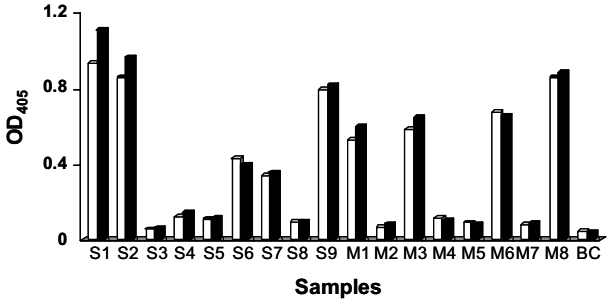

Fig. 3. Detection of GMOs components in samples without GMOs labels with LPH-PCR-ELISA. DNA samples from soybean (S1–9) and maize (M1–8) samples were analyzed for CaMV 35S promoter (solid box) and T-nos terminator (hollow box) with LPH-PCR-ELISA as described in Section 2. The data represented average of triplicated samples.

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