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Detection of *Edwardsiella tarda* by fluorometric or biosensor methods using a peptide ligand

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ABSTRACT

In this study, we identified a peptide ligand for *Edwardsiella tarda* from a phage peptide library and tested two approaches for sensitive detection of the bacteria with the peptide labeled with fluorescein or biotin. At first, the fluorescent peptide was proved to be advantageous in the fluorescence polarization (FP) assay because sensitivity of the assay is maximized when a fluorophore is linked to a small molecule. The FP assay using the fluorescent peptide enabled detection of *E. tarda* in a range from 5.2×10^3 to 2.1×10^5 cells. Second, we devised a new assay method using a quartz crystal microbalance (QCM) biosensor connected to a filter module. When a mixture of *E. tarda* and the biotinylated peptide was injected into the filter module, the *E. tarda*–peptide complex was separated from the unbound peptide by a filter and detected with a streptavidin-coated QCM sensor chip. On injection of samples containing the biotinylated peptide and *E. tarda*, concentration-dependent frequency change was observed in a range from 8×10^2 to 8×10^6 cells. The two approaches are expected to facilitate development of assay methods using other bacteria-binding peptides.

Edwardsiella tarda, an enteric gram-negative bacterium, is known as a causative agent of edwardsiellosis in many freshwater and marine fishes [1]. Because an edwardsiellosis epidemic begins with infection of a few fish and the infection spreads to the surrounding areas within a few days, rapid diagnosis of *E. tarda* infection is critical for the prevention of outbreaks. Enzyme-linked immunosorbent assay (ELISA)[1] techniques have been developed for the diagnosis of edwardsiellosis using polyclonal or monoclonal antibody [2,3]. Recently, rabbit polyclonal antibody was applied to a quartz crystal microbalance (QCM) biosensor for rapid and real-time detection of *E. tarda* [4].

Although antibody has been widely used as a diagnostic probe due to its high affinity and specificity, it has several disadvantages such as high cost of production and low stability. Therefore, the phage-displayed peptide library technique has been applied to identification of peptide ligands that might be used for detection of pathogenic bacteria, including *Bacillus subtilis* [5], *Bacillus anthracis* [6–8], *Salmonella typhimurium* [9], *Pseudomonas aeruginosa* [10], and *Clostridium tyrobutyricum* [11]. However, peptide ligands have not been popularly used as probes for detection of bacteria because of their low affinity.

In this work, a heptameric phage peptide library was subjected to the biopanning of *E. tarda*-binding peptides, yielding two enriched peptide sequences. One of the peptides, showing relatively high affinity and specificity, was synthesized with a fluorescein or biotin label. The fluorescent peptide was proved to be useful for the detection of *E. tarda* by the fluorescence polarization (FP) assay method, which measures a change in the rotational speed of a small fluorescent molecule when bound to a large target molecule. The biotinylated peptide also enabled highly sensitive detection of *E. tarda* by a QCM biosensor and a filter module where the bacteria–peptide complex was separated from the free peptide and detected with a streptavidin (SA)-coated QCM sensor chip.

Materials and methods

Identification of E. tarda-binding peptides

Ph.D.-7 phage-displayed heptameric peptide library was purchased from New England Biolabs (Ipswich, MA, USA). The library consisted of 2.8×10^9 independent sequences and was amplified to a titer of 2×10^{13} plaque-forming units (pfu)/ml. *E. tarda* was grown in 2 ml of tryptic soy broth (Difco) to a late log phase. The bacteria were harvested by centrifugation, suspended in 1 ml of Tris-buffered saline (TBS), and transferred to a 1.5-ml microtube.

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; QCM, quartz crystal microbalance; FP, fluorescence polarization; SA, streptavidin; pfu, plaque-forming units; TBS, Tris-buffered saline; AP, alkaline phosphatase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PEEK, polyether ether ketone.

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Ph.D.-7 peptide library (2×10^{11} pfu in 10 μl) was added to the tube, and the tube was incubated for 1 h at room temperature with shaking. The bacteria were collected by centrifugation at 4000*g* for 1 min and resuspended in 1 ml of TBST (0.1% Tween 20 in TBS). This washing step was repeated three times. After the final washing step, the bound phages were dissociated from the bacteria by adding 1 ml of acidic dissociation solution (0.2 M glycine–HCl, pH 2.2) and shaking for 10 min at room temperature. After centrifugation at 15,000*g* for 5 min, the supernatant, containing dissociated phages, was collected and neutralized with 150 μl of 1 M Tris–Cl (pH 9.6). The neutralized solution was mixed with 30 ml of freshly grown *Escherichia coli* XL-1 Blue, and the mixture was incubated at 37 °C for 4 h with shaking to amplify the recovered phages. The culture was centrifuged at 15,000*g* for 20 min. The supernatant, containing amplified phages, was collected and used for the next round of the biopanning.

After the third and fourth rounds of biopanning, recovered phages were plated with *E. coli* XL-1 Blue to prepare isolated phage plaques. Some phage plaques were randomly selected and grown to prepare their DNA. The DNA was used to determine the nucleotide sequence encoding the random peptide displayed at the N terminus of pIII.

Filter assay method

Biotinylated peptides, P1B and P2B, were purchased from Peptron (Daejeon, Korea). The sequences of P1B and P2B were H_2N-TLYPLDRGGK(biotin)-CO_2H and H_2N-WVWPARLGGK(biotin)-CO_2H, respectively, each comprising one of the enriched sequences (P1 or P2) in the biopanning process and three additional residues (GGK). The biotin group was conjugated to the ε-amino group of the C-terminal lysine to keep the N terminus of the peptides freely protruded from the complex when the peptides were associated with avidin or SA. The peptides were associated with alkaline phosphatase (AP)-linked SA or horseradish peroxidase (HRP)-linked SA, and the resulting peptide–enzyme complex was used for the filter assay method.

Edwardsiella tarda or *E. coli* bacteria (10^6 cells in 0.1 ml of phosphate-buffered saline [PBS]) were mixed with 10 μg of the peptide–enzyme complex and incubated at room temperature for 30 min with shaking. An Isopore membrane (0.4 μm pore and 13 mm diameter, Millipore) was installed in a filter holder and treated with blocking buffer (0.05% Tween-20, 1 mM ethylenediaminetetraacetic acid [EDTA], and 0.25% bovine serum albumin [BSA] in PBS). The mixture of bacteria and peptide–enzyme complex was filtered through the membrane by vacuum. The membrane was washed three times by adding 0.5 ml of wash buffer (0.05% Tween 20 in PBS) over the membrane and applying vacuum again. The membrane was transferred to a 24-well cell culture plate. HRP activity remaining in the membrane was estimated by adding 0.1 ml of 0.1 M Tris–HCl (pH 8.3), 0.16 ml of 0.1 mM luminol, 80 μl of 1.2 mM *p*-iodophenol, and 0.16 ml of H_2O_2 to the well and measuring light intensity with a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). AP activity remaining in the membrane was estimated by adding 0.5 ml of *p*-nitrophenyl phosphate (pNPP) substrate (10 mM pNPP, 0.5 mM $MgCl_2$, and 1 M diethanolamine, pH 9.8) to the well and measuring absorbance of the solution at 405 nm in a 96-well microplate with the microplate reader.

Plate assay method

A fluorescent peptide, P2F, was purchased from Peptron. P2F was synthesized by conjugating a fluorescein to the N-terminal amino group of the P2 sequence via a six-carbon linker. In this case, the small fluorescein group was not expected to interfere with the binding of the peptide because the fluorescent peptide was used by itself without attaching to a large protein. *E. tarda* samples, prepared at different concentrations, were added to immunoplates and incubated at 37 °C for 2 h. A control sample, containing no bacteria, was also prepared and processed in the same way. The immunoplates were washed three times with the wash buffer and treated with the blocking buffer. The P2F peptide solution (0.1 ml of 5 μg/ml solution) was added to each well and incubated at room temperature for 1 h with shaking. PBS was used for dilution of the bacteria and peptide. The immunoplates were washed three times with the wash buffer. After the final washing, 0.1 ml of alkaline dissociation buffer (50 mM KCl and 12 mM NaOH, pH 12.0) was added to each well. Fluorescence intensity was measured at excitation and emission wavelengths of 485 and 528 nm, respectively, with the microplate reader.

FP assay method

Edwardsiella tarda, *E. coli*, and *Yersinia enterocolitica* samples were prepared at different concentrations. Each bacteria sample (50 μl) was mixed with 50 μl of 70 nM P2F peptide, and the mixture was incubated at room temperature for 30 min with shaking. PBS was used for dilution of the bacteria and peptide. FP was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, in a black 96-well plate with a Victor^2 plate reader (PerkinElmer). The FP signal was integrated for 1 s.

QCM biosensor assay method

A filter module was made of a sample injection valve (IDEX Health & Sciences, Oak Harbor, WA, USA), and a 0.5-μm polyether ether ketone (PEEK) frit (IDEX Health & Sciences) and attached to an AMO-QC4 QCM biosensor system (Amogreentech, Gimpo, Korea). The injection valve has six ports: ports 1 and 4 for a sample loop, port 2 for sample injection, port 3 for waste-out, port 5 for a QCM cell, and port 6 for a pump. The injection valve was converted to the filter module by two kinds of modifications. First, a PEEK frit was inserted into port 1 before installing a nut for a sample loop. Second, ports 5 and 6 were exchanged with each other by connecting port 5 to a pump and connecting port 6 to a QCM cell. As a result, when a sample was injected into the valve in the load position, the sample was filtered through the frit and bacteria were retained in the surface facing the valve. When rotating the valve to the inject position, the bacteria were released from the frit and carried to the QCM cell because the buffer flow was reversed.

The AT-cut 10-MHz QCM (8×8 mm square wafer) was purchased from Crystal Sun Life (Tokyo, Japan). SA-coated QCM sensor chips were prepared by the hybrid bilayer membrane method [12]. Each *E. tarda* sample (0.1 ml) was mixed with 0.1 ml of 50 μg/ml P2B peptide, and the mixture was incubated at room temperature for 30 min. The mixture was injected into the filter module in the load position, and 0.5 ml of PBS was again injected to remove unbound P2B peptide from the frit. The bacteria retained in the frit were brought into the QCM cell by rotating the valve to the inject position. The resonant frequency of the QCM was recorded at an interval of 10 s.

Results and discussion

Identification of a peptide ligand

After four rounds of biopanning, 14 and 10 phage clones were randomly selected among the third- and fourth-round phages, respectively. The phages were grown, and their DNA sequences, encoding the random peptides, were determined. Two enriched peptide sequences, TLYPLDR (P1) and WVWPARL (P2), were found

among the 24 phages. The P1 sequence occurred once each in the third- and fourth-round phages, and the P2 sequence occurred twice in the third-round phages.

The enriched sequences were used to prepare biotinylated peptides, P1B and P2B, each containing P1 or P2 sequence followed by GGK residues and a biotin group. The biotinylated peptides were associated with SA–HRP, and the peptide–SA–HRP complexes were used for estimation of binding affinity of the two peptides by the filter assay method. The peptide–SA–HRP complex was mixed with *E. tarda*, and unbound complex was removed by filtering through a 0.4-μm membrane. If the P1B or P2B peptide has a binding affinity to *E. tarda*, some of the peptide–SA–HRP complex would be retained in the membrane because the complex is attached to the bacteria that cannot pass through the membrane. Therefore, affinity of the peptide can be estimated by the HRP activity retained in the membrane.

Some HRP activity was observed in sample 1, containing *E. tarda* and SA–HRP that was not associated with the P1B or P2B peptide (Fig. 1A). However, two other control samples containing only P1B– or P2B–SA–HRP (samples 2 and 3) showed very low enzyme activity. These results show that adsorption of SA–HRP or peptide–SA–HRP to the membrane was not significant, whereas there were some nonspecific interactions between SA–HRP and *E. tarda*. The P1B–SA–HRP showed slightly higher binding than SA–HRP, revealing low affinity of the P1 peptide to *E. tarda*. In contrast, high affinity of the P2 peptide was demonstrated by the 4-fold higher light intensity in sample 5 than in the control (sample 1). Therefore, the P2 peptide was selected for further experiments.

Activity of the P2 peptide was further analyzed with a different enzyme, AP. The P2B peptide was conjugated to SA–AP, and the P2B–SA–AP complex was used to compare affinity of the P2 peptide to *E. tarda* and other enteric gram-negative bacteria, *E. coli*, by the filter assay method. The AP activity retained in the membrane was similar in samples 1, 2, and 3 (Fig. 1B). This means that the SA–AP was nonspecifically adsorbed to the membrane in the three control samples but not to both bacteria. However, when the P2 peptide was attached to SA–AP, the P2B–SA–AP complex showed high affinity to only *E. tarda*. Thus, the P2 peptide was shown to have activity to bind specifically to *E. tarda*.

Detection of E. tarda by plate assay method

The P2F peptide, in which a fluorescein group was attached to the N-terminal amino group of the P2 peptide, was used for the fluorometric detection of *E. tarda* by the plate assay method. Different amounts of the bacteria were adsorbed to immunoplate wells. The P2F peptide was added to the wells, and unbound peptide was removed by washing. The amount of adsorbed bacteria was determined by the fluorescence intensity of the P2F peptide remaining in the well. As shown in Fig. 2, the fluorescence intensity increased in a dose-dependent manner in a range from 10^5 to 10^8 cells. Fluorescence intensity measured with a control sample was approximately 4500, similar to the values at low cell numbers. The relatively high control value may be attributed to nonspecific binding of the P2F peptide as well as a blank value imposed by the instrument and the optical property of the immunoplate. Because the blank value was approximately 2300, when measured with an empty immunoplate, fluorescence from the nonspecific binding was estimated to be 2200.

The lower detection limit of the Synergy HT microplate reader is claimed by the manufacturer to be 1 fmol of fluorescein per well in a 96-well plate [13]. In the plate assay method, the lowest cell number distinguishable from the blank was 7×10^5 cells. Therefore, it can be assumed that approximately 1 fmol of P2F peptide (6×10^8 molecules) was associated with 7×10^5 cells. Although some of the peptide might be retained in the well by nonspecific adsorption, it can be roughly estimated that at least several hundred peptide molecules were attached to each bacterium considering that adsorption efficiency of bacteria to immunoplate wells was reported to be 25 to 50% [14]. In general, enzyme reporters

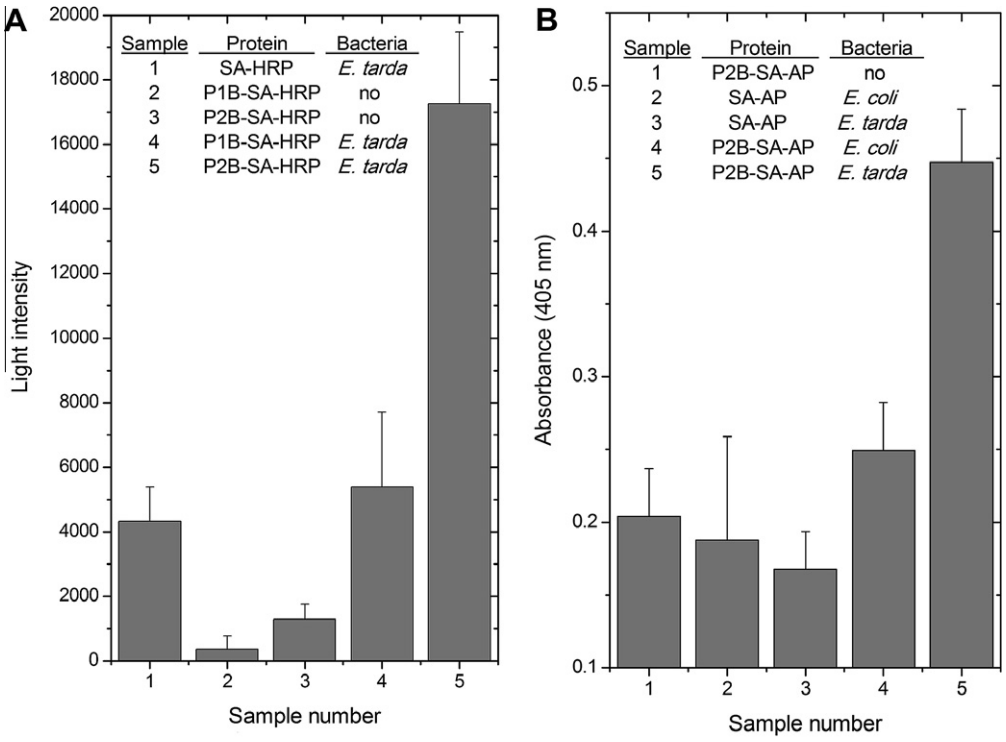

Fig.1. Analysis of the P1 and P2 peptide by the filter assay method. Each sample was prepared by mixing peptide–enzyme complex (protein) and bacteria. SA–HRP and SA–AP denote horseradish peroxidase-linked streptavidin and alkaline phosphatase-linked streptavidin, respectively. Each sample was filtered with a 0.4-μm membrane, and HRP (A) or AP (B) activity remaining in the membrane was measured.

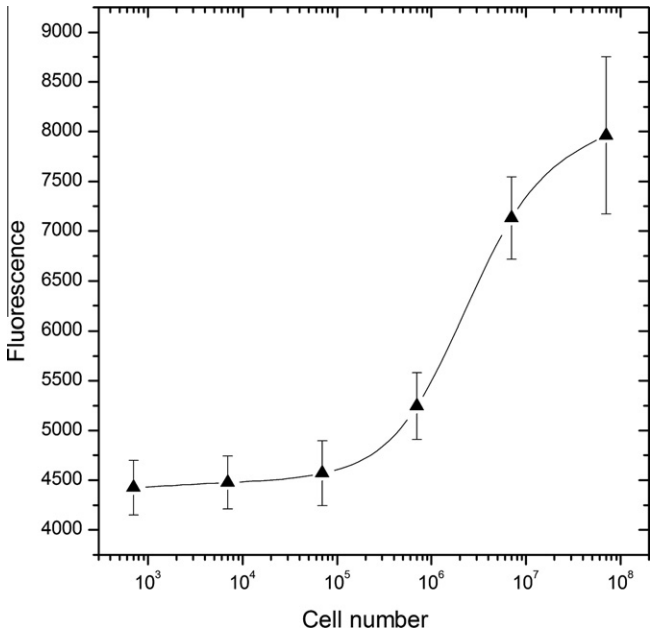

Fig.2. Detection of *E. tarda* with the P2F peptide by the plate assay method. Different amounts of the bacteria were adsorbed to immunoplate wells. The P2F peptide was added to the wells, and unbound peptide was removed by washing. The amount of the adsorbed bacteria was determined by the fluorescence intensity of the P2F peptide remaining in the well.

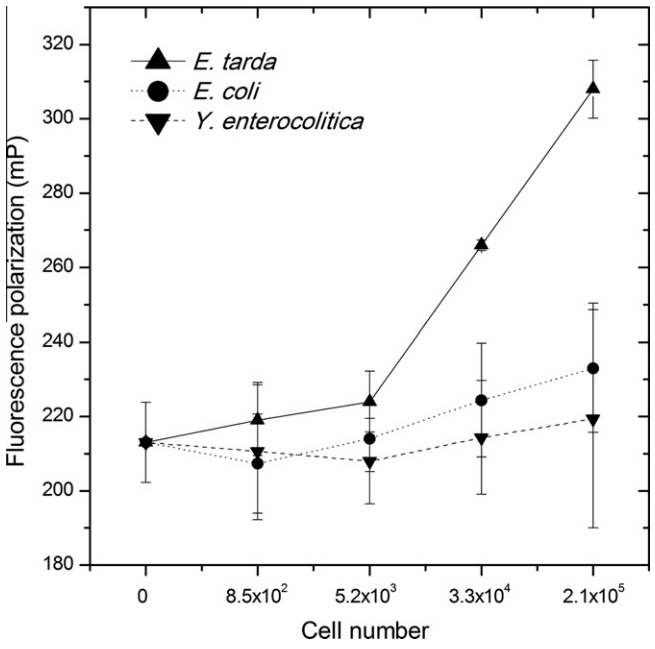

Fig.3. Detection of *E. tarda* with the P2F peptide by the FP assay method. Different amounts of *E. tarda*, *E. coli*, and *Y. enterocolitica* were mixed with the P2F peptide, and the FP value of each mixture was measured.

are expected to enable much more sensitive detections than fluorescent labels because the signal is amplified by the catalytic activity of the enzyme. For example, a typical detection limit of a luminometer is several thousand molecules of luciferase enzyme [15], approximately 10^5-fold lower than that of fluorescein. Therefore, the detection limit of the plate assay method might be improved by orders of magnitude by conjugating the P2 peptide to reporter enzymes instead of fluorescent labels.

Detection of E. tarda by FP assay method

Peptide ligands are especially advantageous over antibody in the FP assay. The FP experiments are performed in solution without solid supports, allowing convenient and sensitive homogeneous assays. The FP assay method uses the change of the rotational speed when a fluorescent molecule is bound to a target molecule. Therefore, sensitivity of the FP assay is maximized when a small molecule is linked to a fluorophore and binds to a big target molecule. Although antibodies have been popularly used in a variety of immunological assay methods, they are not suitable for the FP assay owing to its high molecular weight.

The P2F peptide was used for the FP assay of *E. tarda* and two other enteric gram-negative bacteria, *E. coli* and *Y. enterocolitica*. The FP of the free P2F peptide was approximately 210 mP and increased to 310 mP at 2.1×10^5 cells of *E. tarda*, whereas no significant change was observed with the other two bacteria (Fig. 3). In the FP assay, physically possible FP values range from −330 to 500 mP. In practice, however, these limiting values are rarely attained and typical measured values of FP were in a range of 30 to 300 mP when analyzing interactions between peptide ligands and proteins [16,17]. Therefore, the FP value of 310 mP at 2.1×10^5 cells might be the upper detection limit of the assay. At higher *E. tarda* concentrations, the FP value showed a tendency to decrease, although scattering by bacterial cells contributed to the polarization of the fluorescence as judged by increased FP in a control sample containing only bacteria.

FP of the free P2F peptide was 210 mP, reaching 67% of the upper limit in the typical range of 30 to 300 mP. If FP of the free peptide is already high enough to approach a plateau, the increase of FP on binding to a target will be small, adversely affecting sensitivity of the assay. FP reflects the average angular displacement of the fluorophore, which is dependent on the rate of rotational diffusion during the fluorescence lifetime. Therefore, there are two possible approaches to lower the peptide FP. First, size of the P2F peptide should be minimized to enhance its rotational rate. The six-carbon linker as well as nonessential amino acid residues, if any, can be removed. Second, the peptide FP would be lowered by using fluorescent probes with lifetimes longer than 4 ns of fluorescein such as dansyl dyes (12–20 ns) [18,19] and Re(I) metal–ligand complex (2700 ns) [20].

Detection of E. tarda by QCM biosensor assay method

We devised a novel two-step process using the P2B peptide for the detection of *E. tarda* with a QCM biosensor. In the first separation step, a mixture of *E. tarda* sample and the P2B peptide was injected into the filter module made of a common six-port injection valve and a frit with a pore size of 0.5 μm. The *E. tarda*–P2B complex, with a size of approximately 1 μm, would be retained in the frit and separated from unbound P2B peptides. The second detection step involved reversal of the buffer flow in the frit by rotating the valve. Then the separated *E. tarda*–P2B complex would be released from the frit, brought to a QCM cell, and detected with an SA-coated QCM sensor chip.

Fig. 4 shows the frequency change profiles of each sample after the buffer flow was reversed. Two control samples, each containing only *E. tarda* (8×10^6 cells) or P2B peptide (5 μg), caused no significant frequency change. Without the P2B peptide, the bare *E. tarda* bacteria could not interact with the SA-coated QCM. In the P2B control, the free peptides might be removed to the waste by passing through the frit. In contrast, the samples containing the P2B peptide and different amounts of *E. tarda* bacteria showed concentration-dependent frequency decreases in a range from 8×10^2 to 8×10^6 cells.

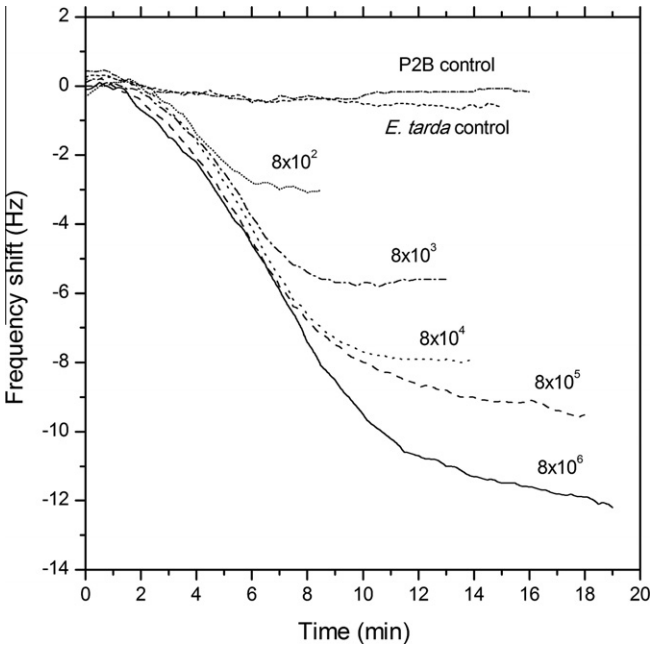

Fig.4. Detection of *E. tarda* with the P2B peptide by the QCM biosensor assay method. Different amounts of *E. tarda* were mixed with the P2B peptide and injected into the filter module in the load position. PBS was further injected into the module to remove unbound peptides. The valve was rotated to the inject position, and frequency was measured at an interval of 10 s.

Oscillation frequency of a QCM decreases from the initial value as mass is deposited on the surface of the crystal. However, unusual frequency increases have been frequently observed in the flexible or loose binding of particles to a QCM surface [21]. Such negative mass effect was explained in terms of the binding nature. When a large particle such as bacteria is attached to a QCM surface by a flexible linkage, movement of the particle might not be in phase with the oscillation of the QCM, resulting in the negative mass effect. In contrast, normal frequency decreases were observed in the multiple and rigid binding of *S. typhimurium* to a QCM containing antibody to somatic O antigen of *Salmonella* [22].

Typical biosensors include a sensing element, capable of binding to a target analyte, immobilized on a sensor surface. Therefore, sensitivity of the biosensor was limited by the binding affinity of the sensing element to the analyte. In our separation detection method, however, each *E. tarda* cell retained several hundred biotin groups on its surface because the cells were saturated with the P2B peptide prior to the injection. Consequently, the detection process was mediated by the multiple tight binding of the biotin groups to SA proteins immobilized on the QCM surface. This eliminated the problem of negative mass effect and enabled a highly sensitive detection. The lower detection limit of this method was 800 cells, corresponding to a mass of approximately 0.5 ng.

Conclusions

Peptide ligands have not been popularly used as probes for the detection of bacteria in spite of their beneficial features such as small size, low price, homogeneity, and high stability. In this article, we have suggested two assay methods by which sensitive detection of bacteria can be achieved with peptide ligands. Fluorescently labeled peptide ligands are applicable to the FP assay because sensitivity of the FP assay is maximized when using a small molecule as a fluorescent probe. The second biosensor method involves two steps: separation of the biotinylated peptide–bacteria complex from the free biotinylated peptide through a filter and detection of the complex with an SA-coated sensor chip. The FP assay is suitable for the high-throughput analysis of relatively clean samples because the result can be biased by contaminants affecting fluorescence. In the analysis of contaminated samples, more reliable results would be obtained by the biosensor method because contaminating molecules are removed in the separation step and only the biotinylated peptide–bacteria complex is brought into the sensor chip with the carrier buffer.

Acknowledgments

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References

[1] B.R. Mohanty, P.K. Sahoo, Edwardsiellosis in fish: a brief review, J. Biosci. 32 (2007) 1331–1344.
[2] P. Swain, S.C. Murkherjee, P.K. Sahoo, P. Das, P. Pattnaik, G. Murjani, B.K. Nayak, Dot–enzyme-linked immunosorbent assay (ELISA) for the diagnosis of *Edwardsiella tarda* infection in fish, Asian Fish Sci. 14 (2001) 89–93.
[3] G. Kumar, G. Rathore, U. Sengupta, D. Kapoor, W.S. Lakra, Production of monoclonal antibodies specific to major outer membrane protein of *Edwardsiella tarda*, Comp. Immunol. Microbiol. Infect. Dis. 33 (2010) 133–144.
[4] S.R. Hong, S.J. Choi, H.D. Jeong, S. Hong, Development of QCM biosensor to detect a marine derived pathogenic bacteria *Edwardsiella tarda* using a novel immobilisation method, Biosens. Bioelectron. 24 (2009) 1635–1640.
[5] J. Knurr, O. Benedek, J. Heslop, R.B. Vinson, J.A. Boydston, J. McAndrew, J.F. Kearney, C.L. Turnbough Jr., Peptide ligands that bind selectively to spores of *Bacillus subtilis* and closely related species, Appl. Environ. Microbiol. 69 (2003) 6841–6847.
[6] D.D. Williams, O. Benedek, C.L. Turnbough Jr., Species-specific peptide ligands for the detection of *Bacillus anthracis* spores, Appl. Environ. Microbiol. 69 (2003) 6288–6293.
[7] J. Brigati, D.D. Williams, I.B. Sorokulova, V. Nanduri, I.H. Chen, C.L. Turnbough Jr., V.A. Petrenko, Diagnostic probes for *Bacillus anthracis* spores selected from a landscape phage library, Clin. Chem. 50 (2004) 1899–1906.
[8] S. Sainath Rao, K.V. Mohan, N. Nguyen, B. Abraham, G. Abdouleva, P. Zhang, C.D. Atreya, Peptides panned from a phage-displayed random peptide library are useful for the detection of *Bacillus anthracis* surrogates *B. cereus* 4342 and *B. anthracis* Sterne, Biochem. Biophys. Res. Commun. 395 (2010) 93–98.
[9] I.B. Sorokulova, E.V. Olsen, I.H. Chen, B. Fiebor, J.M. Barbaree, V.J. Vodyanoy, B.A. Chin, V.A. Petrenko, Landscape phage probes for *Salmonella typhimurium*, J. Microbiol. Methods 63 (2005) 55–72.
[10] S. Carnazza, C. Foti, G. Gioffrè, F. Felici, S. Guglielmino, Specific and selective probes for *Pseudomonas aeruginosa* from phage-displayed random peptide libraries, Biosens. Bioelectron. 23 (2008) 1137–1144.
[11] M. Lavilla, R. De Luis, M.D. Pérez, M. Calvo, L. Sánchez, Selection of high affine peptide ligands for detection of *Clostridium tyrobutyricum* spores, J. Microbiol. Methods 79 (2009) 214–219.
[12] S. Mun, S.J. Choi, Optimization of the hybrid bilayer membrane method for immobilization of avidin on quartz crystal microbalance, Biosens. Bioelectron. 24 (2009) 2522–2527.
[13] BioTek Instruments, Synergy HT Multi-Mode Microplate Reader. Available from: <http://www.biotek.com/products/microplate_detection/synergyht_multimode_microplate_reader.html/>.
[14] B.L. Elder, D.K. Boraker, P.M. Fives-Taylor, Whole-bacterial cell enzyme-linked immunosorbent assay for *Streptococcus sanguis* fimbrial antigens, J. Clin. Microbiol. 16 (1982) 141–144.
[15] Promega Corporation, GloMax-96 Microplate Luminometer. Available from: <http://www.promega.com/ko-kr/products/pm/fluorometers-luminometers-multimode-readers/glomax-96/>.
[16] L.M. Levine, M.L. Michener, M.V. Toth, B.C. Holwerda, Measurement of specific protease activity utilizing fluorescence polarization, Anal. Biochem. 247 (1997) 83–88.
[17] R. Buchli, R.S. VanGundy, H.D. Hickman-Miller, C.F. Giberson, W. Bardet, W.H. Hildebrand, Development and validation of a fluorescence polarization-based competitive peptide-binding assay for HLA-A*0201: a new tool for epitope discovery, Biochemistry 44 (2005) 12491–12507.
[18] P. Johnson, A.C. Thornton, The importance of life-time changes in fluorescence depolarization, Biochem. J. 115 (1969) 725–732.
[19] K. Takeda, K. Yamamoto, Fluorescence lifetime and rotational correlation time of bovine serum albumin–sodium dodecyl sulfate complex labeled with 1-dimethylaminonaphthalene-5-sulfonyl chloride: Effect of disulfide bridges in the protein on these fluorescence parameters, J. Protein Chem. 9 (1990) 17–22.
[20] X.Q. Guo, F.N. Castellano, L. Li, J.R. Lakowicz, Use of a long-lifetime Re(I) complex in fluorescence polarization immunoassays of high-molecular-weight analytes, Anal. Chem. 70 (1998) 632–637.

[21] E. Olsen, A. Vainrub, V. Vodyanoy, Acoustic wave (TSM) biosensor: weighing bacteria, in: M. Zourob, S. Elwary, A. Turner (Eds.), Principles of Bacterial Detection, Springer, New York, 2008, pp. 255–298.

[22] E.V. Olsen, S.T. Pathirana, A.M. Samoylov, J.M. Barbaree, B.A. Chin, W.C. Neely, V. Vodyanoy, Specific and selective biosensor for *Salmonella* and its detection in the environment, J. Microbiol. Methods 53 (2003) 273–285.