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Characterization and Food Application of a Potentiometric Biosensor Measuring β-Lactam Antibiotics

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Abstract β-Lactam antibiotics such as penicillin G, amoxicillin, and ampicillin were determined by a potentiometric biosensor system which exploited penicillinase immobilized on Immobilon cellulose nitrate membrane and a flat-bottomed pH electrode as the biological component and transducer. The optimum reaction buffer for maximum sensitivity was found as 2 mM of sodium phosphate buffer (pH 7.2). The detection limit of the biosensor could be extended to 1 μM of the analytes by increasing the enzyme loading for immobilization to 100 units/ml. The model samples spiked with each of the standard penicillins were measured for their biosensor responses and HPLC peak area, resulting in the relative responses of 82.1–103.5% and 79.5–106.1% for the biosensor method along with HPLC analysis, respectively. This result showed a good precision of the current biosensor method for screening the penicillin compounds.

Key words: Penicillinase, potentiometric biosensor, characterization, β-lactam antibiotics, model samples

Apart from the quantitative analytical methods for chemical contaminants possibly present in various foods [5, 11, 28, 29], the importance of rapid screening methods has been increased due to the elevated inspection demand for the quality control on foods. Hence, thin layer chromatography, *Bacillus megaterium* disc assay, enzyme-linked immunosorbent assay (ELISA), and fluorescence immunoassay (FIA), etc. as rapid screening methods for detrimental organic compounds like antibiotics have been gradually established [8]. Of these, ELISA has been reported as an effective and sensitive method for screening positive samples in broad concentration ranges [7]. This method, however, requires a detector measuring light intensity and involves complicated procedures such as binding of antigen or antibody to a

support, washing of the reaction cell, antigen-antibody complexation and washing, and substrate addition followed by the measurement of light intensity. Hence, it is basically a laboratory-oriented method that is not easily applicable to the *on-site* measurement. As an alternative, the dipstick assay format has been developed. However, it still suffers from low sensitivity and reproducibility according to the types of analyte [4].

As new screening methods for antibiotics, hormones, and pesticides, the optoelectronic, piezoelectric, and electrochemical biosensors have been developed since the beginning of the last decade. Owing to their convenience and rapidity, they are easily applicable to *on-site* analysis [16]. Mellgren *et al.* [9] compared a biosensor method based on biospecific interaction with microbiological, immunochemical, and physical methods for sulfamethazine in raw milk and concluded that the biosensor method was suited for a high-sensitivity detection in a real-time scale. A direct competitive chemiluminescent immunoenzyme biosensor using a thin layer flow cell was developed for making a nano-sensitivity detection on low molecular weight compounds [12]. Piezoelectric immunosensors for determining antibiotics like chloramphenicol [14] and pesticides such as atrazine [22] and 2,4-D [2] have also been developed in a direct-binding or competitive assay format. On the other hand, electrochemical biosensors, which encompass potentiometric and amperometric ones, and have the merits in a convenient system setup and relative inertness to interference caused by coloring substances, are expected to find applicability in the real-time measurement of antibiotics in fermentation broths [19], and in the measurements of atrazine and cyanide, along with heavy metals [4, 27].

Antibiotics, which can be translocated to various foods via many routes such as feed and injection to livestock, are important items for making a quality evaluation in the sector of food hygiene due to their side effects. For example, β-lactam antibiotics including penicillin and cephalosporin, which have a broad-action spectrum against Gram-positive

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and -negative bacteria with a high antimicrobial activity, have also been reported for their high toxicity level on humans and livestock. Of the β-lactam antibiotics, penicillin compounds represent a group of chemicals having a similar chemical structure [13]. According to the substituent groups of the β-lactam ring, natural penicillins such as penicillins G and V, and semisynthetic aminopenicillins like ampicillin and amoxicillin are reported. These compounds are detoxified by a selective hydrolytic cleavage of the β-lactam ring of the cyclic amide induced by penicillinase (β-lactamase). In this case, penicillin is converted to penicilloic acid, resulting in the formation of a carboxyl group which can easily deprotonate H^+ ions to the surrounding medium to induce a pH decrease or potential increase. By using this principle, some studies have been conducted to develop biosensors for screening purposes or for quantitative analysis. These include the development of a silicon-based pH sensor by using pulsed laser deposition technique [18, 20, 21] and of the capacitive penicillin sensor [17], in addition to the study on penicillinase immobilization by a heterobifunctional cross-linking and on the long-term stability of penicillin sensors [24].

The aim of this study was to develop a convenient biosensor system for screening penicillin compounds only with a pH/mV meter and a flat-bottomed pH electrode attached with a penicillinase membrane. The response characteristics of the resulting biosensor were analyzed and the measurements of the penicillin compounds in the spiked model samples from various cold storage foods were carried out by applying the biosensor method and HPLC for comparing the analytical results.

MATERIALS AND METHODS

Enzyme and Reagents

Penicillinase (EC 3.5.2.6, from *Bacillus cereus*) as the biological component, and penicillin G (potassium salt), amoxicillin, and ampicillin as the analytes were purchased from Sigma (MO, U.S.A.). Other reagents were guaranteed or extra-pure reagents obtained from various suppliers and used as given.

System Setup

Enzyme was immobilized onto small pieces (0.9 cm diameter) of Immobilon cellulose nitrate membrane (HAHY 107, Millipore, MA, U.S.A.) by soaking them into the penicillinase solutions of different enzyme activities (2–200 units/ml). Thus, the individual enzyme membrane that was prepared was attached to a flat-bottomed pH electrode (Istek, Korea) and the resulting sensing electrode was connected to the Orion 920A pH/mV meter (MA, U.S.A.) which was interfaced to an IBM-compatible personal computer. System operation was completed with Orion

Data Collect PC Software (version 2.0). To measure the biosensor response, the sensing electrode was first immersed into a beaker containing 18 ml of sodium phosphate buffer. After stabilization of the baseline signal, 2 ml of the analyte or the model sample spiked with a single penicillin compound was injected into the reaction cell. The changes in pH and potential (mV) upon substrate addition were automatically displayed on the PC monitor and the steady-state biosensor signal was taken to calculate the biosensor response.

Preparation of the Model Samples

Pork, beef, chicken, and milk under cold storage were purchased from a discount market in Bundang area (Korea). For the biosensor measurement, 30 g of each solid sample was added into a centrifuge tube installed with an acryl net, followed by centrifugation at 1,000 rpm in a tabletop centrifuge to prepare the corresponding meat drip. The meat drip that was obtained was filtered through a 0.45 μm syringe filter membrane to prepare the analytical sample. Milk was used as an analytical sample after the microfiltration process that was described above. The analytical sample for HPLC analysis was prepared as follows [8]. Ten g of each sample was homogenized with 25 ml of methanol, followed by centrifugation at 3,000 rpm for 20 min. The supernatant was transferred to a 250-ml flat-bottomed flask, and the residue was extracted and centrifuged again as above. The supernatant thus obtained was added to the first supernatant and the final residue was discarded. To the total supernatant, 2 ml of *n*-butanol and 20 ml of ethanol were added, followed by evaporation under a reduced pressure at 40°C. The residue was dissolved in 10 ml of sodium potassium phosphate buffer (pH 6.0). To the dissolved solution, 10 ml of ethyl ether was added and the resulting mixture was agitated for 10 min, followed by centrifugation at 3,000 rpm for 10 min. Seven ml of the water layer from the supernatant was filtered through a 0.45 μm syringe filter membrane to prepare an analytical sample. To the analytical samples of biosensor and HPLC analyses, each penicillin compound at different concentrations was spiked to prepare the model samples.

Analytical Conditions of HPLC

For comparison with the analytical results of the biosensor method, HPLC was done with a HPLC system (model PV-980) of Jasco (Japan) operated with Borwin chromatography software (Rev. 1.2150, Jasco) [23]. The column used was Kromasil C_{18} (5 μm, 4.6 mm i.d.×250 mm), maintained at 30°C, and a UV detector (model UV-975, Jasco) was used at 254 nm. Gradient elution was used in the following condition: for the eluent having a composition of methanol, 0.1 M of sodium acetate (20:80, v/v) was eluted for 10 min at a flow rate of 1.0 ml/min, after which the methanol composition was increased to 40% for 5 min. This composition was maintained up to 40 min, followed by

going back to the original eluent composition to start the next cycle of measurement. The standard solutions of the penicillin compounds were prepared by a serial dilution of the stock solutions dissolved in methanol (20 mM of penicillin G, 4 mM of amoxicillin, and 20 mM of ampicillin) and then 20 μl aliquots were injected into the HPLC system.

RESULTS AND DISCUSSION

Effects of pH and Ionic Strength on the Biosensor Responses

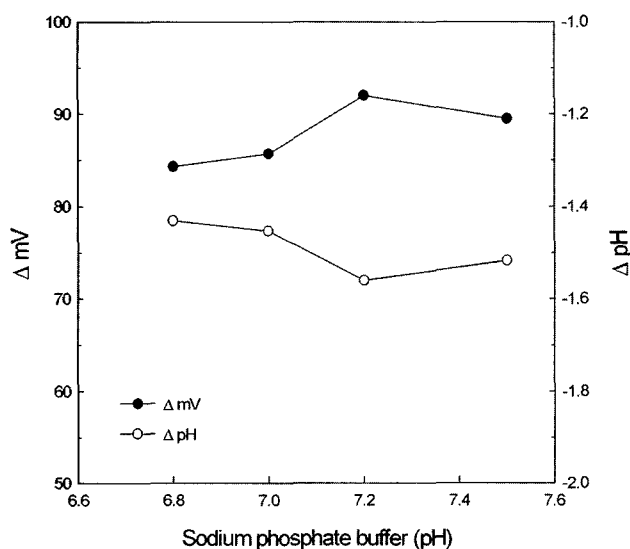It has been reported that the pH and ionic strength of a reaction buffer has a strong influence on the sensitivity of a potentiometric biosensor that measures pH or potential change [30]. Therefore, the effects of pH and ionic strength on the biosensor responses were evaluated (Fig. 1). To find out the effect of pH, sodium phosphate buffers adjusted to pH 6.8-7.5 were used. Compared with the optimum pH of 7.0 for free penicillinase, the biosensor responses in terms of pH and potential change were in the highest values at pH 7.2, amounting to ΔpH of - 1.56 and ΔmV of 92.0. This type of alkaline shift in pH optimum found in the immobilized enzyme has also been reported in the lactate and hypoxanthine sensor of previous reports [6, 15]. The ionic strength of sodium phosphate buffer (pH 7.2) also significantly affected the biosensor responses.

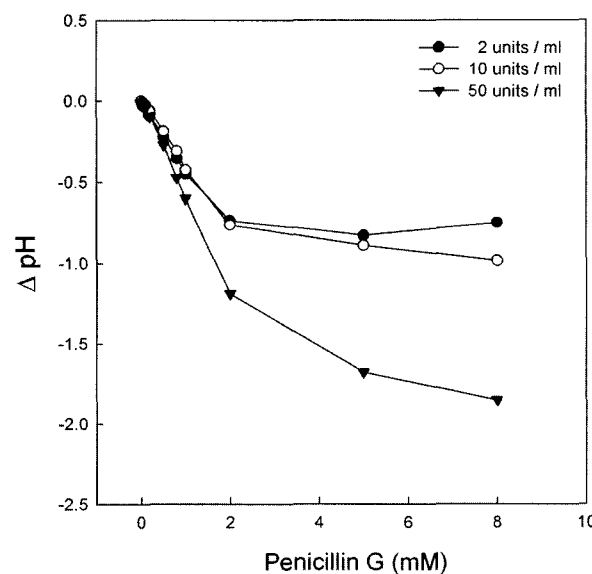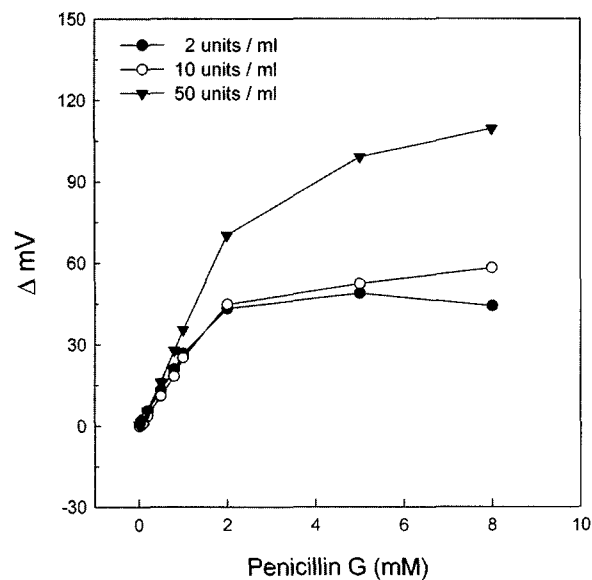Fig. 1. Effects of the pH and ionic strength of the buffer solutions on the biosensor responses.

Fig. 2. Biosensor responses at different enzyme loadings for immobilization.

When buffer concentration exceeded 50 mM, nearly no changes in pH and potential were observed during the enzyme reaction. On the contrary, the biosensor responses were conspicuous by lowering the buffer concentrations below 50 mM [28]. To improve the sensitivity of the biosensor in this study, 2 mM of sodium phosphate buffer (pH 7.2) was used as a reaction buffer for further experiments.

Effects of Enzyme Concentration for Immobilization and Substrate Concentration on the Biosensor Responses
The enzyme loading onto Immobilon cellulose nitrate membrane significantly affected the biosensor responses.

As shown in Fig. 2, the increases in enzyme loading led to the increases in pH and potential change. That is, the enzyme loading of 50 units/ml showed the highest biosensor responses at all substrate (analyte) concentrations. In this case, the potential increase and pH decrease amounted to 115 mV and 1.86, respectively, at the penicillin G concentration of 8 mM. However, no significant changes in the biosensor responses were found between the enzyme loadings of 2 and 10 units/ml. There were no conspicuous increases in the biosensor response over 4.5 mM penicillin G at all enzyme loadings.

The responses of the penicillin sensor at different substrate concentrations were measured at the enzyme loading of 50 units/ml (Fig. 3). In this case, Triton X-100
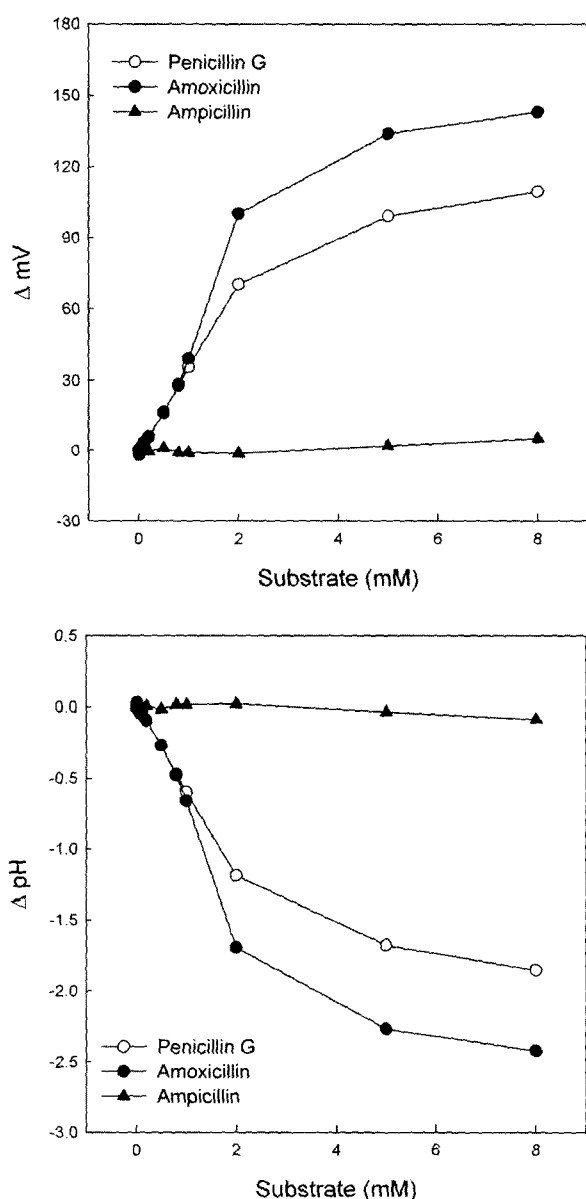

Fig. 3. Biosensor responses at different substrate concentrations. The enzyme loading was 50 units/ml.
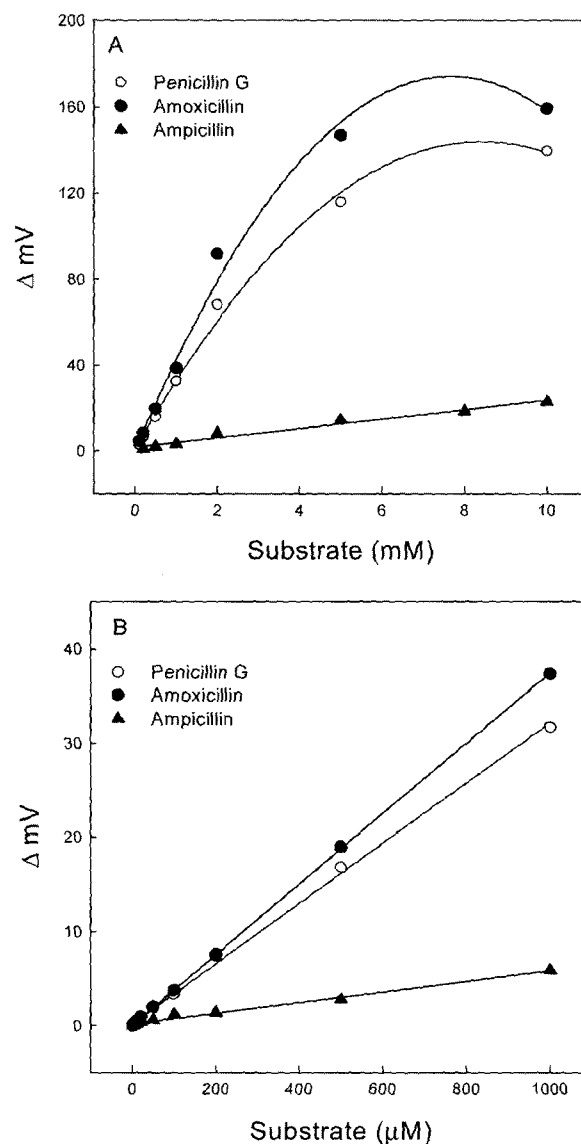

Fig. 4. Calibration curves for the penicillin compounds according to the enzyme loadings of 50 (panel A) and 100 units/ml (panel B).

was added into the reaction buffer to ensure the complete dissolution. As shown in Fig. 3, the biosensor was the most responsive to amoxicillin, followed by penicillin G and ampicillin. The maximum changes in potential and pH at this enzyme loading were 142.9 mV and - 2.42, respectively, for amoxicillin. The changes in potential and pH increased in a concentration-dependent manner up to 2 mM of amoxicillin and penicillin G. Over this concentration, however, the changes in the biosensor response per unit substrate concentration decreased gradually, which implied a saturation process of the active site of the enzyme with these substrates [3, 19, 25]. Nearly no saturation of the enzyme was observed with up to 8 mM ampicillin, which implies that it is a relatively poor substrate with low binding affinity.

Calibration Curves and Reproducibility for the Penicillin Compounds

The calibration curves for the penicillin compounds are shown in the presence of 50 units/ml of the enzyme (Fig. 4, panel A). In all analytes, good correlations were found (r^2 for penicillin G and amoxicillin was 0.9953 and 0.9913, respectively, and r for ampicillin was 0.9746). However, it was impossible to lower the detection limit below 100 μM for the individual substrate and this value was considerably higher than 0.4- 1.3 ppm which is regarded as a reference value suitable for the positive decision-making on the presence of antibiotics in urine or serum of livestock in the screening test [10]. Hence, it was strongly required to decrease the detection limit of the current biosensor. For this purpose, the enzyme loadings for immobilization were increased to 100 and 200 units/ml. As described in Table 1, the biosensor responses occurred in proportion to the enzyme loadings at low substrate concentrations of 10 and 20 μM. However, the increase in the ratios of response decreased abruptly when the analyte concentrations exceeded 20 μM. From this result, it was evident that the amount of enzyme

loadings significantly affected the biosensor response at low analyte concentrations. This fact also suggests that an extension of the detection limit is possible by increasing the enzyme loading. Hence, the enzyme immobilization onto Immobilon cellulose nitrate membrane was done with 100 units/ml penicillinase and the biosensor responses were measured at different substrate concentrations. As shown in the panel B of Fig. 4, linear calibration curves (r for penicillin G, amoxicillin, and ampicillin was 0.9985, 0.9999 and 0.9878, respectively) were obtained up to the analyte concentrations of 1,000 μM and the detection limit of 1 μM was attainable. This value was found to meet the above criterion and was quite favorable when considering the measuring range of 0.01- 3 mM for the penicillin sensors of previous reports [1, 19]. Compared with the indirect detections, such as colorimetric detection after performing a suitable color reaction and iodometric detection based on the consumption of iodine by penicilloic acid, the direct detection on the changes in pH and potential by the current biosensor seems to have some advantages in convenience, rapidity, and simplicity. This method, however, might have some disadvantages in terms of selectivity and susceptibility to the environmental conditions such as ionic strength of the applied buffer system [26].

The reproducibility of the penicillin sensor was estimated by calculating the coefficient of variability (CV, SD/ Mean×100, %) after 7 measurements were repeated with an immobilized membrane. The CVs for 1 mM penicillin G, 0.5 mM amoxicillin, and 4 mM ampicillin were 2.28, 3.40, and 6.20%, respectively. These values seem to be excellent considering that the biosensor might be used for screening of the penicillin compounds in various food samples.
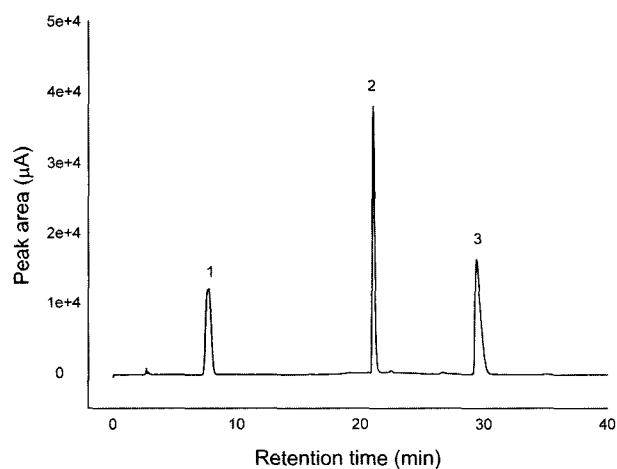
Table 1. Effects of the enzyme loadings for immobilization on the responses of the penicillin sensor.

Penicillin G (μM)	Biosensor response (%)[a]		
	Enzyme loading (units/ml)		
	50	100	200
10	100.0	200.0	350.0
20	100.0	166.7	266.7
50	100.0	118.8	118.8
100	100.0	126.5	130.2
200	100.0	125.4	142.4
500	100.0	113.5	127.0
1000	100.0	107.1	133.8

[a]The biosensor responses at the enzyme loading of 50 units/ml were arbitrarily taken as 100%.

Fig. 5. HPLC chromatograms of amoxicillin (1), ampicillin (2), and penicillin G (3).

The injection concentrations at the retention time for peaks 1, 2, and 3 were 1 mM at 7.7 min, 5 mM at 21.0 min, and 5 mM at 29.3 min, respectively.
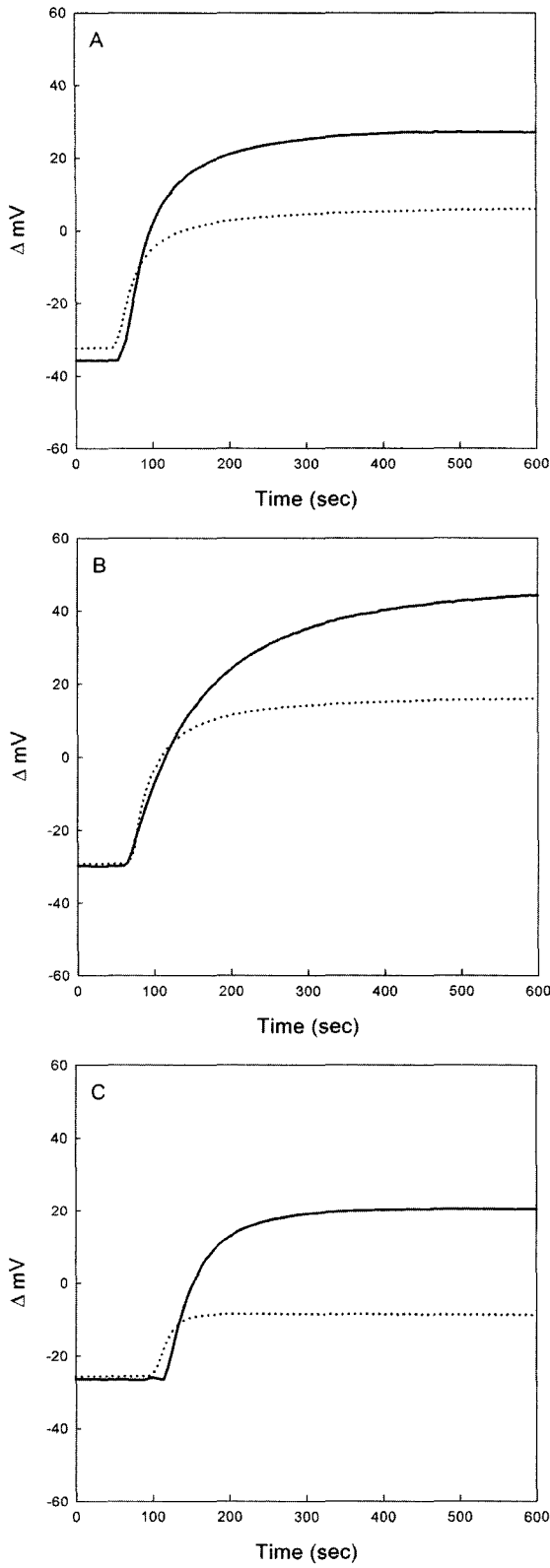
Fig. 6. Response profiles of the analytical samples unspiked and spiked with penicillin G.

Panels A, B, and C represent pork, beef, and chicken, respectively. ...., analytical samples without spiking; —, model samples.
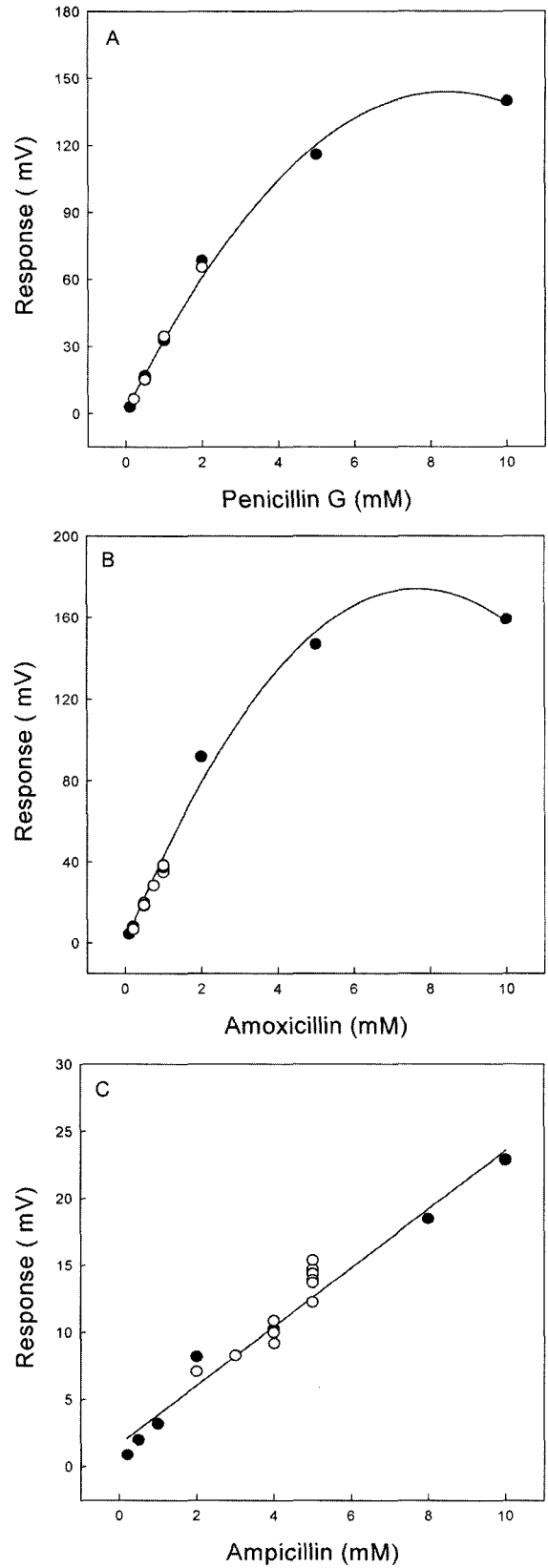
Fig. 7. Comparison of the biosensor responses for the standard samples (●) and model samples (○).

HPLC Analysis of the Penicillin Compounds

Figure 5 shows the HPLC chromatograms of amoxicillin, ampicillin, and penicillin G with the corresponding retention times of 7.7, 21.0, and 29.3 min, respectively.

The penicillin compounds of this study were not found in the analytical samples prepared from pork, etc. when HPLC analysis was completed (data not shown). Therefore, the model samples spiked with predetermined concentrations of each of them were prepared, followed by the measurements of the biosensor responses and HPLC peak area to compare the analytical results.

Analysis of the Model Samples by the Biosensor

Figure 6 represents the response profiles of the analytical samples that were unspiked and spiked with penicillin G in 1 mM, which showed a matrix effect. This effect might be diminished when a buffer solution of higher ionic strength is used. However, in this case, the sensitivity of the biosensor drastically decreased owing to its innate buffering capacity [30]. Therefore, it was necessary to use a low ionic strength buffer and to eliminate the matrix effect simultaneously in making use of a control run. As shown in Fig. 6, the potential change occurred more significantly when the model samples were injected into the biosensor system. Hence, the biosensor responses were regarded as the steady-state potential changes obtained with the model samples minus those with the analytical samples.

Figure 7 shows the comparison of the biosensor responses obtained with the standard and model samples at different analyte concentrations. The response of the model samples converged quite well to the calibration curves of the standard samples in the case of penicillin G and amoxicillin (panels A and B of Fig. 7). Although not so significant, a small deviation compared with the standard samples was found in the ampicillin case (panel C of Fig. 7). To show this result quantitatively, the ratios of the biosensor responses of the model samples against those of the standard samples are described (Table 2). The relative responses at different analyte concentrations were 95.5–103.5, 82.1–96.2, and 84.4–100.7%, respectively, in the case of penicillin G, amoxicillin, and ampicillin. This fact implied that the biosensor could measure the penicillin compounds in the model samples nearly to the concentrations of spiking.

Analysis of the Model Samples by the Comparative HPLC

When the HPLC chromatograms of the model samples spiked with the penicillin compounds were compared with those of the standard samples, the peaks of penicillin G, amoxicillin, and ampicillin were found in both cases and matched correctly in the chromatograms (data not shown). Figure 8 shows the comparison of the HPLC peak area obtained with the standard and model samples at different
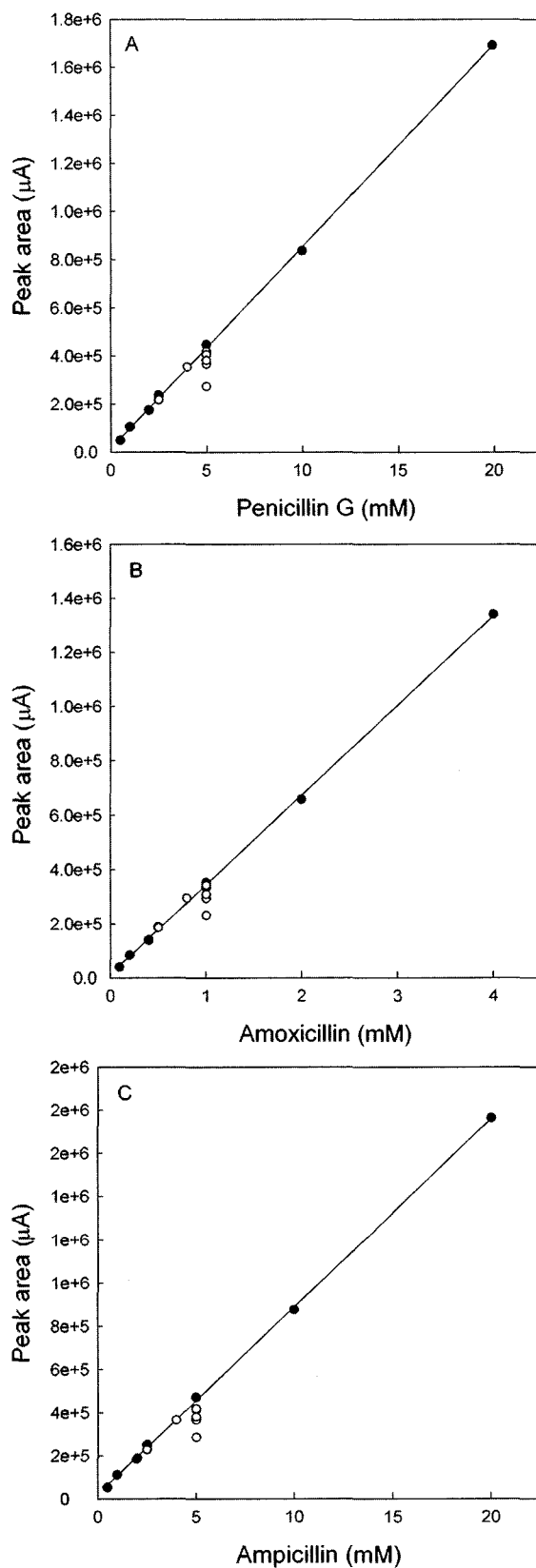

Fig. 8. Comparison of the HPLC peak area for the standard samples (●) and model samples (○).

Table 2. Comparison of the relative responses for the penicillin compounds obtained by the biosensor method and HPLC.

Substrate	Biosensor method		HPLC	
	Spiked concentration (mM)	Relative response (%)[a]	Spiked concentration (mM)	Relative response (%)[a]
Penicillin G	0.2	98.6	2.5	92.4
	0.5	99.2	4.0	101.4
	1.0	103.5	5.0	84.0
	2.0	95.5		
Amoxicillin	0.2	82.1	0.5	98.5
	0.5	96.2	0.8	106.1
	0.75	87.4	1.0	86.1
	1.0	96.1		
Ampicillin	2.0	84.4	2.5	91.2
	3.0	100.7	4.0	99.1
	4.0	94.4	5.0	79.5
	5.0	97.1		

[a]Percentage of the responses of the model samples against those of the standard samples.

analyte concentrations. Like the case of the biosensor method, the peak area of the model samples also converged to the calibration curves of the standard samples. The degree of convergence found in the HPLC analysis seemed to be as comparable as that of the biosensor method. As shown in Table 2, the relative responses in the HPLC analysis at different concentrations of penicillin G, amoxicillin, and ampicillin were 84.0–101.4, 86.1–106.1, and 79.5–99.1%, respectively. Judged from the results of Figs. 7 and 8, and Table 2, the potentiometric penicillin sensor seemed to have both sensitivity and precision as a rapid screening method for the penicillin compounds.

Acknowledgments

REFERENCES

1. Cullen, L. F., J. F. Rusling, A. Schleifer, and G. J. Papariello. 1974. Improved penicillin selective enzyme electrode. *Anal. Chem.* **46:** 1955–1961.
2. Halamek, J., M. Hepel, and P. Skladal. 2001. Investigation of highly sensitive piezoelectric immunosensors for 2,4-dichlorophenoxyacetic acid. *Biosens. Bioelectron.* **16:** 253–260.
3. Höbel, W., A. Papperger, and J. Polster. 1992. Penicillinase optodes: Substrate determinations using batch, continuous flow and flow injection analysis operation conditions. *Biosens. Bioelectron.* **7:** 549–557.
4. Keay, R. W. and C. J. McNeil. 1998. Separation-free electrochemical immunosensor for rapid determination of atrazine. *Biosens. Bioelectron.* **13:** 963–970.

5. Kijk. P. J. 1994. Confirmation of chloramphenicol residues in bovine milk by gas chromatography/mass spectrometry. *J. AOAC Intl.* **77:** 34–40.
6. Kim, N., R. Haginoya, and I. Karube. 1996. Characterization and food application of an amperometric needle-type L-lactate sensor. *J. Food Sci.* **61:** 286–290.
7. Kolosova, A. Y., J. V. Samsonova, and A. M. Egorov. 2000. Competitive ELISA of chloramphenicol: Influence of immunoreagent structure and application of the method for the inspection of food of animal origin. *Food Agric. Immunol.* **12:** 115–125.
8. Korea Food and Drug Administration. 2000. Test methods for residual compounds in the meat-processed foods. *KFDA Notice 2000-18,* pp. 326–388.
9. Mellgren, C., A. Sternesjo, P. Hammer, G. Suhren, L. Bjorck, and W. Heeschen. 1996. Comparisons of biosensor, microbiological, immunochemical, and physical methods for detection of sulfamethazine residues in raw milk. *J. Food Prot.* **59:** 1223–1226.
10. Ministry of Agriculture and Fisheries. 2001. Indication of residual analysis in meats. *MAF Notice 2001-5.*
11. Norma, P., G. Rey, N. Mario, D. Gilberto, L. Hectot, E. Irma, and M. Zenaida. 2002. Liquid chromatographic determination of multiple sulfonamides, nitrofurans and chloramphenicol residues in pasteurized milk. *J. AOAC Intl.* **85:** 20–24.
12. Osipov, A. P., N. V. Zaitseva, and A. M. Egorov. 1996. Chemiluminescent immunoenzyme biosensor with a thin-layer flow-through cell. Application for study of a real-time biomolecular antigen-antibody interaction. *Biosens. Bioelectron.* **11:** 881–887.
13. Pam, L., L. S. Rosa, and T. M. Mark. 1996. Electrochemiluminescence-based detection of β-lactam antibiotics and β-lactamases. *Anal. Chem.* **68:** 2426–2431.
14. Park, I.-S., D.-K. Kim, N. Adanyi, M. Varadi, and N. Kim. 2004. Development of a chloramphenicol sensor based on thiol or sulfide mediated self-assembled antibody monolayers. *Biosens. Bioelectron.* **19:** 667–674.
15. Park, I.-S., N. Kim, B.-S. Noh, and B.-D. Choi. 2002. Characteristics of FIA-type hypoxanthine sensors prepared

by some immobilization methods. *Food Sci. Biotechnol.* **11:** 122–126.

16. Park, S.-H., D.-H. Lee, K.-H. Oh, K. Lee, and C.-K. Kim. 2002. Detection of aromatic pollutants by bacterial biosensors bearing gene fusions constructed with the *dnaK* promoter of *Pseudomonas* sp. DJ-12. *J. Microbiol. Biotechnol.* **12:** 417–422.

17. Poghossian, A., M. Thust, M. J. Schoning, M. Muller-Veggian, P. Kordos, and H. Luth. 2000. Cross-sensitivity of a capacitive penicillin sensor combined with a diffusion barrier. *Sens. Actuat. B* **68:** 260–265.

18. Poghossian, A., M. Thust, P. Schroth, A. Steffen, H. Luth, and M. J. Schoning. 2001. Penicillin detection by means of silicon-based field-effect structures. *Sens. Mater.* **13:** 207–223.

19. Rusling, J. F., G. H. Luttrell, L. F. Cullen, and G. J. Papariello. 1976. Immobilized enzyme-based flowing-stream analyzer for measurement of penicillin in fermentation broths. *Anal. Chem.* **48:** 1211–1215.

20. Schoning, M. J., L. Beckers, A. Schaub, W. Zander, J. Schubert, S. Mesters, P. Kordos, and H. Luth. 1996. A novel silicon-based pH sensor prepared by pulsed laser deposition technique, pp. 781–784. *In* R. Puers (ed.), *Proc. Eurosensors X (Vol. 3).* Leuven 8-11 September.

21. Schoning, M. J., D. Tsarouchas, A. Schaub, L. Beckers, W. Zander, J. Schubert, P. Kordos, and H. Luth. 1996. A highly long-term stable silicon-based pH sensor using pulsed laser deposition technique. *Sens. Actuat. B* **35:** 228–233.

22. Steegborn, C. and P. Skladal. 1997. Construction and characterization of the direct piezoelectric immunosensor for atrazine operating in solution. *Biosens. Bioelectron.* **12:** 19–27.

23. Supelco. 2003. Technical Library of Supelco, Chromatograms/Analytical Techniques/Liquid Chromatography/Chloramphenicol (http://www.sigmaaldrich.com).

24. Thust, M., M. J. Schoning, J. Vetter, P. Kordos, and H. Luth. 1996. A long-term stable penicillin-sensitive potentiometric biosensor with enzyme immobilized by heterobifunctional crosslinking. *Anal. Chim. Acta* **323:** 115–121.

25. Tran-Minh, C. 1996. Biosensors in flow-injection systems for biomedical analysis, process and environmental monitoring. *J. Mol. Recog.* **9:** 658–663.

26. Van Opstal, M. A. J., R. Wolster, J. S. Blauw, P. C. Van Krimpen, W. P. Van Bennekom, and A. Bult. 1990. Determination of penicillin in pharmaceutical formulations by flow injection analysis using an optimised immobilized penicillinase reactor and iodometric detection. *J. Pharm. Biomed. Anal.* **8:** 49–60.

27. Volotovskky, V. and N. Kim. 2003. Ion-sensitive field effect transistor-based multi-enzyme sensor for alternative detection of mercury ions, cyanide and pesticide. *J. Microbiol. Biotechnol.* **13:** 373–377.

28. Waley, S. G. 1992. *Chemistry of β-Lactams*, pp. 198-228. Blackie Academic and Professional, New York, U.S.A.

29. Water, C. V. and N. Haagsma. 1991. Analysis of chloramphenicol residues in swine tissues and milk: Comparative study using different screening and quantitative methods. *J. Chromatogr.* **566:** 173–185.

30. Xie, X., A. A. Suleiman, and G. G. Guilbault. 1992. A fluorescence-based fiber optic biosensor for the flow-injection analysis of penicillin. *Biotechnol. Bioeng.* **39:** 1147–1150.