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Food Chemistry 97 (2006) 355–360
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Chemiluminescent imaging detection of staphylococcal enterotoxin C_1 in milk and water samples

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Received 19 November 2004; received in revised form 5 May 2005; accepted 7 May 2005

Abstract

A sensitive, simple and rapid technique for high throughput simultaneous detection of staphylococcal enterotoxin C_1 (SEC_1) has been developed. The proposed method has the advantage of showing the specificity of enzyme-linked immunosorbent assays (ELISA), sensitivity of luminol-based enhanced chemiluminescence (ECL) assay, and high throughput of chemiluminescence (CL) imaging assay. It was based on a standard sandwich immunoassay format; 96-well ELISA plates were used as solid phase material. A commercial high-sensitivity cooled CCD camera has been applied to image the weak CL. Under the optimum conditions, the increased CL intensity was proportional with the concentration of SEC_1 in the range of 8.0–125.0 ng ml^{-1} and the detection limit was 0.5 ng ml^{-1} (3σ). The relative standard deviation (RSD) for eight parallel measurements of 25.0 ng ml^{-1} SEC_1 was 0.06. The proposed method has been successfully applied to the determination of SEC_1 in milk and water samples. The results obtained compared well with those by ELISA.

Keywords: SEC_1; Enhanced chemiluminescence; ELISA; Imaging

1. Introduction

Foodborne microbial diseases affect a large number of people each year. One of the most frequent diseases is gastroenteritis, which is caused by the ingestion of food contaminated with staphylococcal enterotoxins (SEs). SEs is a family of major serological types of heat stable enterotoxins (SEA through SEE and SEG through SEJ). These toxins cause toxic shock-like syndromes and have been implicated in food poisoning and several allergic and autoimmune diseases. SECs are a group of highly conserved proteins with significant immunological cross-reactivity. The three antigenically distinct SEC subtypes are SEC_1, SEC_2, and SEC_3. As low as 100 ng SEs are sufficient to cause symptoms of intoxication in humans (Balaban & Rasooly, 2001). These biological effects make the detection of these toxins very important from the standpoint of public health concerns. Some methods have been developed for the determination of SEs in different matrixes.

Liquid chromatography and mass spectrometry were traditional methods for detection of these toxins. However, these methods requiring significant sample preparation steps to eliminate interference from other components of the sample matrix (Kientz, Hulst, & Wils, 1997; Ligler et al., 2003; Rasooly & Ito, 1999). Immunoassays have been growing in popularity and acceptance because of the reduction in the requirement for extensive sample preparation. Radioimmunoassays (RIAs) used in the early 1970s were selective and

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.05.008

relatively sensitive but required the use of radioactive tracers of the toxins (Lam, Wan, Boulet, & Le, 1999). The enzyme-linked immunosorbent assay (ELISA) technique for SEs detection has also been developed leading to the production of a commercial assay for four of the SEs. ELISA gives many desirable features for the detection of SEs including high selectivity and the use of non-radioactive reagents. However, carcinogenic reagents are often required in colorimetric assay (Giletto & Fyffe, 1998). PZ (Piezoelectric crystal) sensor has been reported for the analysis of SEC_2 and SEB in different samples. The fabricated PZ sensor can be used for SEB determination in the range of 2.5–60 μg/ml. For most potent SEs the minimum intoxication level is about 200 ng in a portion of 100 g of food. Therefore, methods of detection need to be sensitive enough to measure toxin concentrations as low as nanograms per gram of food, which for liquid samples translates to nanograms per milliliter. However, the sensitivity of piezoelectric crystal sensor is too low (Gao, Chao, Chao, & Li, 2000; Lin & Tsai, 2003). PCR (polymerase chain reaction) has been used for the determination of SEC_1 gene from fresh cheese. Eventhough there have been many reports of applications of the PCR to food samples, the method is not yet widely used in food laboratories because of the lack of a simple and reliable method for quantification of the PCR products (Mäntynen, Niemelä, Kaijalainen, Pirhonen, & Lindström, 1997). An electrochemical method has been reported for the analysis of SEC_1. The sensor is composed of a three-electrode system and a layer of antibody of staphylococcal enterotoxin C_1 (SEC_1). The antigen of SEC_1 is detected by an electrochemical method at a constant antibody concentration. However, it suffers from electrode-fouling problem (Dong, Luo, Feng, Li, & Gao, 2001). Fluoroimmunoassays have been developed to detect high and low molecular weight toxins, respectively, in complex samples (Goldman et al., 2002; Rowe, Scruggs, Feldstein, Golden, & Ligler, 1999). Fluorescence-based biosensor has been developed for simultaneous analysis of multiple samples (Ligler, 2000). However, these methods are often costly, complicated and requiring an excitation source, or suffer from the disadvantages of non-specific radiation. The detection and monitoring of toxins in clinical fluids, environmental samples, food, and drinking water require new approaches.

CL analysis is becoming increasingly important in various fields for its high sensitivity, rapidity, simplicity, feasibility, and low cost both in instrument and material. In the routine clinical laboratory, CL analysis is now commonly used for immunoassay and DNA probes assays (Dodeigne, Thunus, & Lejeune, 2000; Kricka, 1999, 2003; Navas & Jiménez, 1999; Sun et al., 2004; Surugiu, Danielsson, Ye, Mosbach, & Haupt, 2001). When used in immunoassay, horseradish peroxidase (HRP) was employed as label frequently, and it was detected with the luminol-based enhanced chemiluminescence (ECL) system, ECL is the co-oxidation of luminol and a substrate called an enhancer by hydrogen peroxide in the presence of HRP. ECL as a detection system has definite advantages such as a high sensitivity and a short assay time (Ramos, Torijas, & Navas Díaz, 2001). Along with the development of CL, significant progress has been made in techniques to measure CL. When CL is coupled with imaging detectors, the CL-based imaging assay provide simple, sensitive and high throughput means of detection, and therefore has been successfully applied to immunoassay (Carretero, Fernandez, Bowie, & Worsfold, 2000; Cheek, Steel, Torres, Yu, & Yang, 2001; Creton & Jaffe, 2001; Huang, Huang, Fan, & Lin, 2001; Maus & Wightman, 2001; Miura, 2001). To the best of our knowledge, however, there is a lack of information concerning CL imaging assay of SEC_1 in the literature. In this work, a chemiluminescent imaging assay for the SEC_1 was described. The proposed method has the advantage of showing the specificity ELISA (non-chemiluminescent assays), sensitivity of ECL system and high throughput of CL imaging assay. The presented method was simple and sensitive. In addition, the CL imaging system allows immunoassays on 96-well plates or more than 384-well plates to be performed simultaneously.

2. Materials and methods

2.1. Reagents and solutions

SEC_1, two monoclonal antibodies against SEC_1 were kindly donated by Prof B.Q. Jin (Department of Immuno, The Fourth Military Medical University, Xi'an, China). Bovine serum albumin (BSA) was obtained from Sigma, USA. H_2O_2, Na_2CO_3, $NaHCO_3$, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, NaCl, KCl, Tween 20, NaH_2PO_4 and Tris-(hydroxymethy) aminomethane were obtained from Shanghai Chemical Plant (Shanghai, China). Luminol and PIP (*p*-iodophenol) were obtained from Kangpei Technology Company (Xi'an, China).

Unless otherwise stated all chemicals and reagents used in this study were of analytical grade quality.

All solutions were prepared in deionized, distilled water. The coating solution was 0.05 $mol l^{-1}$ carbonate buffer, pH 9.6, containing 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ per liter. 0.15 $mol l^{-1}$ PBS buffer, pH 7.4 was prepared by dissolving 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 8.0 g NaCl, 0.2 g KCl in 1 l water. 96-well plates were rinsed with PBST solution (PBST: PBS solution containing 0.05% (v/v) Tween-20). SEC_1 antigen and antibody were diluted with PBSTB solution (PBSTB: PBST containing 0.1% (w/v) BSA). Substrate buffer was 0.1 $mol l^{-1}$ Tris–HCl solution, pH 8.5. A 5.0×10^{-2} $mol l^{-1}$ luminol stock solution was prepared by dissolving 9.32 g luminol in 20 mL 0.1 $mol l^{-1}$ NaOH

solution and then dilution to 1 l with water. H_2O_2, luminol, PIP working solution was prepared by appropriate dilution with 0.1 mol l^{-1} pH 8.5 Tris–HCl buffer solution daily. SEC_1 calibrators at a concentration of 8.0 to 125.0 ng ml^{-1} were prepared by appropriate dilutions of the SEC_1 stock solution with PBSTB solution.Appropriate safety precautions were exercised when handling toxin preparations. All solutions, glassware, etc., containing bacteria or toxic analytes were handled by personnel wearing gloves. All equipment and instruments were disinfected with a 20% bleach solution and were rinsed with distilled water. Analyte containing solutions were also treated with 20% bleach prior to disposal. Contaminated disposables (test tubes, pipette, used 96-well plates) were placed in biohazard containers and later incinerated.

2.2. Instrumentation

The plate was imaged using a Fluorchem™ IS-8800 system (Alpha Innotech, CA, USA). The Fluochem imaging system is a powerful digital system ideal for instant photography of a wide variety of samples. Thermoelectric cooling of the CCD camera allows imaging of low light samples in UV-illuminated, chemiluminescent and fluorescent applications. The instrument is controlled by Alpha Ease FC software. Imaging analysis and archiving treatment were performed with Alpha Ease FC software running under Windows 2000. The ELISA 96-well plates used for the assay were obtained from Corning Incorporated (Corning, USA).

2.3. Procedures

A typical ''sandwich type'' immunoassay was used. A pair of antibodies that recognize different epitopes of same antigen was used to capture and detect a certain antigen. The assay protocols were the following: (a) the 96-well plates were coated with SEC_1 monoclonal antibody (100 μl/well) overnight at 4 °C. All further steps were performed at 37 °C. These wells were washed three times with PBST solution; (b) addition of SEC_1 standard or sample (100 μl/well), incubation for 30 min, washed four times; (c) addition of HRP-conjugated anti-SEC_1 monoclonal antibody (100 μl/well) diluted 1000-fold in 0.05 mol l^{-1} carbonate buffer pH 9.6. After a final washing step, the chemiluminescent substrate was added. After three minutes, imaging process was commenced. CL imaging measurements were performed in Fluorchem™ IS-8800 system. The background value was obtained by imaging an equally sized region outside the region of interest and was subtracted from each measurement. During these studies, the exposure time was optimized to 10 min. The intensity of the spots was determined using the Spot Denso function of the software, which combines the pixel intensities. The wells were individually analyzed and the intensities (AVG) were plotted as a function of analytes concentration to yield the calibration curve. IDV is the sum of all the pixel values after background correction: IDV = $\sum$(each pixel value-BACK), AREA is the size (in pixels) of the region enclosed by the box, ellipse, or free hand writing, AVG is the average value after background correction of the pixels enclosed, AVG = IDV/AREA, BACK is the background value that will be subtracted from all the pixels in the object.

2.4. Model milk and water samples

Soymilk and water were chosen because they were food that has been implicated in SE food intoxication. To simulate milk and water samples contaminated with SEC_1; water and milk samples were spiked with known concentrations of SEC_1 at an ng ml^{-1} level. After the addition of SEC_1, samples were incubated with the food matrix for a minimum of 2 h and were tested using a sandwich immunoassay format.

3. Results and discussion

3.1. Optimization of the CL imaging conditions

CL imaging depends on both assay procedure and the characteristics of substrates. CL emission intensity is sensitive to a variety of environmental factors such as temperature, solvent, ionic strength, solution pH and other species present in the system. The effects of the concentrations of luminol, PIP, H_2O_2 and CL detection buffer solution were examined.

In this CL imaging system, the detection buffer solution is medium of enzyme reaction and also is medium of the CL reaction. There are two factors, which determine the overall response in this system: the influence of pH on the enzyme activity and the effect of pH on the generated CL signal. The optimal pH value for HRP is 6–7. However, the CL reaction between luminol and H_2O_2 shows the maximum CL intensity at pH 10–11. Therefore, the effect of medium pH on the immunoassay was investigated. The result (Fig. 1) shows that optimal pH was 8.5. The result showed that the CL emission in the Tris–HCl buffer was stable than in other buffers such as Na_2CO_3–$NaHCO_3$, KH_2PO_4–K_2HPO_4. 0.1 mol l^{-1} Tris–HCl buffer gave the biggest signal to noise ratio, and the reproducibility of the ECL.

As the CL reagent, luminol concentration affects the ECL intensity. The effect of luminol concentration was investigated. The experiment results showed that (Fig. 2) the CL intensity increased from 2.5×10^{-5} to 7.5×10^{-4} mol l^{-1} and reached the maximum value at the luminol concentration of 7.5×10^{-4} mol l^{-1}. The effect of H_2O_2 concentration on the CL intensity was

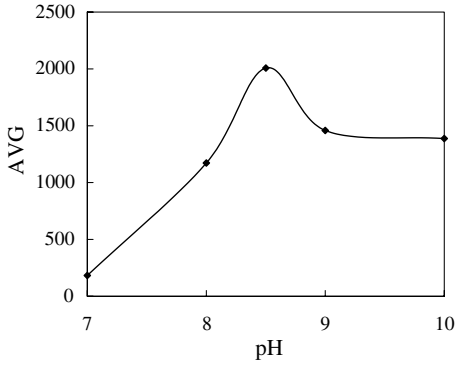

Fig. 1. Effect of buffer pH on CL intensity: luminol, 7.5×10^{-4} mol l^{-1}; H_2O_2, 3.0×10^{-3} mol l^{-1}; PIP, 1.0×10^{-3} mol l^{-1}; SEC_1, 25.0 ng ml^{-1}; exposure time, 10 min.

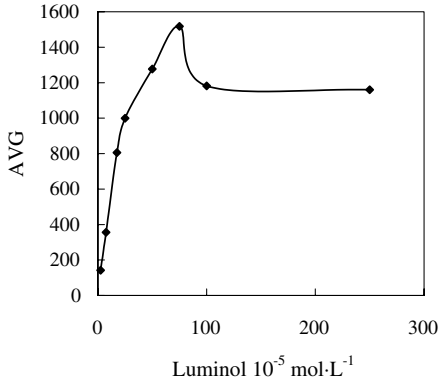

Fig. 2. Effect of luminol concentration on CL intensity: H_2O_2, 3.0×10^{-3} mol l^{-1}; PIP, 1.0×10^{-3} mol l^{-1}; SEC_1, 25.0 ng ml^{-1}; exposure time, 10 min.

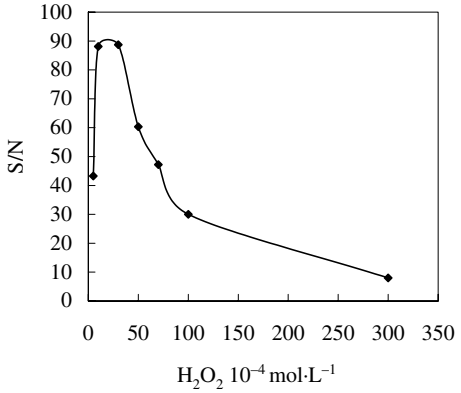

Fig. 3. Effect of H_2O_2 concentration on CL intensity: luminol, 7.5×10^{-4} mol l^{-1}; PIP, 1.0×10^{-3} mol l^{-1}; SEC_1, 25.0 ng ml^{-1}; exposure time, 10 min.

also investigated (Fig. 3). The experimental results showed that when H_2O_2 concentration was 3.0×10^{-3} mol l^{-1}, CL signal with biggest *S*/*N* (signal to noise ratio) could be obtained. Under optimum conditions, the CL emission of luminol–H_2O_2 system was enhanced upon addition of PIP. With an increasing PIP concentration, the CL emission increased and reached a maximum value at 1.0×10^{-3} mol l^{-1} (Fig. 4). Thus 0.1 mol l^{-1} (pH 8.5) Tris–HCl buffer solution containing 3.0×10^{-3} mol l^{-1} H_2O_2, 7.5×10^{-4} mol l^{-1} luminol and 1.0×10^{-3} mol l^{-1} PIP was selected as the CL detection solutions. The effect of exposure time was investigated in the range of 3–30 min. The results showed that the best exposure time was 10 min, which was selected as the optimum time for determining SEC_1.

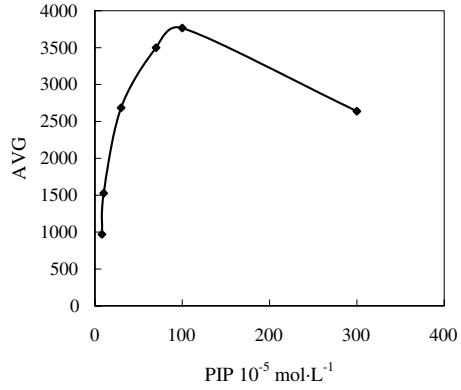

Fig. 4. Effect of PIP concentration on CL intensity: luminol, 7.5×10^{-4} mol l^{-1}; H_2O_2, 3.0×10^{-3} mol l^{-1}; SEC_1, 25.0 ng ml^{-1}; exposure time, 10 min.

3.2. Analytical characteristics

Under the selected conditions, CL response to SEC_1 concentration was linear in the range of 8.0–125.0 ng ml^{-1} with the regression equation of $I = 359.71[SEC_1]$ (ng mL^{-1}) $- 2845.9$ ($n = 5$, $r^2 = 0.9976$) and the detection limit was 0.5 ng ml^{-1} (3σ). ELISA (non-chemiluminescent assay) was used as contrastive method. The relative standard deviation (RSD) was 0.06 for the determination of 25.0 ng ml^{-1} SEC_1 ($n = 8$) when chemiluminescent imaging assay was used. The RSD was 0.07 when ELISA (non-chemiluminescent assay) was used.

3.3. Sample analysis

The present CL imaging system was applied to the assay of SEC_1 in milk and water samples. The results were

Table 1
Results of analysis of SEC_1 in milk and water

Sample no.	Proposed method (ng ml^{-1})	ELISA (ng ml^{-1})	Difference (%)
Milk1	54.0	53.0	+1.9
Milk2	25.0	26.0	−3.8
Milk3	27.0	26.0	+3.8
Water1	12.0	12.3	−2.4
Water2	12.6	12.5	+0.8

Table 2
Results of recovery test

Sample no.	Original (ng ml^{-1})	Added (ng ml^{-1})	Total(ng ml^{-1})	Found (ng ml^{-1})	Recovery (%)
Milk1	0	25.0	25.0	27.0	108
Milk2	0	50.0	50.0	46.0	92
Milk3	0	50.0	50.0	54.0	108
Water1	0	25.0	25.0	24.0	96
Water2	0	25.0	25.0	25.2	101

given in Table 1, which agreed well with those obtained by the ELISA (non-chemiluminescent assay).

3.4. Recovery test of SEC_1 in milk and water samples

The feasibility of applying the proposed immunoassay to measure toxin levels in a complex matrix was studied. This was conducted by adding various levels of SEC_1 into three milk samples and two water samples. The results of recovery test were shown in Table 2.

4. Conclusions

The ability to measure accurately and precisely very low amounts of toxins in clinical fluids, environmental samples, food, and drinking water is very important. As low as 100 ng SEs are sufficient to cause symptoms of intoxication in humans. The reported methods mainly include ELISA, RIA, and fluoroimmunoassays assay. ELISA is conventional detection method for SEs and offers high specificity. In this paper, traditional ELISA was successfully coupled to CL imaging system. Therefore, the method offers not only the advantages of ELISA (non-chemiluminescent assay) but also the advantages of CL imaging. High sensitivity is obtained by the powerful detection system. As low as 0.5 ng ml^{-1} SEC_1 could be detected. In contrast, at least 8 ng ml^{-1} of SEC_1 was required to produce an unambiguous signal in ELISA (non-chemiluminescent assay). The proposed method is sensitive, simple and rapid and can reliable measure SEC_1 in food. Therefore, these merits should make it easily popular and used for various real applications.

Acknowledgment

This study was supported by a Grant from the Science and Technique Ministry of China (2003BA310A05).

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