Amperometric Detection of Staphylococcal Enterotoxin B in milk using Magnetic Bead-Based Immunoassay on Disposable Screen Printed Carbon Electrode Strip

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ABSTRACT

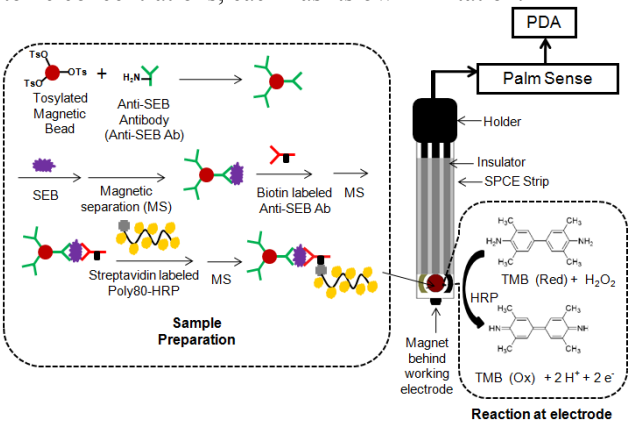
A novel approach for the sensitive detection of Staphylococcal enterotoxin B (SEB) in milk is reported. The method is based on using a sandwich enzyme-linked immunoassay with SEB specific antibodies on magnetic beads. For capture, a SEB-specific polyclonal antibody was covalently linked with tosylated magnetic beads and used to capture SEB toxin from milk samples. To produce a signal, the captured toxins were reacted with a biotin labeled SEB-specific antibody which was then conjugated with streptavidin labeled poly80 horseradish peroxidase (poly80-HRP) enzyme. The sandwiched toxin was then trapped on the surface of a screen-printed carbon electrode (SPCE) strip which had a small cylindrical magnet fixed behind the working electrode. The electro-chemical signal were generated using enhanced 3,3',5,5'-Tetramethylbenzidine (TMB) substrate at a potential +150 mV *vs*. silver (Ag) reference electrode. The estimated lowest detection of SEB toxin concentration was found to be 3 pg/ml from SEB spiked milk samples. The results were very consistent and reproduced on 10 different SPCEs and also on same SPCE, which offers future promise for the rapid, specific and cost-effective detection of toxins.

Keywords: SEB toxin, milk, Magnetic beads, Poly80-HRP, SPCE strip, and Amperometric detection.

1 INTRODUCTION

Bacterial toxins contribute to millions of food-related illnesses in the United States each year [1], especially in dairy and fruit-based products [2]. Additionally, bacterial toxins can be produced in purified form and used as deliberate food adulterants and biological weapons [3]. *Staphylococcus aureus* is a Gram-positive bacterium that produces enteric toxins, called staphylococcal enterotoxins (SEs). These toxins are a leading cause of food-borne gastroenteritis with symptoms including nausea, vomiting and diarrhea [4]. Heat processing and normal cooking temperature can inactivate or kill bacterial cells, but SE is monomeric, heat stable proteins. To date, 10 emetic SEs have been identified: A, B, C1, C2, C3, D, E, H, I, G, and J. Among these, SEA, B, C, and D are the most common food poisoning agents and SEB is categorized as a select agent of importance in biological defense [5] because of its high rate of morbidity and ease of dissemination. Therefore, rapid, sensitive and inexpensive methods are needed to detect trace levels, at or below 1 ng/ml (1 part per billion), of enterotoxins to ensure the safety of processed food.

Several immunobased methods have been reported for the detection of toxins [6] and each of these methods has been demonstrated in food matrix. Although current technologies are useful for the detection of toxins at below toxic concentrations, each has its own limitation.



Scheme 1: A schematic diagram of the apparatus and immunosensing process of magnetic bead based SPCEM immunosensor system. The immunosensing method was developed for assay preparation and used for amperometric detection of SEB toxin from milk samples, as is shown on the left panel of the diagram.

Horseradish peroxidase (HRP) is a common reporter enzyme used in commercially available ELISA test kits with 3,3',5,5'-Tetramethylbenzidine (TMB) and other a chromogenic substrates. The ability of HRP to produce electrochemical signals through TMB-mediated amperometry has allowed the development of electronic immunosensors based on similar approaches previously applied to chromogenic ELISA platforms [7]. Recent work has also shown that for the use of labeling with Poly-HRP versus HRP may be advantageous in the generation of the signals [8]. Here we present an approach to capture and quantify SEB in milk samples that combines amperometric detection with an inexpensive and disposable immunosensing strip. As illustrated in Scheme 1, we evaluated the use of poly-HRP as the reporter HRP, by labeling through use of a biotinylated antibody and

streptavidin labeled poly-HRP. The system was designed for the rapid, sensitive, and specific detection of toxins, based on the antibody-antigen sandwich model, magnetic beads covalently linked with specific antibodies, commercially available aqueous TMB substrate, and disposable SPCEs.

2 EXPERIMENTAL

2.1 Instrumentation

Cyclic voltametry and amperometric determination were performed with a potensiostat Parstat 2273 advanced electrochemical system, (AMETEK Princeton Applied Research, Oak Ridge, TN, USA) and PalmSense/Personal Digital Assistant, (Palm Instruments BV, BZ, Houten, Netherlands). An iPAQ hx2000 series pocket PC from Hewlett Packard (Palo Alto, CA) was used for data collection and calculation. SPCEs were purchased from the University of Florence (Italy). Neodymium magnets were purchased from Magcraft (Vienna, VA) and the magnetic separator (Dynal MPC®-S, Dynal MPC® 9600) from Invitrogen (Carlsbad, CA.).

2.2. Chemicals

Tosylated M-280 Dynabeads (2.8 μm in diameter) and Sheep *anti*-SEB IgG, sheep *anti*-SEB/biotin and purified SEA toxins were obtained from Toxin Technology (Sarasota, FL). Purified SEB toxin was provided by Dr. Gregory Bohach (University of Idaho, Moscow). Streptavidin/poly80 HRP conjugates were from Fitzgerald Industries International, RDI Division, Concord, MA. Milk (1% low fat) was purchased from a local retailer (commercially pasteurized). Enhanced K-Blue TMB substrate was purchased from Neogen (Lansing, MI). The buffer: Phosphate Buffered Saline (PBS) was prepared from a 10x autoclaved stock (10x PBS: 100 mM phosphate buffer, 1.37M NaCl, 27 mM KCl, pH 6.8), PBST buffer (1x PBS with 0.05% Tween-20) and PBSTB buffer (1x PBST were prepared by adding 3% bovine serum albumin (BSA) to the buffers. Deionized water (12.8 MΩ) was collected from Labconco, water Pro PS.

2.3. Preparation of SPCE with Magnet (SPCEM)

The SPCE strips used in this study consist of a graphite working electrode (diameter of 3 mm), a silver pseudo-reference electrode, and a graphite counter electrode printed on a planar polyester substrate (of thickness 450 micron) with the dimensions of 0.8 cm x 4.5 cm. A cylindrical neodymium magnet (3.0 x 3.0 mm) was fixed to the opposite side of the working electrode with epoxy to hold magnetic beads on the working electrode as shown in scheme 1.

2.4. Preparation of SEB assay in milk

Coupling of tosylated magnetic beads (MBs) with anti-SEB monoclonal antibodies and blocking with BSA were performed as described in Invitrogen protocol [9] and stored in 2 ml PBSTB (~ 3 mg MB/ml) at 4 °C prior to use. Anti-SEB antibody MBs (4 µl) were mixed with milk (100 µl) pre spiked with different concentrations of SEB and milk alone (negative control) in 300 µl PCR tubes. Each sample was mixed gently by pipeting the mixture up and down and then placing on a rotator for 40 min at room temperature (rt). The tubes were then placed into the magnetic rack, and MBs were allowed to separate for 2 min before the milk was removed and MBs were washed one time with 150 µl PBST buffer. A solution (1 µg/ml) of biotinylated *anti*-SEB polyclonal antibody in PBSTB (100 µl) was added to each sample tube and placed on rotator for 30 min. The MBs were separated and then washed with 150 µl PBST buffer (1X) and then 100 µl of streptavidin labeled poly80 HRP conjugate solution (1:10,000) of dilution was added to each sample tube. MBs were resuspended by pipeting up and down several times and assay tubes were then incubated at rt for 30 min with rotation. After incubation each tube of MBs was washed three times with 150 µl of PBST, and then resuspended to a total final volume of 10 µl of PBST.

2.5. Immunosensing Procedure

A volume of 10 µl sample (MB suspension) was applied to the SPCEM strip upon the working electrode surface. After 15 seconds, the MBs were washed with water and 60 µl of TMB solution were dropped over MBs, covering all three electrodes for the electrochemical reaction. The SPCEM strip was then incubated 30s at rt. A fixed potential of 150 mV *vs*. Ag/reference electrode was applied and amperometric experiment was performed. A mild water jet was applied on the electrodes, to remove the beads after completion of experiment and the electrodes were then washed two times with 0.1M hydrochloric acid (HCl) followed by three times with water to make ready for the next amperometric measurement. Two kinds of current signals were measured: (i) Response current (RC; the sum of the current signals collected per 0.1s during the following 60s); (ii) signal/noise (s/n) ratio of RC (ratio of average RC when SEB was present and when the SEB was absent of last 10 sec.). Each experiment was run in triplicate and data are reported as the mean current signals and ± standard deviation (S.D.).

3 RESULTS AND DISCUSSION

3.1. Voltammetric Studies of SPCE

HRP catalyzes the TMB reaction as shown in scheme 1. The enzymatic activity of this reaction can be measured by amperometric detection of the reduction in current generated by TMB (ox) at an appropriate working electrode. Electrical characteristics of the SPECM were determined by running a non-enzymatic TMB redox voltammogram in the presence of magnetic beads on the working carbon electrode. Fig. 1a shows the voltammogram of TMB in the potential range of – 350 to + 800mV; the gray line shows the voltammogram obtained in the presence of the TMB substrate with H_2O_2 and the black line shows

the voltammogram in the presence of HRP, TMB and H_2O_2. A working potential of 150 mV versus Ag and TMB substrate: 1X PBS buffer (pH 5.0) (1:1) were selected for measurement of HRP enzymatic activity. Two electron redox behaviors were observed for TMB with oxidation potential at + 200 and +400 mV. The addition of HRP (0.1 Uml^{-1}) to substrate solution resulted in the conversion of TMB(red) into TMB(ox). Consequently, decreases in oxidation potential and current were observed. These conditions were optimized for the enzymatic activity and for SPCEM.

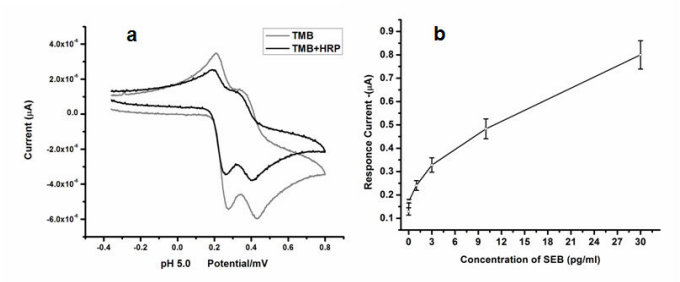


Figure 1: (a) Cyclic voltammograms (CV) of the SPCEM. The typical CV was obtained in TMB substrate in PBS buffer (1:1 v/v), which was used as a supporting electrolyte for the SPCEM. Magnetic beads were included in both scans. The gray line is the voltammogram with only the TMB solution and magnetic beads, and the black line is the voltammogram for the magnetic beads, the TMB solution and HRP. (b) Average response current between 50 – 60 sec. generated by the electrochemical reaction for detection of SEB using poly80-HRP immunoassay method.

3.2. Analysis of SEB in Milk

The milk samples were spiked with 0, 0.3, 1.0, 3.0, 10.0, 30.0 and 100.0 pgSEB / ml. The spiked samples were equilibrated for 30 min and directly analyzed following the amperometric procedure as explained in the experimental section. When the TMB/H_2O_2 substrate mixture is incubated in the presence of HRP the electrochemical oxidation of TMB (shown in scheme 1) is accomplished enzymatically. Therefore, the enzymatic reaction product, TMB(Ox) can be detected through its reduction at the surface of SPCEM. The result of above reaction was expressed in terms of response current as shown in fig. 1b. The response current value for the 0 control samples was not different for the 0.3 pg/ml sample but a clear difference is noted at 1 pg/ml of higher. Almost one and half fold difference was observed at 3 pg/ml sample as compared with 0 pg/ml control sample. A four and half fold difference was observed at 30 pg/ml sample with mean standard deviation (SD) of 6.2%. Although not included in the figure 1, we also analyzed two samples containing higher amounts of SEB (300 and 500 pg /ml in milk). These samples produced more than 6 time difference in RC. Figure 2 shows a typical response current graph, observed in our experiments. Although, during the initial 20 sec. of the electrochemical reaction. RC shows some variable value but after that it appears to stabilize and become constant. This is likely due to the formation of an equilibrium between TMB (Red) and TMB(Ox) after enzymatic reaction.

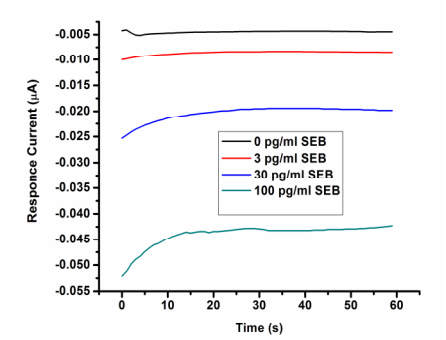


Figure 2: Typical graph of response current obtained from 0, 3, 30, and 100pg/ml SEB detection over 60 sec. The difference of current response from baseline (0 pg/ml SEB) to 3 or 30 or 100 is used as s/n ration for measuring the detection of SEB concentrations.

3.3. Specificity of SEB on immunosensing strips

Quantitative sensitivity and specificity of this assay was determined with 7 different samples. To determine the specificity of this immunoassay method, approximately 100 pg/ml samples of SEA or mouse IgG were spiked into the milk and samples were analyzed. Milk, PBSTB buffer, and magnetic beads alone were used as negative controls while magnetic beads with HRP served as a positive control (fig. 3). As shown in bar **1** in Fig. 3 the positive control and shows gave an RC of ~ -1.8376 μA while the SEB assay gave a current of ~ -1.0732 μA (Bar **5**. Fig. 3). The current obtained with milk, magnetic beads only or PBSTB buffer (Bars 2, 3 and 4 respectively) result in little or no current. SEA, and mouse IgG, (Bar 6, and 7 respectively) also gave resulted in little or no current. Indicating that there was no obvious cross- reaction of the SEB specific immunosensing strip with other protein.

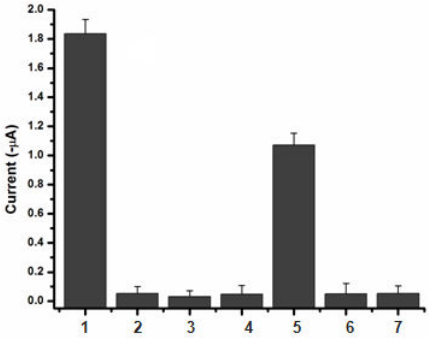
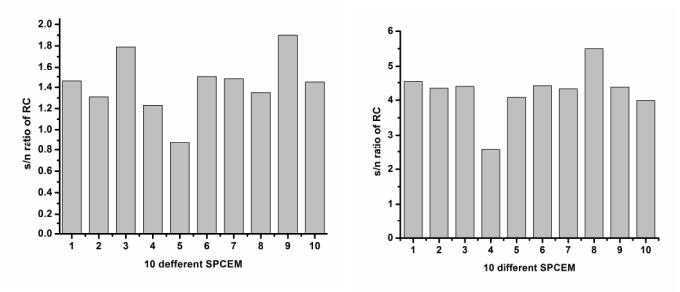


Figure 3: Specificity of SEB detection by the magnetic bead based immunoassay on SPCEM strip. **1**: HRP labeled magnetic beads (+ve control), **2**: 0 pg SEB/ml in milk, **3**: magnetic beads without *anti*-SEB antibody in buffer (-ve control), **4**: PBSTB buffer only (-ve control), **5**: 100 pg/ml SEB in milk, **6**: 100 pg/ml SEA in milk, **7**: 100 pg/ml mouse IgG in milk.

3.4. Reproducibility Test

Ten new SPCEs were prepared to check the reproducibility of our immunoassay for SEB toxin detection. Two concentrations of SEB (3.0 and 30 pg SEB/ml) in milk were selected and analyzed on different SPCEMs as reported in the experimental section. Based on the signal/noise ratio of response current as shown in Figure 4, the readings for both 3 and 30 pg/ml were relatively consistent with a reproducibility of 80%.



(a) (b)

Figure 4: Signal / noise ratio of response current obtained from 10 different SPCEMs. (a) analysis of 3 pg SEB/ml in milk and (b) 30 pg SEB/ml in milk.

4 CONCLUSION

Here, we describe a rapid, specific, and sensitive electrochemical technique for the detection of SEB in milk. The technique is based on a sandwich amperometric immunoassay. The low cost disposable SPCEM immunosensing strips and poly80 HRP were the key elements of this detection system. Amperometric detection was selected because of its many benefits, including its adaptability for use in a miniaturized and portable system. One of the important advantages of this method is that approximately 3 pg/ml of SEB in milk sample can be detected in around 2 h. This method has a potential for further applications and provides the basis for rapid and sensitive toxin detection in a food matrix.

5 ACKNOWLEDGEMENTS

The authors gratefully acknowledge the U. S. Department of Agriculture (2006-34479-17058) for financial support

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