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A novel method for rapid production and purification of exfoliative toxin A of *Staphylococcus aureus*

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Abstract

The exfoliative toxins of *Staphylococcus aureus* are the causative agents of the scalded-skin syndrome. Previously described methods of toxin production and purification require large quantities of culture medium, take a long time and often produce low yields of toxin. A novel method of toxin production and purification using a dialysis sac to separate the culture medium from the staphylococci is described. This method produces up to 12 mg of crude toxin per ml of bacterial cell culture bathing the surface of the dialysis sac within 36 h and almost 10 mg of purified toxin per ml of cell culture within 3 days, in contrast to previous procedures that took over a week to produce 0.1–1.0 mg ml^{-1} crude toxin and less than 0.01 mg ml^{-1} purified toxin. This rapid method of toxin production should speed up future research into the pathogenesis of the staphylococcal scalded-skin syndrome.

Keywords: Methodology; Exfoliative toxin A; Exfoliative toxin B; Dialysis sac; *Staphylococcus aureus*

1. Introduction

The exfoliative toxins (ET) of *Staphylococcus aureus* are the causative agents of the staphylococcal scalded-skin syndrome, a blistering skin disorder that particularly affects infants and young children [1]. The condition is characterised by the formation of large fragile superficial blisters, which rupture to leave extensive areas of denuded skin. Its severity varies from a localised blister to generalised exfoliation affecting the entire body surface [2]. Two toxins affecting humans (ETA and ETB) with 25% sequence homology have been identified and sequenced, and their three-dimensional structures have been elucidated [1–4]. Recently, desmoglein-1, part of the epidermal desmosomal structure that links cells together, has been identified as one substrate for ETA [5].

Different methods of production and purification for the exfoliative toxins have been described previously [6–18]. Most procedures require the growth of bacteria in large volumes of culture medium, the purification time lasts 10 days or more and only small quantities of the final purified toxin are produced, usually less than 0.01 mg pure toxin per ml of cell culture supernatant [11,13,14,16,17]. A novel method of high-yield toxin production and purification using a dialysis sac to separate the culture medium from the staphylococci is described. The recent identification of the toxin substrate is likely to generate more research involving the toxins. This novel and rapid method of toxin production should speed up research into the pathogenesis of the staphylococcal scalded-skin syndrome.

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2. Materials and methods

2.1. Staphylococcal strains and maintenance

An ETA-producing strain SSS681 of *S. aureus* isolated from a baby with pemphigus neonatorum was used for this study [19,20]. The strain was maintained on tryptic soy agar (TSA), which was made up of Tryptone (Oxoid, UK) 17 g, Soya peptone (Oxoid, UK) 3 g, Agar No. 1 (Oxoid, UK) 15 g in 1 l distilled water and sterilised by autoclaving at 121°C for 15 min [18].

2.2. Bacterial cell culture

Bacterial cell culture of *S. aureus* was carried out using TSKG culture medium, which was made up of Tryptone (Oxoid, UK) 17 g, Soya peptone (Oxoid, UK) 3 g, K_2HPO_4 2.5 g, glucose 5 g, and Novobiocin (Sigma, UK) 500 μg in 1 l distilled water and sterilised by autoclaving at 110°C for 20 min [18].

The inoculum for cell culturing onto the dialysis sac was prepared by transferring six single colonies of *S. aureus* from each TSA medium plate into separate 25-ml Universal bottles containing 10 ml of TSKG culture medium and incubated overnight on an orbital shaker at 37°C. Bacteria were harvested by centrifugation ($4000 \times g$ for 10 min) and the cells washed twice in cold sterile 10 mM Tris–HCl with 1 mM EDTA buffer (TE buffer) and resuspended in 10 ml of TE buffer.

2.3. Bacterial cell culture using dialysis sac

One hundred millilitres of TSKG culture medium were added to a length of 30×5 cm dialysis sac, which was then clipped at both ends, placed in a 1 l conical flask (Fig. 1) and sterilised by autoclaving at 110°C for 20 min. Two millilitres of *S. aureus* inoculum were then added to the flask containing the dialysis sac and incubated at 37°C in an orbital shaker (150 rpm) for 20–24 h. Ten such flasks were used for every batch production, which is equivalent to a total of 1 l of culture medium inside the dialysis sacs.

2.4. Harvesting the culture supernatant

Between 2 and 3 ml of bacterial culture grown on the surface of the dialysis sac were collected from each flask, pooled to make approximately 25 ml and centrifuged at $10\,000 \times g$ for 30 min at 4°C. The supernatant was passed through a 0.22-μm pore size filter to remove any remaining bacteria and, at this stage, could be stored at −20°C for at least a year without affecting the purification of ETA.

2.5. ETA purification

The supernatant was then dialysed against 4 l of cold 20 mM Tris–HCl buffer (pH 7.2). The dialysed solution was loaded onto a DEAE Sepharose column (3×6 cm) and washed with 100 ml of 20 mM Tris–HCl (pH 7.2). The eluting proteins were monitored at 280 nm using a UV cord (Pharmacia, UK) and the unbound fraction collected and concentrated to approximately 1 ml using an Amicon ultrafiltration unit with a PM10 filter. Gel filtration chromatography was then carried out using a Pharmacia FPLC system on a Superose 12 column (1×30 cm) equilibrated with 20 mM Tris–HCl (pH 7.2). Proteins were eluted using the same buffer at a rate of 1.0 ml min^{-1} and 1-ml fractions were collected. The fractions containing ETA were identified by SDS–PAGE and Western blot analysis, pooled and concentrated using an Amicon Centriprep 10 concentrator to 1 ml. Buffer exchange was carried out at this stage by adding 15 ml of 20 mM sodium acetate buffer (pH 5) to the Centriprep 10 concentrator and the volume reduced again to 1 ml – this was repeated twice. Finally the ETA toxin was purified by ion exchange chromatography using a Mono-S column (1×5 cm) on a Pharmacia FPLC system. The column was equilibrated with 20 mM sodium acetate buffer (pH 5) and the ETA eluted using a 0–1 M NaCl gradient. Fractions of 1 ml were collected, the ETA identified using SDS–PAGE analysis and concentrated to 1 ml using the Amicon Centriprep 10 concentrator.

2.6. SDS–PAGE

A 10–15% gradient SDS–PAGE was run on the PhastSystem (Pharmacia: PhastSystem development Technique file 210), with broad-range molecular mass markers from Bio-Rad (UK).

2.7. Western blot

Anti-ETA antibody was developed and characterised as described previously [21]. Protein samples were resolved on discontinuous SDS–PAGE using the Mini Protean system (Bio-Rad) by the Laemmli method [22] and electrophoresed for 45 min at 200 V. The proteins on the SDS–PAGE gel were electrophoretically transferred overnight at 30 mA onto a nitrocellulose membrane using a Semi-Dry Transblot apparatus (Hoeffer Scientific Instruments, UK). The membrane was washed in phosphate-buffered saline (PBS) and then incubated with 3% (w/v) bovine serum albumin (BSA) in PBS for 15 min. Anti-ETA IgG (1 μg ml^{-1}) in 1% (w/v) BSA in PBS with 0.1% (v/v) Tween 20 (PBS–Tween) was added to the membrane and incubated for 45 min. The membrane was washed twice in PBS–Tween for 10 min. Anti-sheep IgG conjugated to horseradish peroxidase (HRP) (Sigma) (1 μg ml^{-1}) was then added to PBS–Tween and incubated for 45 min. The membrane was washed as described above and developed using the Sigma DAB development system (Sigma) by adding one tablet each of 3,3-diaminobenzidine tetra-

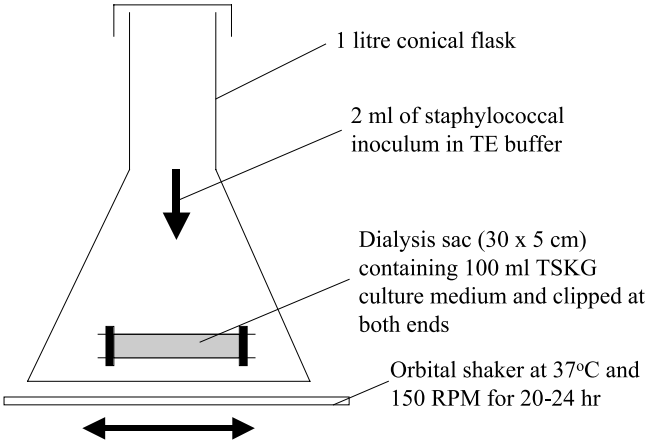

Fig. 1. Diagram showing the method for exfoliative toxin production by culturing *S. aureus* on the surface of a length of dialysis sac containing the culture medium inside it.

hydrochloride and urea/hydrogen peroxide to 5 ml of distilled water. Detection sensitivity was 100 ng.

2.8. Measuring ETA using double-antibody capture ELISA

Anti-ETA antibody conjugation with HRP was accomplished using the Pierce Immunpure Plus Activated Peroxidase Kit (Pierce, UK). Maxi Sorb microtitre plates (Nucc, UK) were coated with 100 μl of 1 μg ml^{-1} anti-ETA IgG in PBS for 1 h at 37°C. The plates were washed three times with PBS–Tween, incubated with 3% (w/v) BSA in PBS–Tween for 1 h at 37°C and then washed twice in PBS–Tween. Pure ETA, serially diluted to obtain a standard curve from 1 to 1000 ng ml^{-1} in a final volume of 100 μl per well was incubated at 37°C for 2 h, along with 100 μl per well of ETA samples from different stages of purification. The wells were washed three times in PBS–Tween, anti-ETA IgG conjugated to HRP (10 ng ml^{-1}) was added and the plate incubated for a further 2 h at 37°C. It was then washed twice with PBS–Tween and developed using *o*-phenylenediamine dihydrochloride (Sigma) 20 mg in 10 ml of 0.15 M phosphate citrate buffer (pH 5) plus 1% (v/v) H_2O_2 and read at 450 nm. The detection limit was 100 pg for the pure ETA. Dilutions of unknown samples of ETA were done so that values fell within the standard curve. Each experiment was carried out at least three times using different microtitre plates and in triplicate.

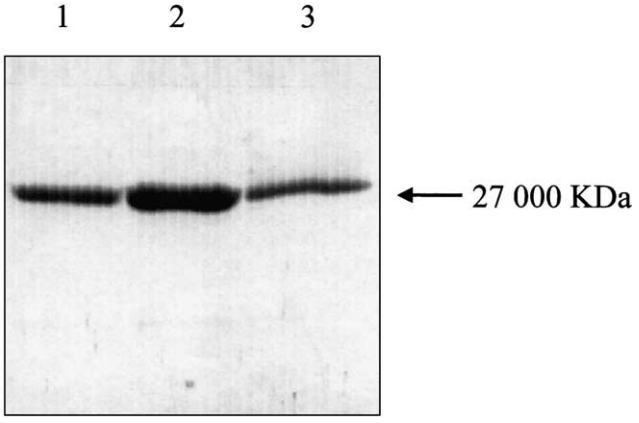

Fig. 2. 12.5% SDS–PAGE of pure ETA protein after Mono-S ion exchange chromatography from three different batches of purification (lanes 1, 2 and 3).

2.9. N-Terminal sequencing

N-Terminal sequencing was carried out on the purified ETA protein using an automated sequencer (Perkin-Elmer) at the NIMR (Mill Hill, London) by Dr A. Atken.

3. Results

Incubating 2 ml of staphylococcal cell culture inoculum with 100 ml TSKG culture medium in the 1 l conical flask without using the dialysis sac to separate the staphylococci from the medium produced only 0.1 mg crude toxin per ml of supernatant in the flask. In contrast, the use of dialysis sac as described in Section 2 (Fig. 1) increased the yield to 12 mg of crude toxin per ml of culture supernatant on the surface of the dialysis sac and 10 mg ml^{-1} of toxin after purification. The identity of the final toxin product was confirmed by three different methods. Firstly, SDS–PAGE confirmed the presence of a protein with a molecular mass of 27 kDa consistent with the predicted molecular mass of the toxin (Fig. 2) [9–13]. Secondly, Western blot analysis with specific ETA antibodies [21] identified the purified protein as ETA. Finally, N-terminal sequencing identified the N-terminal amino acids of ETA as EVSAEEIKKH [9–12]. The final toxin product (Fig. 2) was pure enough to develop antitoxin antibodies and perform crystallisation studies.

4. Discussion

Several different production methods have been described for ETA, including the use of a salt-free yeast diffusate/casamino acid culture medium with small quantities of Novobiocin [18] in the absence of CO_2 [10], and a 20–24-h incubation period [16,17]. The method of toxin production used here differs from existing methods in that dialysis sac was used to separate the culture medium from the bacteria and their extracellular products. The dialysis sac prevents contamination of highly charged and hydrolysed proteins in the complex culture medium inside the dialysis sac with the extracellular toxin produced by the staphylococci grown on the surface of the sac [14–16]. As a result, the purification procedure was simplified from a minimum of six to only three steps because the complication of removing the culture medium proteins and peptides from the toxin preparation was no longer an issue. In addition, reducing the total volume of bacterial cell culture supernatant on the surface of the dialysis sac to only 2–3 ml per flask (compared to previously reported volumes of up to 50 l [11,14,16,17]) produced a very concentrated crude toxin preparation, which was easier to handle.

Two main purification procedures for the toxins have been described previously – one based on ion exchange chromatography [7,11,12] and the other on isoelectric focusing [8,15–17]. The DEAE–ion exchange was preferred in this case because it removed a larger number of contaminating proteins. Subsequent gel filtration produced a toxin preparation with only one minor contaminating protein as observed with SDS–PAGE. For some applications, toxin of this purity would be sufficient [23]. However, further purification was easily achieved using Mono-S ion exchange chromatography, which gave a single band on SDS–PAGE.

The described method of toxin production and purification using a dialysis sac is quicker than previous procedures, taking only a few days instead of weeks [7–17]. Furthermore, the production of 12 mg of crude toxin per ml of bacterial cell culture bathing the surface of the dialysis sac in each conical flask is much higher than previous reports of 0.01–1.0 mg ml^{-1} [11,13,14,16,17], as is almost 10 mg purified toxin per ml of cell culture [11,13,14,16,17] compared to 5–10 mg per 3 l supernatant (1–3 μg ml^{-1}) [17], 100–210 mg per 50 l supernatant (2–4 μg ml^{-1}) [14], and 3–5 mg l^{-1} (3–5 μg ml^{-1}) [16].

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