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Chemisorption of Thiolated *Listeria monocytogenes*-specific DNA onto the Gold Surface of Piezoelectric Quartz Crystal

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Piezoelectric (PZ) crystal biosensor system was used to detect the DNA of food pathogenic *Listeria monocytogenes*. *L. monocytogenes*-specific DNA was multiplied via the polymerase chain reaction using LM1 oligonucleotide (5'-TTACGAATTAAAAAGGAGCG-3') and LM2 oligonucleotide (5'-TTAAATC AGCAGGGGTCTTT-3') as primers. DNA fragment of 161 bp, which was specific only for *L. monocytogenes*, was observed. To obtain a large amount of single-stranded DNA containing an SH group used for coupling to the gold electrode chemisorptively, LM1 oligonucleotide containing a mercaptohexyl group was utilized as a single strand PCR primer. The PCR product was immobilized onto the gold electrode of PZ crystal, and hybridization was monitored in quartz crystal microbalance (QCM) system by injecting the antisense single-stranded DNA of 161 nucleotides obtained via the single strand PCR using the unmodified LM2 primer. Approximately 70 Hz of frequency drop was observed in the QCM system in the case of two consecutive injections of 5 μg of the antisense single-stranded DNA.

Key words: Listeria monocytogenes-*specific DNA, piezoelectric quartz crystal, chemisorption, QCM.*

Nucleic acid probe assays using DNA hybridization could be utilized for the detection and identification of microorganisms. Colony hybridization method consists of lysing cells, transferring the DNA onto a membrane such as nitrocellulose paper, and hybridizing DNA probes labeled with radioactivity or fluorescence to specific sequences of the bound DNA. Although this method is precise compared to the polymerase chain reaction (PCR) technique, cumbersome labeling with radioactivity or fluorescence is required.

Piezoelectric quartz is a type of crystal, which elicits a unique resonant frequency under oscillator circuit in a given power. The resonant frequency shifts when trace amount of a material is bound to the crystal surface as suggested in the classic equation describing the relationship of frequency to mass:

$$\Delta F = -2.3 \times 10^{6} F^{2} \Delta m/A$$

where ΔF is the change in fundamental frequency of the coated crystal, F is the fundamental frequency of the crystal, A is the coated area, and Δm is mass deposited.[1] Quartz crystal microbalance (QCM) is a very sensitive mass measuring device because the resonance frequency of the quartz decreases upon the increase of a given mass on the QCM on a nanogram level.

Recently, the specificity of DNA hybridization has been utilized in biosensor systems with piezoelectric (PZ) crystals.[2-4] Single-stranded DNA probe was electroblotted onto the surface of a gold-plated quartz crystal. The crystal sensor was dipped into the hybridization solution, and the surface mass increase was measured based on the decrease in the resonant frequency of the crystal.[2] After chemisorption of 10-mer oligonucleotide probe, having a mercaptopropyl group at the 5'-phosphate end, onto the surface of a gold-plated quartz crystal, DNA hybridization was also measured.[3]

To obtain the relatively long single-stranded DNA containing sulfhydryl groups, which can be immobilized onto the gold plate of quartz crystal, a new method utilizing PCR with a primer containing a mercaptohexyl group at the 5'-phosphate end has been developed. In this research, we tested the PZ crystal biosensor system for detecting DNA hybridization using the PZ crystal coated with a single-stranded DNA probe, which is specific for *L. monocytogenes* and contains a mercaptohexyl group at the 5'-phosphate end.

Materials and Methods

Materials. *Eco*RI restriction enzyme was from Promega. *Taq* DNA polymerase for PCR was purchased from Perkin Elmer (USA). TA cloning kit was from Invitrogen (USA), and other chemicals were obtained from Sigma and Aldrich. LM1 oligonucleotide primer (5'-TTACGAATTAAAAAGGAGCG-3'), LM2 oligonucleotide primer (5'-TTAAATCAGCAGGG GTCTTT-3'), and LM1 primer modified with a mercaptohexyl group (SH-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-) at the 5'-phosphate end were purchased from Bioneer Co. (Cheongju,

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Abbreviations: PZ, piezoelectric; QCM, quartz crystal microbalance.

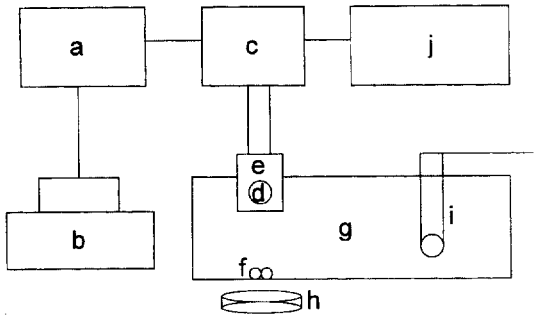

Fig. 1. Schematic diagram of the apparatus for a sequence-specific DNA-immobilized sensor system based on quartz crystal microbalance analysis. (a) frequency counter; (b) personal computer; (c) oscillator circuit; (d) gold electrode; (e) quartz crystal; (f) magnetic bar; (g) glycerol bath; (h) magnetic stirrer; (i) heater; (j) power supply.

Korea). All other chemicals were of analytical grade.

Culture media. *L. monocytogenes*, which was provided by Dr. Chung Ho Ryu (Department of Environmental Management, Taejon Junior College), was grown in Luria-Bertani agar medium (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar).

Apparatus. The piezoelectric crystals used were AT-cut quartz wafer (Seiko EG and G, Japan) of $8 \times 8 \times 0.18$ mm dimensions placed between two gold electrodes of 5 mm diameter. The resulting crystals had a fundamental resonant frequency of 9 MHz with reproducibility of ±0.1 Hz. An oscillating circuit was built in-house and used to drive the crystal. The oscillator was powered by a regulated power supply set at 5 V dc. The frequency of the vibrating crystal was monitored through a universal frequency counter (Dagatronics Co., Korea). The crystal, which functions as working electrodes was dipped into a reaction cell placed in a 25°C glycerol bath with a circulation system. Schematic diagram of the apparatus for a sequence-specific DNA-immobilized sensor system based on quartz crystal microbalance analysis is shown in Fig. 1.

Subcloning of the PCR product. PCR was carried out to amplify the DNA specific for *L. monocytogenes*. LM1 and LM2 primers, whose sequences are found in the downstream region of *hlyA* gene and unique in *L. monocytogenes*,[11] were selected as a 5'-primer and a 3'-primer, respectively, for 40 cycles of PCR. After a colony of *L. monocytogenes* was suspended in 100 μl of H_2O, the suspended solution was boiled for 5 min and centrifuged in a microcentrifuge at 12,000 rpm for 25 s. Ten microliter of the supernatant was used as a DNA template for PCR. Denaturation was done for 1 min at 95°C, annealing for 1 min at 55°C, and polymerization for 2 min at 72°C. After PCR, the amplified target DNA was subcloned into the pCRII vector using the TA cloning kit.

Preparation of QCM surface. The crystal was soaked in 1.2 M NaOH for 30 min, washed with distilled water, and immersed in 1.2 M HCl for 5 min. Twenty microliter of concentrated HCl was placed on the gold electrodes of the crystal for 2 min, with special care to keep the acid from touching the electrode lead. The crystal was washed with distilled water and dried in an oven at 100°C for 20 min.[5] QCM crystals were used immediately after preparation.

DNA immobilization onto the gold plate of QCM. Modified LM1 with a mercaptohexyl group was used as a PCR primer for the preparation of the sense single-stranded DNA probe to be immobilized onto the gold plate of the QCM crystal. The QCM crystal was immersed in an aqueous solution containing the LM1 single-stranded DNA probe with a mercaptohexyl group for 12 h at 25°C. The crystal was air-dried in a hood, washed with distilled water, and air-dried again.

Hybridization and measurement of frequency shift. The crystal coated with the single-stranded DNA probe containing the mercaptohexyl LM1 oligonucleotide was dipped into a reaction cell containing 7.5 m*l* distilled water at 25°C. The resonance frequency of the crystal was determined after a steady baseline was obtained. The steady state resonant frequency was read after injection of the antisense single-stranded DNA of 161 nucleotides obtained through the single strand PCR using the unmodified LM2 as a primer.

Results and Discussion

PCR for amplification of the *L. monocytogenes*-specific DNA. *L. monocytogenes* in various foods could be detected through PCR using primers specific for the bacteria.[6-11] In this experiment, LM1 and LM2 oligonucleotides, whose sequences are found in the downstream region of *hlyA* gene and specific for *L. monocytogenes*, were selected as a 5'-primer and a 3'-primer, respectively, for the 40 cycles of PCR. The region between LM1 and LM2 sites is very unique compared to the regions between the primers used in the previous research papers.[7-10] To identify the PCR product, samples were electrophoresed on 2% agarose gel. Lane 1 of Fig. 2 indicates the Stratagene's Kb DNA ladder. Lanes 2-4 show the amplified DNA fragments in the PCR with the LM1 and LM2 primers using the chromosomal DNA from *L. monocytogenes*, *E. coli*, and *Salmonella typhimurium* as DNA templates, respectively. No amplified DNA fragment was detected using the chromosomal DNA from *E. coli* and *S. typhimurium*. Approximately 160 bp DNA fragment was observed only in the case of *L. monocytogenes* (Fig. 2, lane 2). The size of the PCR-amplified DNA was found to be the same as that of the calculated DNA based on the genomic DNA sequence in the downstream region of *hlyA* gene of *L. monocytogenes*. The linear pCRII vector containing T-cohesive tail was ligated with A-tailed PCR product DNA amplified by *Taq* DNA polymerase. Lanes 2 and 3 of Fig. 3 show *L. monocytogenes* chromosomal DNA and the amplified DNA fragment using the chromosomal DNA from *L. monocytogenes*, respectively. The pCRII vector containing the PCR product was digested with *Eco*RI, and the insert was resolved on 2% agarose gel (Fig. 3, lane 4). DNA fragment of 161 bp was found to be subcloned

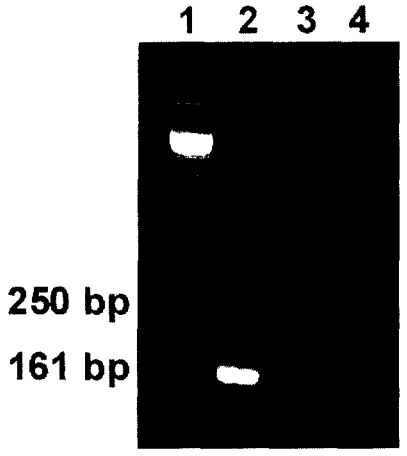

Fig. 2. PCR amplification of the *Listeria monocytogenes*-specific DNA using LM1 and LM2 primers whose sequences are found in the downstream of *hlyA* gene. Lane 1, molecular weight markers; lane 2, *Listeria monocytogenes* DNA template; lane 3, *E. coli* DNA template; lane 4, *Salmonella typhimurium* DNA template.

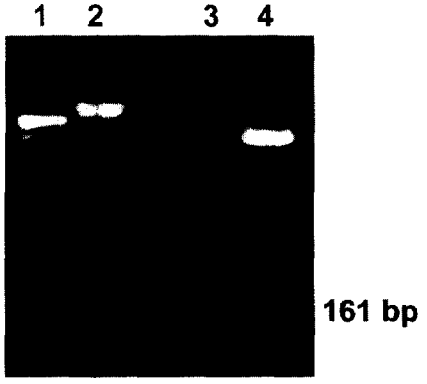

Fig. 3. Subcloning of the *Listeria monocytogenes* PCR fragment into the TA cloning vector. Lane 1, molecular weight markers; lane 2, *Listeria monocytogenes* chromosomal DNA; lane 3, PCR product using LM1 and LM2 primers; lane 4, subcloned TA vector digested with *Eco*RI.

into the pCRII vector. The 161 bp PCR product in this recombinant vector was used as a DNA template for PCR to obtain large amounts of the sense single-stranded DNA containing the mercaptohexyl LM1 oligonucleotide and the antisense single-stranded DNA containing the LM2 oligonucleotide.

Chemisorption of mercaptohexyl DNA onto the gold electrode of the crystal. DNA was not found to be coupled to PZ quartz crystal using several immobilization methods such as the glutaraldehyde coupling, the triazine coupling, the epoxy coupling, and the UV-crosslinking methods (data not shown).[12,13] Therefore, the chemisorption method of DNA containing an SH group to the gold electrode of the PZ crystal was used. One way to obtain a large amount of the single-stranded DNA containing an SH group used for coupling to gold electrode chemisorptively is to synthesize oligonucleotides containing SH groups and use these as primers for PCR. Mercaptohexyl LM1 oligonucleotide was used as a

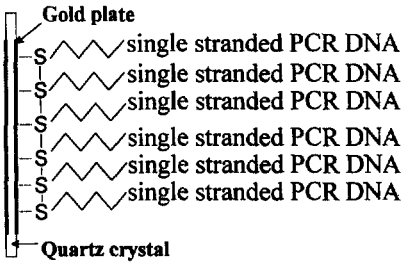

Fig. 4. Schematic diagram of chemisorption of mercaptohexyl DNA onto the gold electrode of the crystal.

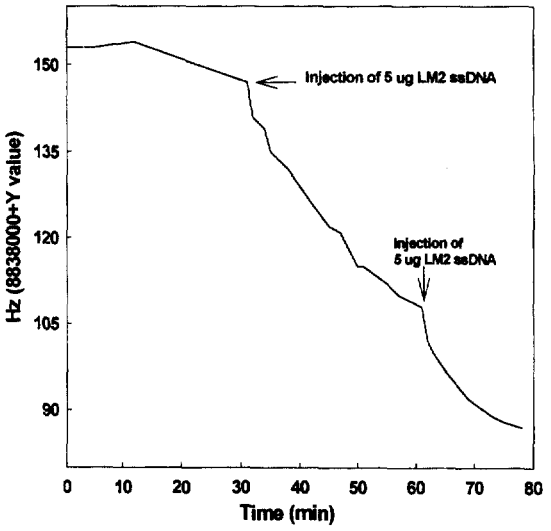

Fig. 5. Sequence-specific hybridization of the mercaptohexyl LM1 single-stranded PCR DNA coupled onto the gold plate with the LM2 single-stranded PCR DNA at 25°C.

primer for the single strand PCR to multiply the 161 nucleotide product containing an SH group. The PCR product was immobilized onto the gold electrode of PZ crystal. The sulfhydryl groups are adsorbed on the gold plate and may have disulfide bridge within them (Fig. 4).[3,4,14-16] The C6 (hexyl) group works as a linker to prevent steric hinderance for DNA hybridization.

Hybridization of mercaptohexyl LM1 single-stranded DNA coupled on the gold plate with LM2 single-stranded DNA. In order to detect *L. monocytogenes*-specific DNA, hybridization was monitored in QCM system by injecting the antisense single-stranded DNA of 161 nucleotides obtained through the single strand PCR using the unmodified LM2 primer. Variable frequency drops were observed in QCM system depending on the amounts of the immobilized SH-modified single-stranded DNA and the injected antisense single-stranded DNA, type of the hybridization solution, and hybridization temperature. Approximately 40 Hz frequency drop was observed within 30 min by the first injection of 5 μg of the antisense single-stranded LM2 DNA in H_2O (Fig. 5). Additional 30 Hz frequency drop was observed by the second injection of the same amount of the above single-stranded DNA. In the case of the control experiment, by injecting sufficient amount of nonspecific DNA such as denatured pUC19

DNA, less than 10 Hz frequency drop was monitored. It has been reported that 1 Hz frequency drop corresponds to 1 ng of the hybridized DNA.[3] However, only 70 Hz frequency drop was monitored by the injection of 10 μg of the antisense single-stranded DNA (Fig. 5). One of the reasons for the low response is that water was used as the hybridization solution in this case, even though salt is required for efficient hybridization.[17] Because in the presence of some salt, our lab-made system became very unstable and affected by the surrounding environmental conditions such as electricity stability, temperature, and humidity, a steady baseline could not be obtained within a short time. Glycerol bath was also used to dampen the environmental influences, which may destabilize the system. In the case of water bath to be used for the next research on *S. typhimurium*, sensitivity could be increased even though stability is reduced to some extent (manuscript in preparation).

PZ DNA sensor could be used for monitoring food-pathogenic bacteria by utilizing pathogenic bacteria-specific PCR products, if the efficiency of DNA immobilization onto the gold electrode of the PZ crystal and the stability of QCM system for DNA are improved.

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