Biosensors and Bioelectronics 49 (2013) 99–104

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel thermometric biosensor for fast surveillance of β-lactamase activity in milk

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ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form
12 April 2013
Accepted 2 May 2013
Available online 9 May 2013

Keywords:
β-lactamase
Antibiotics
Milk
Enzyme thermistor
Enzyme immobilization

ABSTRACT

Regulatory restrictions on antibiotic residues in dairy products have resulted in the illegal addition of β-lactamase to lower antibiotic levels in milk in China. Here we demonstrate a fast, sensitive and convenient method based on enzyme thermistor (ET) for the surveillance of β-lactamase in milk. A fixed amount of penicillin G, which is a specific substrate of β-lactamase, was incubated with the milk sample, and an aliquot of the mixture was directly injected into the ET system to give a temperature change corresponding to the remained penicillin G. The amount of β-lactamase present in sample was deduced by the penicillin G consumed during incubation. This method was successfully applied to quantify β-lactamase in milk with the linear range of 1.1–20 U mL^{-1} and the detection limit of 1.1 U mL^{-1}. The recoveries ranged from 93% to 105%, with relative standard deviations (RSDs) below 8%. The stability of the column equipped in ET was also studied, and only 5% decrease of activity was observed after 60 days of use. Compared with the conventional culture-based assay, the advantages of high throughput, timesaving and accurate quantification have made this method an ideal alternative for routine use.

1. Introduction

In the dairy industry, β-lactam antibiotics are widely used as veterinary medicine and even dietary supplement to improve the milk yield. But their overuses often lead to antibiotic contamination in milk, which may break the limitation of food safety standard. In order to pass the inspection before entering the market, β-lactamases (EC 3.5.2.6, BLA) that can specifically catalyze the hydrolytic degradation of β-lactam antibiotics have been used to destruct the antibiotic residue since 1980s (Koryckadahl et al., 1985). However, this illegal practice brings new risk to consumers' health. First, β-lactamases could reduce the efficacy of patients undergoing antibiotic therapy, as well as increase the general risk of antibiotic resistance (Kantiani et al., 2009; Pengov and Kirbis, 2009). Second, addition of β-lactamases would make it possible to conceal and thus encourage the overuse of antibiotics during milk production, storage and transport. Last, there is a concern that inadequately purified β-lactamases commonly used in dairy industry may contain bacterial DNA which would expose consumers to another uncertain threat. Therefore, estimation of β-lactamases in milk is of great significance.

Despite of the same capability of catalyzing the hydrolytic degradation of the amide bond present in the four-membered ring of β-lactam antibiotics (Abraham and Chain, 1940), β-lactamases can be classified into four classes based on their molecular structure or function (Bush and Jacoby, 2010). The molecular classification system divides β-lactamases according to the amino acid sequence; while the functional classification takes into account their substrate and inhibitor specificity profiles. Several conventional methods have been developed for β-lactamase analysis, i.e. immunoassay (Chambers et al., 2001; Hujer et al., 2002, 2009), spectrophotometric detection (Gao et al., 2003; Perret, 1954; Xing et al., 2005), and culture-based testing (Cui et al., 2007; Goyal et al., 2008; Livermore and Yuan, 1996). These techniques are sensitive and reliable, but the disadvantages of requiring laborious and time-consuming manipulation limit their use in routine high-throughput screening. Improved methods, such as chromogenic substrate assays (Jorgensen et al., 1982; O'Callaghan et al., 1972), electrochemiluminescence (Liang et al., 1996), HPLC (Kunied et al., 1993), and MALDI-FTMS Spectrometry (Xu et al., 2010), have been reported. And more recently, sensors based on biocompatible hydrogels (Yang et al., 2007, 2008), gold nanoparticles (Liu et al., 2007), and oligonucleotide microarrays (Rubtsova et al., 2010) have also been proposed. High sensitivity and specificity were achieved by these strategies. However, most of them rely on complex equipment and skilled workers, which result in high inspection cost. As a result, a simple, fast, inexpensive, and reliable system for detecting β-lactamase in milk which can meet the

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http://dx.doi.org/10.1016/j.bios.2013.05.005

high-throughput testing requirements of the dairy industry is highly desirable.

The enzyme thermistor (ET) is a flow-injection calorimetric biosensor that directly detects the heat evolved during enzyme-catalyzed reaction (Danielsson and Mosbach, 1987, 1988; Danielsson et al., 1981; Ramanathan and Danielsson, 2001). Its universal detection principle permits a label-free strategy which simplifies the assay and instrument design. Furthermore, the advantage of non-contact measurement decreases signal interference and dispels the risk of sensor contamination, and hence significantly improves operational stability and reduces the recalibration rate. Numerous sensing applications have been developed based on ET, including enzyme activity determinations (Torabi et al., 1999; Xie et al., 1994, 1997), clinical metabolite monitoring (Harborn et al., 1997; Ramanathan et al., 2001), tracking fermentation processes (Mandenius et al., 1981; Rank and Danielsson, 1992), environmental analysis (Borrebaeck and Mattiasson, 1979; Pirvutoiu et al., 2001; Preininger and Danielsson, 1996) and antibiotics (Decristoforo and Danielsson, 1984; Lawung et al., 2001). Specially, it has also been applied in the detection of urea and lactate in adulterated milk (Chen et al., 2011; Mishra et al., 2010).

Here we present a new screening method for the determination of β-lactamases in milk by monitoring the conversion of penicillin G using ET biosensor. Penicillin G added as tracer is specifically hydrolyzed by the β-lactamase, thus the amount of β-lactamase in milk could be deduced by determining the penicillin G consumed during incubation. Due to the complexity of milk samples, a number of modifications are applied to the ET device to improve signal stability and simplify sample pretreatments, and excellent detection limit of 1.1 U mL^{-1} and high throughput of 5 min per injection have been achieved, which make the proposed biosensor a rapid, sensitive, and highly stable detection system that can facilitate continuous analysis of milk β-lactamase in dairy industry.

2. Experimental section

2.1. General principle of the procedure

The enzymatic reaction involved in the process is shown in Fig. 1, where ΔH is the enthalpy change of the reaction. This reaction took place in both the incubation step and ET analysis step, as shown in the principle diagram. During the incubation, β-lactamase present in the test sample catalyzed the degradation of the added penicillin G, which caused a decrease to the penicillin G concentration. And then, the mixture was injected into the ET system to determine the remained penicillin G after incubation. The ET system was equipped with an enzyme column, where a large amount of β-lactamase was pre-immobilized to realize a fast and complete conversion of the penicillin G flowing through, hence a stable temperature signal response and accurate quantification. The β-lactamase activity in milk is positively correlated with the consumption of penicillin G during incubation, thus can be calculated from the original penicillin G added and the remained penicillin G determined by ET.

2.2. Instrument setup and operation

The thermometric detection system consisted of an HPLC pump (Waters 510, USA), a six-port injection valve (Type 50 from Rheodyne, Cotati, CA, USA), an ET unit (Omik Bioscience, Lund, Sweden) and a computer for recording and analyzing the data (Fig. S1, in Supplementary file). The ET unit was constructed and operated as previously described (Ramanathan and Danielsson, 2001). Briefly, an enzyme column and a reference column were mounted in an aluminum block insulated with polyurethane foam in order to minimize the effects of external temperature fluctuations. The operating temperature was set to 30 °C and the columns were allowed to equilibrate with the carrier buffer of 0.1 M phosphate buffer, pH 7.0. The flow rate and the injection volume were optimized to be 1.0 mL min^{-1} and 350 μL, respectively. With this system, specific catalysis reaction would take place in the enzyme column when a substrate solution flows by. And the resulted temperature rise was immediately recorded by a thermal probe equipped at the exit of enzyme column, with a sensitivity of 10^{-5} °C (Danielsson and Mosbach, 1988).

2.3. Reagents and materials

Penicillin G (Potassium salt, 1500–1750 U mg^{-1}) of USP grade and β-lactamase used for immobilization in enzyme column were purchased from Sigma Chemical Co. (USA). The β-lactamase standard (4460 kU mL^{-1}) used to prepare calibration solutions and spiked samples was acquired from National Institutes for Food and Drug Control (China). The deionized water was collected from a Milli-Q water purification system (Millipore Corp., Bedford, MA). All other chemicals were of analytical reagent grade or better. Trisoperl controlled pore glass (CPG) beads (125–140 μm particle diameter and 50 nm pore size) were bought from VitraBio (Germany). The serial dilutions of β-lactamase standard and penicillin G were prepared freshly every day, with 0.1 M phosphate buffer, pH 7.0.

2.4. β-lactamase immobilization

The β-lactamase (~5000 U) was covalently immobilized on CPG beads using a glutaraldehyde coupling scheme as follows: 500 mg of CPG was incubated with a 2.5% glutaraldehyde solution prepared in phosphate buffer (0.1 M, pH 7.0 unless otherwise indicated) for 1 h under reduced pressure and then at normal pressure for another hour. After thoroughly washed with deionized water, the brick-red colored activated CPG was immediately mixed with β-lactamase (6 mg dissolved in 20 mL) in phosphate buffer. The coupling was allowed to proceed overnight at 4 °C under mild agitation. The CPG was then washed with phosphate buffer, and subsequently incubated with a lysine solution (5 mg mL^{-1}) to block all the unreacted aldehyde groups. Finally, the immobilized enzyme preparation was washed exhaustively with phosphate buffer before being packed into the columns. When not in use the enzyme activated CPG was stored in 75% ethanol at 4 °C.

2.5. Matrix-matched calibration curve

The spiked calibration solutions were prepared using milk product that contained no β-lactamase. Freshly prepared β-lactamase standards were pipetted to the blank milk samples to obtain a serial concentration of 0.0, 4.0, 6.0, 10.0, 14.0, 18.0 and 20.0 U mL^{-1}. Then, 20 μL of 10 mM penicillin G solution was added to 2 mL of each solution, followed by incubated at 25 °C for 3 h under mild agitation. The calibration preparations were injected into the ET without any treatment both before and after incubation.

2.6. Sample preparation

All the dairy product samples (57 liquid milk products) were bought locally. Each milk sample was analyzed using a subtraction method. In detail, three test tubes were labeled as A, B and C. In each tube, 2 mL of milk sample was added and treated according to the label as follows: (A) Mixing 2 mL sample with 20 μL

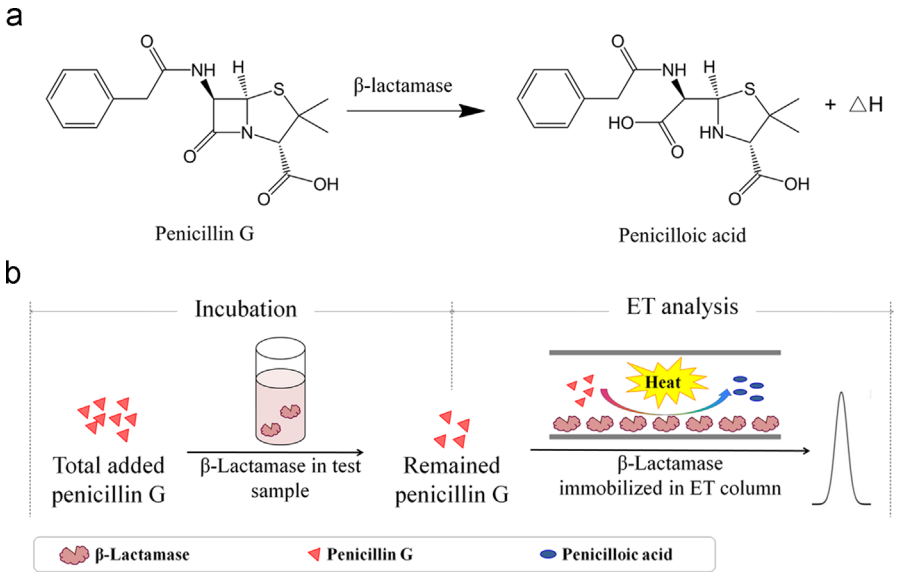

Fig. 1. Schematics of (a) the hydrolytic degradation of the four-membered β-lactam ring in penicillin G catalyzed by β-lactamase and (b) the principle of whole analysis procedure.

phosphate buffer and then immediately analyzed, (B) mixing 2 mL sample with 20 μL of 10 mM penicillin G standard solution and then immediately analyzed and (C) mixing 2 mL sample with 20 μL of 10 mM penicillin G standard solution, followed by a 3 h incubation prior to being analyzed. The presence of β-lactamase was defined as a difference between the response signals of (B) and (C), while (A) showed the sample background.

3. Results and discussion

3.1. Assay optimization

The effects of pH, running temperature, flow rate and sample volume on the sensitivity, accuracy and linear range of the β-lactamase assay were investigated. The ET assay performed satisfactorily in the pH range of 6.0–7.0. However, pH of 7.0 was chosen in order to optimize the buffering capacity of the phosphate buffer thereby minimizing sample to sample pH variation which improved assay reproducibility. A running temperature of 30 °C was chosen despite the fact that β-lactamases showed higher activity at 37 °C, because a more stable baseline was achieved at 30 °C. This is presumably due to an increase in temperature fluctuations as the difference between the running temperature and the ambient temperature increases. A series of flow rate experiments were also conducted and 1.0 mL min^{-1} was found optimum to get sharp peak signals as well as sufficient contact time between the immobilized enzyme and the substrate. The influence of injection volume was also studied (Fig. S2, in Supplementary file). The response signal increased up until the sample size reached 350 μL, after which peak tailing began to appear. Peak tailing complicates data analysis and increases assay cycle time. Thus a sample volume of 350 μL was chosen for further experiments.

Milk contains a complex mixture of fats, proteins and sugars, which gives rise to non-specific signal effects and makes analysis of milk challenging. In addition, the presence of fat and casein micelles effects sample diffusion rates and increases the risk of clogging. Typically these issues are addressed by developing complicated milk pretreatment regimes, which are even more tedious than the analytical procedure itself. In this work, we have spent considerable effort incorporating design features into the ET system so as to obviate the need for any milk sample pretreatment. This includes the adoption of flow pipelines with low nonspecific adsorption, CPG carrier particles with large diameters, and thin layer membrane frits with proper porosity. Combined these improvements have resulted in an ET system that entirely eliminates the need for any milk sample pretreatment whatsoever. As evidenced in Fig. S3 (Supplementary file), penicillin G substrate displayed comparable gradient curves in 10%, 50% and 100% milk without any sample pretreatment, and no significant matrix influence was observed. Moreover, other treatment methods were also evaluated as shown in Fig. S4 (Supplementary file). Centrifugation and filtration only resulted in a 10% decline in matrix background, thus giving very similar sensitivity (1.0–1.5 U mL^{-1}) and reproducibility (RSD less than 8%) as that without sample treatment.

However, these routine pretreatments, centrifugation, filtration and dilution, did increase column life that is of considerable practical interest. Therefore, a number of studies were performed in an effort to extend column life. The most successful of these protocols involved washing the column with a high ionic strength solution (0.1 M phosphate buffer, 0.5 M NaCl) for 10 min after every 100 injections. Using this protocol, the immobilized β-lactamase enzyme column retained full activity for a period of 30 days, and was reduced by only 5% after 60 days of operation. In addition, when to be stored, the column was thoroughly flushed with 75% ethanol for 20 min prior to being kept in 75% ethanol during storage. The high ionic strength and ethanol column treatments presumably removed non-specifically bound hydrophilic and hydrophobic material, respectively. Storing columns in ethanol eliminates bacterial growth and does not have any detectable affect on the activity of the immobilized β-lactamase.

3.2. Calibration of β-lactamase in milk

Calibration solutions spiked with 0, 4.0, 6.0, 10.0, 14.0, 18.0 and 20.0 U mL^{-1} β-lactamase were prepared in milk matrix (free of β-lactamase), and penicillin G standard was added to give a final concentration of 0.1 mM. Each solution was tested by ET both before and after 3 h incubation, and their actual sensor signals were recorded in Fig. 2a. Since the signal response was positively associated with the amount of penicillin G, all calibration solutions before incubation gave the same peak height that corresponded to the total added penicillin G. During incubation, β-lactamase present in the calibration solutions catalyzed the hydrolysis of penicillin G,

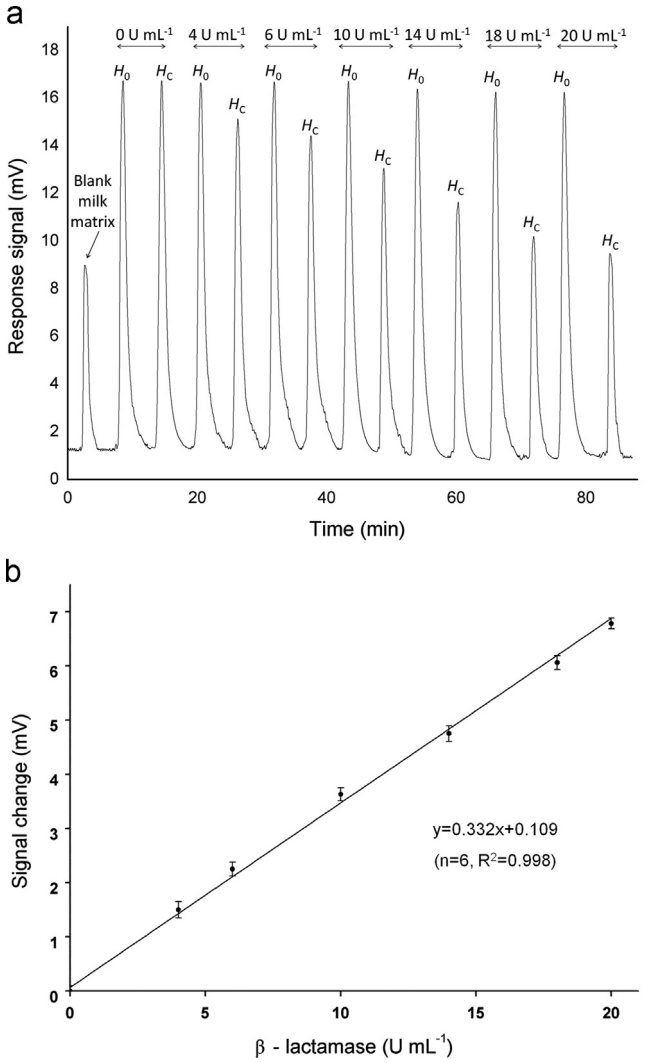

Fig. 2. Milk spiked β-lactamase calibration curve. (a) The actual signal responses for each β-lactamase concentration recorded by ET and (b) calibration curve plotted as the peak height change vs. β-lactamase concentration. H_0, peak height before incubation; H_C, peak height after incubation.

thereby resulting in a peak height decline. The blank milk matrix also generated a thermal signal, mainly ascribed to nonspecific interactions with the enzyme column. And it is notable that the peak height of the solution containing 20.0 U mL^{-1} after incubation was nearly the same as that of blank milk matrix, indicating that almost all the added penicillin G had been degraded.

The calibration curve was plotted with the peak height change vs. β-lactamase concentration as shown in Fig. 2b. The peak height change corresponding to the consumption of penicillin G during incubation was calculated using the equation

$$\Delta H = H_0 - H_C$$

where ΔH is the peak height change, H_0 is the peak height of calibration solution before incubation, and H_C is the peak height of the same solution after incubation (as labeled in Fig. 2a). A good linear response (R^2=0.998) was obtained for β-lactamase quantification in the range of 1.1–20.0 U mL^{-1} with minimum detection limit of 1.1 U mL^{-1} (3 times the blank background signal). The intra- and inter-assay coefficients of variation were less than 8.0% and 12%, respectively.

As a comparison, calibration of β-lactamase in PB was also investigated. Surprisingly β-lactamase activity using penicillin G as a substrate was much higher in milk than in buffer (Fig. S5, in Supplementary file). A concentration of 40 U mL^{-1} β-lactamase was required to hydrolyze 90% of the added penicillin G in buffer, while only 20 U mL^{-1} was required in milk. This highlights the need to use spiked milk samples for accurate calibration. It is unclear why β-lactamase has higher activity in milk, but presumably this is merely a function of the biologically friendly environment the milk matrix provides as compared to the austere environment buffer offers. Known amounts of β-lactamase were added to milk samples and measured to evaluate the recovery and precision (C.V.%) of the assay (n=6). Excellent recoveries in the range of 93–105% were obtained with C.V.% values of less than 8% (Table 1).

Table 1
The recovery and precision studies for determination of β-lactamase in milk (n=6).

β-Lactamase added (U mL^{-1})	β-Lactamase measured (U mL^{-1})	Recovery (%)	C.V. (%)
4	4.2	105	7.8
6	5.6	93	6.2
10	10.5	105	5.5
18	17.6	98	3.1
20	20.4	102	3.4
50[a]	52.1	104	4.7

[a] This sample was measured with the ET biosensor after a 5-fold dilution.

3.3. Determination of β-lactamase in real milk samples

The β-lactamase assay was employed to detect the presence of β-lactamase in commercial milk samples. The assay strategy is summarized in Fig. 3, and the detailed procedure is described in the experimental section. Each milk sample is quantified by 3 individual measurements in order to ensure test accuracy. Measurement A provides a background of the sample that is used to identify the non-specific signal components. Measurement B presents the total amount of penicillin G added in the sample. Measurement C shows the amount of penicillin G that remains in the sample after incubation. The β-lactamase concentration is determined by calculating the difference between the peak height of B (H_B) and the peak height of C (H_C), which corresponds to the penicillin G consumption during incubation. If H_C is equal to H_B, then the milk sample contains less than 1.1 U mL^{-1} β-lactamase. If H_C is less than H_B, then the milk sample contains detectable β-lactamase, the amount of which can be determined by the calibration curve. While any samples exceed the linear range, a further dilution is necessary for accurate quantification.

Using this method, 57 commercial liquid milk products collected from local market were analyzed in 7 batches with each containing 8 or 9 test samples. In each batch, a quality control sample with known amount of β-lactamase (4, 10 or 20 U mL^{-1}) was randomly inserted into the sequence and measured in order to ensure test accuracy. A portion of the actual sensor signals was presented in Fig. 4. In addition, two samples of each batch were randomly selected (14 samples in total) and evaluated with the conventional microbiological assay, and the results correlated well. Among the 57 samples, no detectable level of β-lactamase was found. This is in good agreement with another recent monitoring report (Xu et al., 2010). Since pasteurization does not visibly affect β-lactamase activity (Xu et al., 2010), it is reasonable to deduce that the illegal addition of β-lactamase is mostly eliminated in the dairy industry in China.

3.4. Comparison of ET and microbiological assays

To further validate the ET β-lactamase assay result, a comparison was made between our assay and an established microbiological assay, the modified cylinder plate method (Cui et al., 2007). This culture based method is a labor-intensive process. It defines

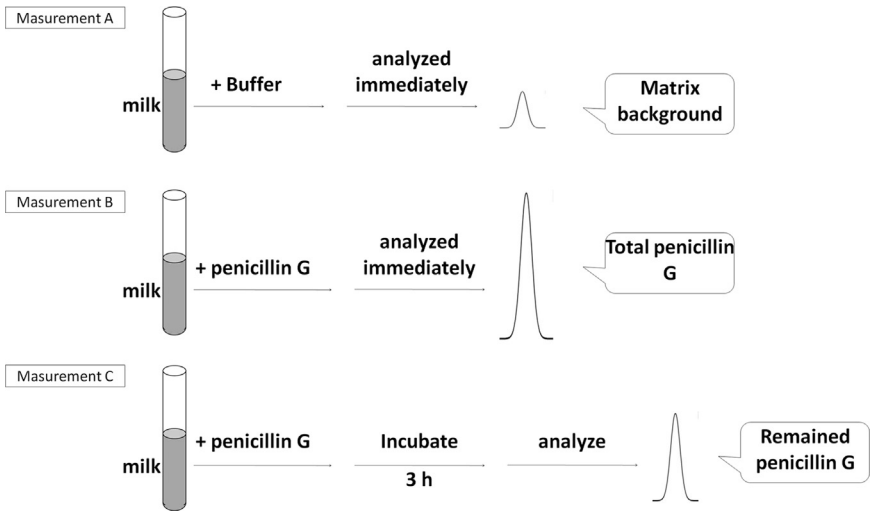

Fig. 3. Schematics of the three-measurement strategy used for determination of β-lactamase in milk products.

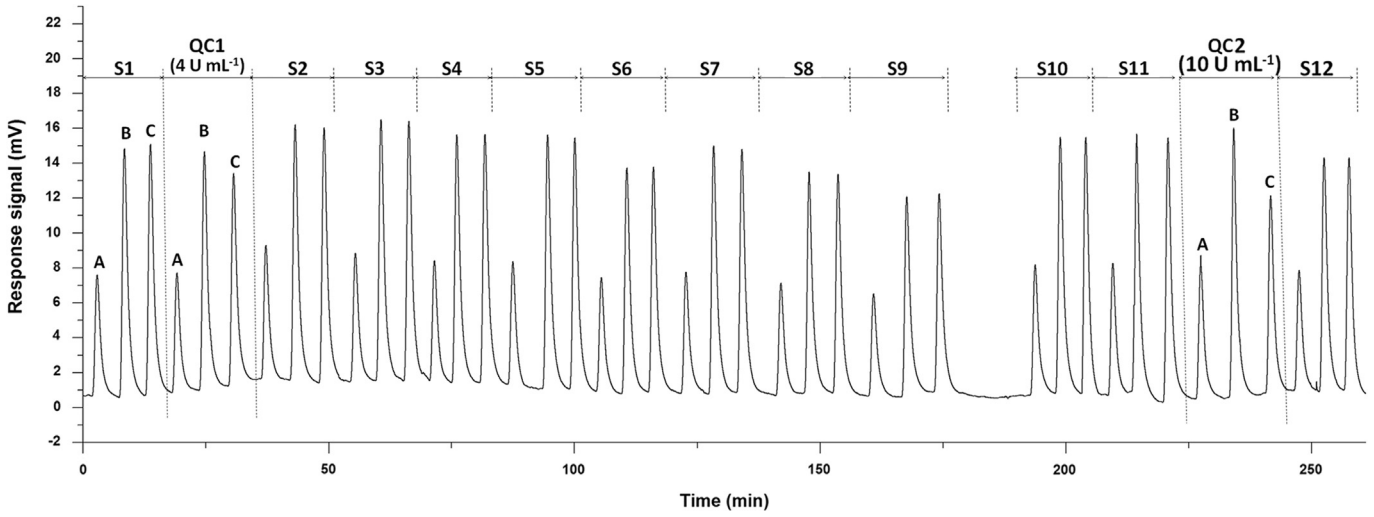

Fig. 4. The actual sensor signals of commercial milk products. Batch one (9 test samples, S1–S9, and one quality control sample, QC1) and a portion of Batch two (S10–S12 and QC2) were presented. For each sample, peak A, B, C corresponded to the matrix background, the total added penicillin G, and the remained penicillin G, respectively.

β-lactamase activity as a difference between the inhibitory zones of the test samples and control samples, and provides results roughly classified as negative (<4 U mL^{-1}), positive (4–20 U mL^{-1}) or extremely positive (>20 U mL^{-1}). Spiked milk samples containing 4, 10, 20, and 40 U mL^{-1} β-lactamase as well as a commercial milk sample were analyzed simultaneously using the ET and the cylinder plate method. The results obtained are in good agreement as presented in Table 2. The analysis time for the ET biosensor is 5 min per injection, whereas with the cylinder plate method, up to 24 h is needed for one batch of test. Moreover, a higher sensitivity is observed for ET biosensor. To sum up, the ET method has a number of advantages over the traditional culture based method including: (i) the ability to provide quantitative results, (ii) the greatly shortened assay time and (iii) easy automation for high throughput analysis.

Table 2
Comparison of results obtained from ET biosensor and microbiological assay for determination of β-lactamase in milk ($n=6$).

β-Lactamase added (U mL^{-1})	β-Lactamase measured (U mL^{-1})		Recovery (%)		C.V. (%)	
	ET	Microbiol.	ET	Microbiol.	ET	Microbiol.
0	ND	ND	–	–	–	–
4	4.1	Positive	102	–	7.5	–
10	9.8	Positive	98	–	5.8	–
20	19.5	Positive	99	–	2.1	–
40[a]	42.0	>20	105	–	4.3	–

[a] This sample was quantified with the ET biosensor after a 5-fold dilution.

4. Conclusions

In conclusion, we have proposed and demonstrated a flow injection ET biosensor for fast screening β-lactamase in milk without sample pretreatment. The method involves the following steps: addition of penicillin G, incubation, and ET analysis directly. Elimination of sample pretreatment speeds up and simplifies the assay protocol. The enzyme column equipped in ET biosensor shows a high stability, losing only 5% activity after 60 days of use. And no clogging was detected even after hundreds of injections. This method was successfully applied to determine β-lactamase in commercial milk products, providing the results in good agreement with an established culture-based assay. The reported

method is fast, sensitive, highly reproducible, cost effective, and easy automatable to provide quantitative results, making it an ideal solution for high throughput β-lactamase surveillance.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Projects 81001248).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.05.005.

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