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Short communication

Rapid whole protein quantification of staphylococcal enterotoxin B by liquid chromatography

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ABSTRACT

Food poisoning caused by *Staphylococcus aureus* is one of the most important foodborne diseases in the world. The ability of these bacteria to produce one or more enterotoxins in milk and dairy products is linked to staphylococcal food poisoning. Enterotoxin B (SEB) is an exotoxin produced by *S. aureus* and is one of the compounds most frequently involved in staphylococcal food poisoning worldwide. In this work, 20 samples of milk collected from restaurants have been studied for the presence of *S. aureus* enterotoxigenic strains. All the isolates from milk samples have been analysed by liquid chromatography-coupled with diode array detector for the rapid identification and quantification of SEB as intact protein. Limit of detection and limit of quantification values were 0.5 and 1 μg/mL, respectively. *S. aureus* was found in 35% of analysed samples but only one of them was an enterotoxigenic strain, which produced staphylococcal enterotoxin B at levels of 3.6 μg/mL.

1. Introduction

Staphylococcus aureus is considered one of the major borne-pathogens throughout the world (Chen, Hsiao, Chiou, & Tsen, 2001). Enterotoxigenic *S. aureus* may produce one or more of a family of enterotoxins, known as staphylococcal enterotoxins (SEs). These bacteria are a leading cause of gastroenteritis resulting from the consumption of contaminated food. Staphylococcal food poisoning (SFP) is due to the absorption of SEs preformed in the food. Among these toxins, enterotoxin B (SEB) is one of the most prevalent compounds in staphylococcal food poisoning (Casman, 1965). SEB is a 23–29 kDa protein, which is stable to heat, proteolytic digestion and pH change (pH 4–10).

Among the foods implicated in SFP, milk and dairy products, especially handled foods, play an important role, since enterotoxigenic strains of *S. aureus* have been frequently isolated in them (Soriano, Font, Moltó, & Mañes, 2002a). In 1914, enterotoxigenic staphylococci were associated, for the first time, with foodborne illness, due to the repeated ingestion of unrefrigerated milk (Barber, 1914). Food contamination is usually caused by one or a combination of factors including cross-contamination, poor hygiene and temperature abuse during the preparation of the foods. The contamination of food with staphylococci can take place at various stages ranging from production to the sale and distribution.

The presence of enterotoxigenic staphylococci and their toxins in foods has mainly been detected by use of the staphylococcal enterotoxin-reversed passive latex agglutination (SET-RPLA) kit (Labib, Hedström, Amin, & Mattiasson, 2009; Soriano, Font, Rico, Moltó, & Mañes, 2002b) followed by other immunological techniques, such as enzyme-linked immunosorbent assay (ELISA), immunoaffinity-based time-resolved fluorescence (Peruski, Johnson, & Peruski, 2002), fluorescent latex microparticle immunoassay (Medina, 2006), immunomagnetic-electrochemiluminescent detection (the ORIGEN system) (Kijek, Rossi, Moss, Parker, & Henchal, 2000), magnetoelastic detection (Ruan, Zeng, Varghese, & Grimes, 2004), immunoaffinity-based multichannel sensors (Yacoub-George et al., 2002), and paramagnetic beads (Garber, Kodumudi, Venkateswaran, & O'Brien, 2010). During recent years, different separation procedures coupled to mass spectrometric detectors have been used by several authors to increase the yield and recovery of the peptides. (Callahan, Shefcheck, Williams, & Musser, 2006; Hennekinne et al., 2009; Kawano et al., 2000; Nedelkov, Rasooly, & Nelson, 2000; Rasooly & Do, 2009). In this work, we applied liquid chromatography coupled with diode array detector (HPLC-DAD) for the rapid identification and quantification of intact SEB in isolates from milk samples.

2. Materials and methods

2.1. Chemical and reagents

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany) and analytical-grade trifluoroacetic acid (TFA) was

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supplied by Sigma–Aldrich (St. Louis, MO). Deionised water for LC mobile phase was prepared by reverse osmosis with a Milli-Q water purification system (Millipore, Molsheim, France). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corporation, Danbury, CT) ultrasonic bath. Phenex-RC and Millipore 10 K centrifugal filters were supplied by Phenomenex (Madrid, Spain) and Millipore, respectively. The stock standard solution of SEB was purchased from Sigma–Aldrich.

2.2. Sample collection and bacterial identification

A total of 20 milk samples were collected from restaurants in the final stages of preparation. These samples were studied to detect the presence of *S. aureus* strains and their capacity to produce enterotoxins. Milk samples, (25 mL) were diluted with 225 mL of buffered peptone water (CM 509, Oxoid, Basingstoke, UK). The samples were further diluted with buffered peptone water, and 0.1-mL portions of various dilution levels were spread on the surfaces of Baird Parker (BP) agar (CM 275, Oxoid) supplemented with tellurite and egg yolk emulsion (SR 275, Oxoid). Plates were incubated at 37 °C for 24–48 h. Suspected colonies were subjected to Gram staining, examined microscopically, and identified with the API Staph system (BioMérieux, Marcy ÍEtoile, France). To increase the production of enterotoxins, isolated staphylococcal strains were prepared by inoculating seven colonies from Baird Parker agar into 10 mL of tryptone soya broth (CM 129, Oxoid). After 18–24 h of growth at 37 °C with shaking, the cultures were centrifuged and the supernatant was filtered with 0.22-μm hydrophilic regenerated cellulose filter membranes, and the filtrate was retained for an assay of the toxin content. *S. aureus* strains were studied for their ability to produce enterotoxins with the proposed method by HPLC-DAD and, as a confirmatory test, also according to the SET-RPLA methodology.

2.3. HPLC-DAD analysis

Bacteria cultures identified as *S. aureus* were analysed with HPLC-DAD. Culture filtrates were concentrated and desalted using 10 k NMWL Millipore centrifugal filter units. Samples were centrifuged at 9000 rpm and washed twice with 0.1% TFA in ACN. HPLC-DAD analysis was performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Tokyo, Japan). A Jupiter C18 reversed-phase analytical column (150 × 2 mm, 3 μm) column was used (Phenomenex, Torrance, CA) with a flow rate of 0.2 mL/min. The volume injected of standards and sample solutions was 20 μL. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in ACN. HPLC conditions were set up using a gradient that started at 20% B with a linear gradient of 20–40% B in 10 min, then changed to 60% B at 20 min, and back to 20% B at 30 min. SEB was detected at 205 nm and 23.5 min. Enterotoxin identification was performed by injection of the samples in triplicate and comparing the retention time and UV spectra of purified extracted samples to pure standards and fortified samples.

3. Results and discussion

In order to optimise the developed HPLC-DAD method, limit of detection (LOD) and limit of quantification (LOQ) were calculated according to $s/n = 3$ and $s/n = 10$, respectively. LOD and LOQ values were 0.5 and 1 μg/mL, respectively. The technique of standard additions was used to calculate the recovery of this method. No enterotoxigenic bacteria cultures were added with SEB standard solution at five levels in a range of 1–50 μg/mL in triplicate. Mean recoveries of fortified cultures ranged from 89.1 ± 2.7% to 101.6 ± 2.1% for concentrations tested. Standard and fortified matrix curves showed good linearity, with coefficients of correlation (r^2) greater than 0.997 and no matrix effect was detected. For repeatability and reproducibility, five series of samples spiked at 5 μg/mL were compared with SEB standards signal. The RSD obtained for intraday variation ($n = 5$) was 3.1%. The inter-day variation showed an RSD value of 7.3%. These values were lower than 20%, confirming the good reproducibility and repeatability of this technique (European Union, 2002).

Out of 20 milk samples examined, 9 (45%) revealed typical colonies of *S. aureus* on BP agar. The high percentage of contaminated milk samples found in this work is in accordance with previous studies in milk samples (Sospedra, Rubert, Soler, Soriano, & Mañes, 2009). All analysed samples were collected from restaurants where possible infringements of hygienic practice in the handling and cleaning of milk containers can increase the microbial contamination of these kind of samples. Due to its high level of nutrients, milk provides a suitable growth medium for several bacteria. Only 35% of the isolates were identified as *S. aureus* species by the coagulase test API Staph system. One of these isolates was enterotoxigenic. Results obtained by HPLC-DAD were confirmed by SET-RPLA and SEB was the only enterotoxin detected by both methods.

S. aureus can produce enterotoxins in milk samples usually because the food has not been kept hot enough or cold enough (Mattick et al., 2003; Scott & Bloomfield, 1990). Staphylococci are present in the nasal passages and throats and on the hair and skin of approximately 50% of healthy individuals. Although food handlers are frequently implicated in the transmission of this pathogen to food, equipment and environmental surfaces can also be sources of contamination (Bhatia & Zahoor, 2007). Huong et al. (2010) studied the presence of *S. aureus* and their toxins in

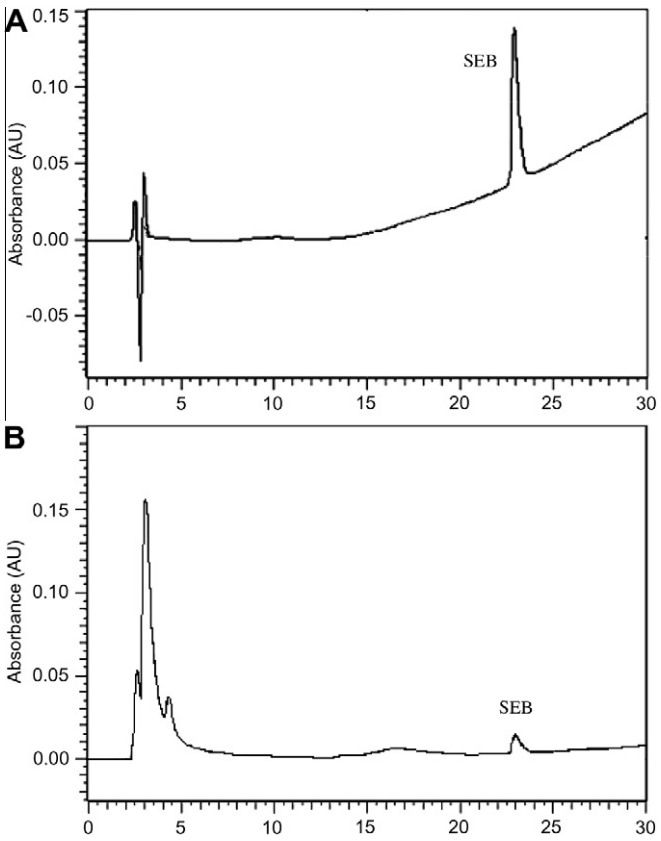

Fig. 1. HPLC-DAD chromatograms; (A) bacteria culture spiked with intact SEB standard (B) enterotoxin B-positive culture isolated from milk.