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GST–Fbe can recognize β-chains of fibrin(ogen) on explanted materials

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Abstract

Staphylococcus epidermidis, a coagulase-negative staphylococcus (CoNS), is one of the leading pathogens of nosocomial infections, particularly associated with foreign body infections. Adherence of *S. epidermidis* to fibrinogen deposited on the surfaces of implants is important for the development of foreign body infections. A gene (*fbe*) encoding a fibrinogen-binding protein from *S. epidermidis* (Fbe) was identified by shotgun phage display. A portion of *fbe* was cloned into a GST-fusion vector. Affinity to glutathione–Sepharose by the GST-tag and affinity to fibrinogen–Sepharose by the Fbe part were applied to purify the recombinant Fbe. The purity and efficacy of the methods used in protein purification was compared. Furthermore, the potential physiological role of Fbe was studied by the interaction between GST–Fbe and components extracted from explanted materials in vitro.

Keywords: Purification; *Staphylococcus epidermidis*; Fibrin; Fibrinogen

1. Introduction

Staphylococci are known as pathogens in humans. The emergence of antibiotic resistance and the increase in the prevalence of nosocomial infections with these microorganisms have been accumulated in recent years. A better understanding of the pathogenesis of life-threatening staphylococcal infections is urged. *Staphylococcus epidermidis*, an important pathogen of coagulase-negative staphylococci (CoNS), can cause several diseases, especially infections associated with indwelling medical devices. Antibiotic resistance is commonly reported in most clinical isolates of CoNS. Many of them are multi-resistant to the usual anti-staphylococcal agents, such as β-lactam drugs, erythromycin, clindamycin, tetracycline, and gentamicin. More than 80% of clinical isolates are resistant to methicillin and other semi-synthetic penicillin, making the treatment of these infections more difficult and complicated [1].

The infections caused by *S. epidermidis* share some common features, e.g., most of them are nosocomial, often resistant to various antimicrobial agents, and usually involve an indwelling foreign body. Infections caused by *S. epidermidis* can result in prolongation of hospital stay. In some cases, the infections are difficult to treat and removal of indwelling foreign material is needed [2].

Medical devices have been used extensively in

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modern health. Biomaterials have many applications, from a simple intravenous catheter to a more complex artificial organ. Due to the nature of biomaterial, initial implantation of a medical device is associated with a rapid-form conditioning film of the implant by host components from the fluid in a certain order [3]. The final composition of such a film may differ according to the type of material, insertion site and duration of the implant. In short-term implants, fibrinogen (Fg) was constantly present, whereas fibronectin (Fn) and von Willebrand factor were present occasionally [4], while in long-term implants, Fn is more commonly found. To the pathogens, such a layer of conditioning film provides potential sites to initiate colonization.

Fg, a dominant glycoprotein in plasma and extracellular matrix, is composed of three pairs of peptide chains, $A\alpha B\beta\gamma_2$. The overall molecular mass of this molecule is 340×10^3–67×10^3 for α-chain (α1–610), 55×10^3 for β-chain (β1–461) and 48×10^3 for γ-chain (γ1–411). It plays an important role in blood clotting, wound healing etc. However, as a constant component of the conditioning film on the implant surfaces, Fg was also found to mediate bacterial adherence. Bacterial adherence to implants of *S. aureus*, another important pathogen of staphylococci, has been found to be promoted by Fg [5–11]. The interactions between *S. epidermidis* and Fg have been found to be in a heterogeneous manner. Some strains bind strongly to the Fg-coated surface, while some bind to Fg moderately or weakly [12]. A phage display library has been constructed based on chromosomal DNA of *S. epidermidis* strain HB isolated from a patient with osteomyelitis. A clone of phagemid was identified by panning such a library against immobilized Fg. Subsequently, the complete sequence of the gene (*fbe*) encoding a Fg-binding protein of *S. epidermidis* was obtained resulting in an open reading frame of 3276 nucleotides. A more extensive phage display library was constructed. Nine phagemids were enriched by panning against Fg. Alignment of these insertions revealed an overlap within amino acid residue of 269 and 599 [12].

Efforts have been made to purify the Fg-binding protein (Fbe) in its native form. Results indicated that native Fbe was sensitive to protein extraction and difficult to obtain in sufficient amounts. Thus, a portion of *fbe* encoding amino acid residue 87 to 646, which covered the putative Fg binding sites (residue 269 to 599), was cloned into a glutathione S-transferase (GST)-fusion expression system. The properties of Fbe were studied in its fusion form with GST [13–15]. Here, we focus on the methods of purification on the recombinant protein and interactions between Fbe and components absorbed on implanted foreign materials.

2. Experimental

2.1. Construction of GST–Fbe

A DNA fragment of *fbe* gene (nucleotide 261 to 1938) was amplified by polymer chain reaction (PCR) and cloned into a GST fusion vector pGEX-4T3 (Amersham Pharmacia Biotech, Uppsala, Sweden). This fusion expression system utilizes an affinity tag consisting of GST at the N-terminal end of the recombinant protein, which can bind to glutathione–Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden). This plasmid encodes a thrombin cleavage site between GST of 29×10^3 relative molecular mass and the C-terminal fused protein. The clone procedure has been described previously [13]. In brief, upper primers with additional *Bam*HI sites and lower primers with additional *Eco*RI sites were used to amplify the fragment corresponding to amino acids 87 to 646. The amplified fragment digested with *Bam*HI and *Eco*RI was ligated into plasmid pGEX-4T3 digested with *Bam*HI and *Eco*RI. The competent cells were prepared from *E.coli* strain TG1 (La Jolla, CA, USA) by HEPES (1 m*M* pH 7.0) and stored at −70 °C in small portions at 1×10^9 CFU/ml until used. Electroporation was done at room temperature in 0.1 cm cuvette (Bio-Rad, USA), at a setting of 2.5 kV, 2.5 μF, 200 Ohm for 2.5 ms using a Gene Pulser (Bio-Rad, USA). The transformants obtained by electroporation were selected on Luria broth (LB) agar plates containing ampicillin at 100 μg/ml. Restriction enzymes, T4 DNA ligase, Taq DNA polymerase and DNA purification kits were purchased from Promega.

2.2. Purification of proteins

2.2.1. Purification of GST–Fbe by affinity to glutathione–Sepharose 4B

E. coli strain TG1 harboring recombinant plasmid was grown in 2XYT broth with 100 μg/ml ampicillin. As a control, TG1 strain harboring pGEX-4T3 plasmid was also cultivated and purified in parallel. The preculture was grown at 30 °C overnight and diluted 1:20 in fresh 2XYT broth plus 100 μg/ml ampicillin to OD_{600}=1.0. GST–Fbe, was induced by isopropyl thio galactoside (IPTG, 1 m*M* to final concentration, La Jolla, CA, USA). Different induction times were applied to the cultivation—2 h, 4 h and overnight. The cells were harvested by centrifugation at 8000 rpm, resuspended in Tris–EDTA buffer (pH 7.4). The density of the cells was adjusted to OD_{600}=1.0. Ten milliliters of cell suspension was lysed by lysozyme to a final concentration of 0.1 mg/ml at 37 °C for 1 h. The lysate was incubated at room temperature for 1 h with additional 1% Triton X-100. The sample was applied to the glutathione–Sepharose 4B column. The bound GST–Fbe was eluted by affinity elution of reduced glutathione following the protocol recommended by the manufacturer. The chemicals used in this study, unless indicated, were purchased from Sigma, St Louis, MO, USA.

2.2.2. Purification of GST–Fbe by affinity to fibrinogen–Sepharose

Fg–Sepharose was prepared by coupling 70 mg human Fg to 3.5 g CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) following the procedure recommended by the manufacturer. Bacterial lysate prepared as mentioned above was applied to Fg–Sepharose, washed with phosphate-buffered saline (PBS) and eluted with additional 1 *M* NaCl.

2.2.3. Purification of GST–Fbe by fast protein liquid chromatography (FPLC)

The translated amino acid sequence of portion of Fbe was submitted to ExPasy for the theoretical pI prediction (http://us.expasy.org/tools/). The experimental pI was determined by running a sample on PhastGel IEF 3–9 (Amersham Pharmacia Biotech, Uppsala, Sweden). For FPLC® purification, proteins obtained by affinity chromatography, were dialyzed against buffer A (Tris–HCl, 20 m*M*, pH 7.4) and applied to a MonoQ HR5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution was done by salt gradient.

2.2.4. Determination of protein concentration

Protein concentrations of the samples were performed using the Bio-Rad DC Protein Assay Kit (Bio-Rad, USA) following the protocol recommended by the manufacturer.

2.3. Generation antibodies

Rats were immunized with 3×20 μg of GST–Fbe, with a 2-week interval. Serum samples were taken 2 weeks after the last immunization. Freund's complete adjuvant was used at the first time and incomplete the second and third times.

2.4. Extraction of proteins from explanted materials

To extract components from explanted biomaterials, polyethylene catheters subcutaneously implanted in rats (SIC), tips of peripheral venous catheters from patients (PVC), and tips of central venous catheters from patients (CVC), were extracted with 2% sodium dodecyl sulfate (SDS) at 95 °C for 10 min as described by Francois [4]. All human materials used in this study were collected from Huddinge Hospital, Stockholm, Sweden.

2.5. Interaction between GST–Fbe and components absorbed on the explanted materials

Components in the extract of explanted catheters (SIC, CVC, and PVC) were separated by 10% Homogeneous Tris–HCl Precast Gel for PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Serially diluted Fg was included as a control to determine the sensitivity and specificity of antibodies against Fg. Extraction from polyethylene catheters coated with Fg (FgCC) in vitro was included as a control of coating efficiency of Fg on catheters. Proteins separated on SDS–polyacrylamide gel electrophoresis

(PAGE) were visualized by silver staining. Fg-related components were detected by Western blotting probing the transferred nitrocellulose membranes with horseradish peroxidase-conjugated rabbit IgG against Fg (Fg–HRP or Fn–HRP, DAKO, Denmark, diluted 1000 times in PBS+0.05% Tween 20). Components recognized by GST–Fbe were detected by probing the membranes with GST–Fbe (10 μg/ml). Bound GST–Fbe was subsequently detected by rat-anti-GST–Fbe (diluted 1000 times in PBS+0.05% Tween 20). The bound rat antibodies were detected by anti-rat-HRP (DAKO, Denmark, diluted 1000 times in PBS+0.05% Tween 20). All bound HRP-conjugated antibodies were substrated by 4-chloro-1-naphthol tablets (Sigma) as recommended by the manufacturer.

3. Results and discussion

3.1. GST–Fbe was purified in fusion with glutathione S-transferase

A transformant was isolated with a plasmid, pPL46, encoding a fusion protein composed of GST and a portion of the fibrinogen binding protein corresponding to the DNA fragment originally cloned in the phage display system. This protein was called GST–Fbe [13]. As shown in Fig. 1, induced for 2 h gave the similar yield as 4 h, but induced for 4 h gave a higher yield in cell density. While induced overnight, the yield of GST–Fbe was dropped. Thus, induction time with IPTG was set to be 4 h.

The GST–Fbe from the lysed cell culture could be purified from glutathione–Sepharose with affinity to the fusion partner, GST-tag and from Fg–Sepharose with the affinity to the subcloned portion of Fbe. As a control, GST was purified from lysate of TG1 strain harboring pGEX-4T3 by glutathione–Sepharose (lane 1, Fig. 1) but not by Fg–Sepharose (data not shown). Samples prepared from glutathione–Sepharose and Fg–Sepharose were further purified by FPLC under the designed condition. The pI of GST–Fbe was predicted to be 4.43. The experimental pI was with pI 4 and 5 on IEF 3–9 gel. Thus, the optimal condition for FPLC was set to be dialyzed against Buffer A (40 m*M* Tris–HCl at pH 7.4). A peak was eluted at a salt concentration of approximate 29% of Buffer B (Buffer A plus 1 *M* NaCl) as shown in Fig. 2. The amount of co-purified proteins from host strains was less than 10% on the sample prepared by affinity chromatography on glutathione–Sepharose.

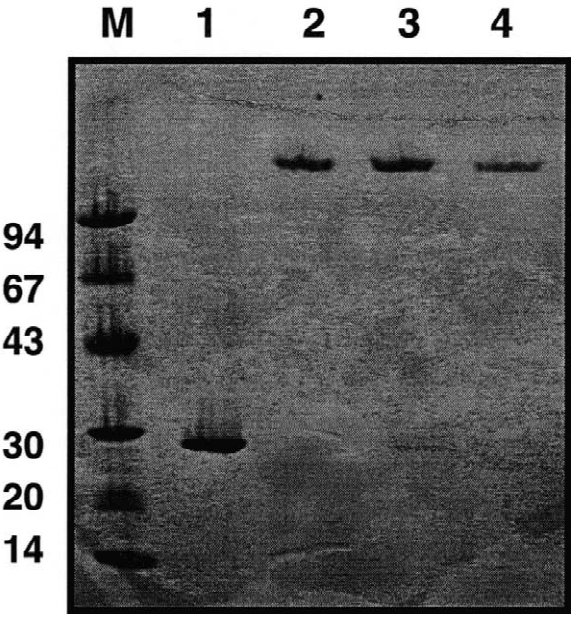

Fig. 1. Induction of GST–Fbe by IPTG Lane M, marker. Lane 1, GST purified from glutathione Sepharose. Lane 2, GST–Fbe purified from culture induced with IPTG for 2 h. Lane 3, induction for 4 h and Lane 4, induction overnight.

3.2. Comparison of methods to purify GST–Fbe

Proteins of GST–Fbe purified from glutathione–Sepharose, Fg–Sepharose and FPLC could interact with soluble or immobilized Fg in capture-enzyme-linked immunosorbent assay (ELISA) in a similar way (refer to [13]). Despite the similarity, purification on glutathione–Sepharose was simpler and gave a higher yield than purification on Fg–Sepharose. Purification on FPLC did not contribute further to the purity of the proteins (as shown in Fig. 2). The minor amount of co-purified proteins from host cells did not affect the interaction between GST–Fbe and Fg. Thus, proteins purified from glutathione–Sepharose were used for antibody production and other studies. In addition, the ability of GST–Fbe purified from Fg–Sepharose indicated that fusion with GST did not

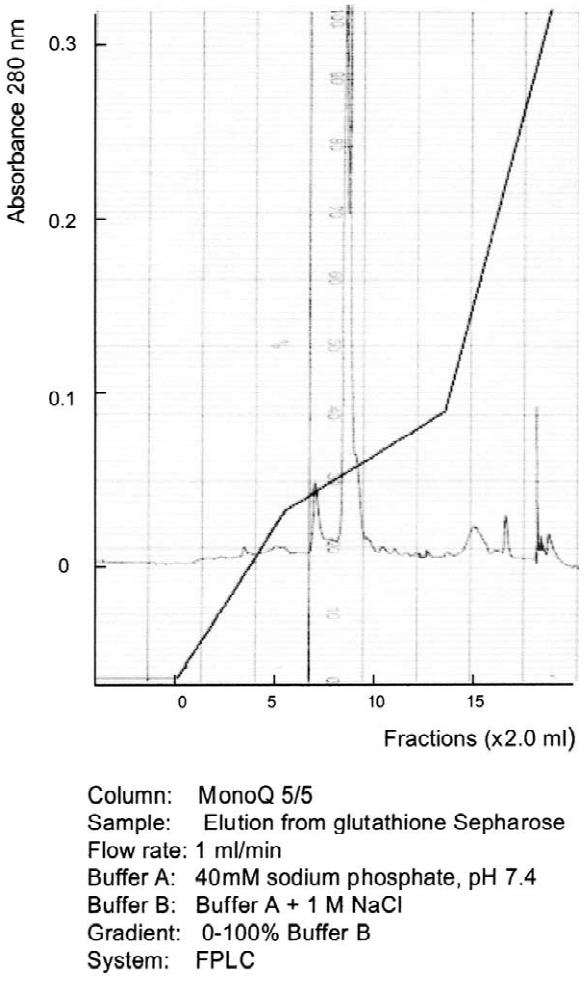

Fig. 2. FPLC on sample eluted from glutathione–Sepharose. The running condition was applied as described.

alter the Fg affinity of the protein of interest. In a further kinetic study of the interaction between fluorescent-labeled Fbe and Fg with a fluorescent correlative spectrometer, further purification of GST–Fbe on FPLC can give a better performance in obtaining a mono-form of labeled GST–Fbe (data not shown).

3.3. GST–Fbe can recognize the beta-chains of Fg from the explanted materials

Three types of explanted materials have been subjected to studies on the components adsorbed onto them—SIC from rats, PVC and CVC from patients. Silver staining revealed more than 30 bands from these materials representing host components—extracellular matrix (ECM), plasma proteins and/or host cells, adsorbed on polymer surfaces as shown in lane 1 in Fig. 3A. Derivatives of fibrin(ogen) were found in all extracts of these samples, particularly its β-chains. In the extract from SIC, two bands were detected by anti-Fg–HRP—one was smaller than 16 kDa, and the other was of similar size as β-chains of Fg as shown in lane 1 in Fig. 3B. In the extract from PVC, two bands were detected by anti-Fg–HRP—one was larger than 83 kDa in size, and the other one was of similar size as β-chains of Fg (lane 2 in Fig. 4A and B). In the extract from CVC, one band was detected, which was of similar size as β-chains of Fg (lane 3 in Fig. 4A). Further studies on extract from PVC showed that only one of these fibrin(ogen)-derived proteins, its β-chains, was recognized by GST–Fbe, as shown in lane 5 in Fig. 4B, whereas in a control, β-chains of Fg were recognized by GST–Fbe [13], also shown in lane 4 in Fig. 4B.

In the previous functional studies of Fbe, we have shown that Fbe binds to the β-chains of Fg [13]. Recently, Davis et al. had reported that the binding region of Fbe was located at the N-terminal of the β-chains, overlapping the thrombin cleavage site of fibrinopeptide B [16]. As a dominant component in plasma, Fg was found present not only constantly but also actively promoted bacterial adherence in explanted materials [4,17]. β-chains of Fg are found to be more resistant to enzyme degradation [18]. A recent report shows that 70% of the fibrinopeptide B remains intact in the clots of whole blood [19]. Implanted biomaterials are covered by plasma components from the host fluids, in which Fg seems to be abundant. This is supported by our findings in extracts from implanted materials (e.g., PVC, CVC, and SIC) and by other researchers [4,5,17]. In studies on mutants of *S. epidermidis* lacking *fbe*, less bacterial adherence to Fg was reported, which suggested that Fbe might be the major Fg adhesin of *S. epidermidis* [15]. The results obtained in this study showed the β-chains-like Fg components absorbed on the surface of explanted materials could be recognized by GST–Fbe. Therefore, binding to Fg, particularly its β-chains, may significantly contribute to the adherence of *S. epidermidis* to implants in vivo.

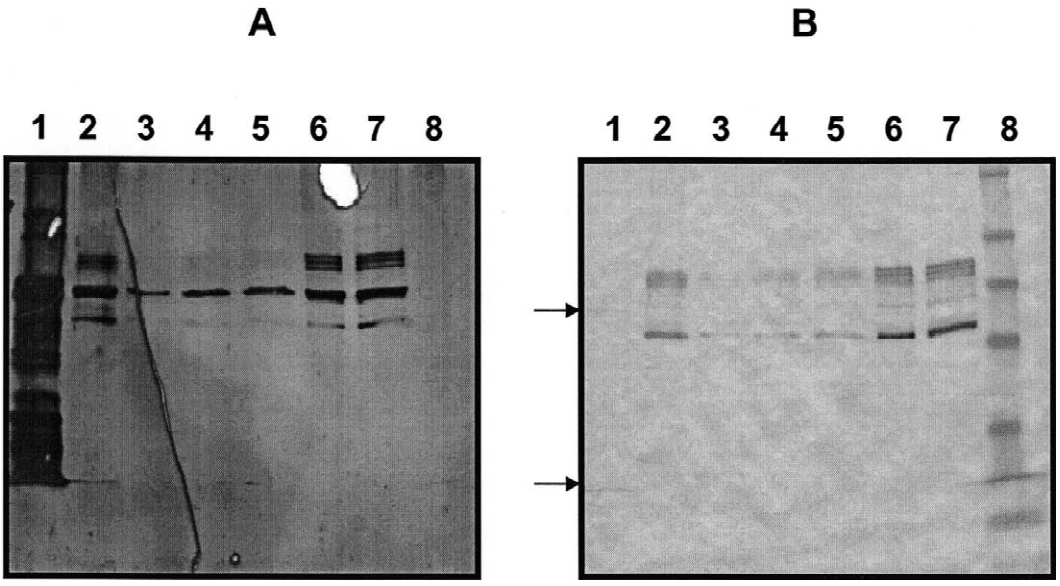

Fig. 3. Extract of SIC from rats. (A) Silver staining. Lane 1, extraction from subcutaneously implanted catheters (SIC) in rats. Lane 2, extraction from Fg-coated catheters. Lane 3–7, standard of Fg at 0.1 mg, 0.3 mg, 0.5 mg, and 1 mg, respectively. Lane 8, pre-stained marker (176, 83, 62, 47, 32, 25 and 16×10^3, respectively). (B) Immunodetected by anti-Fg–HRP. Same order as A.

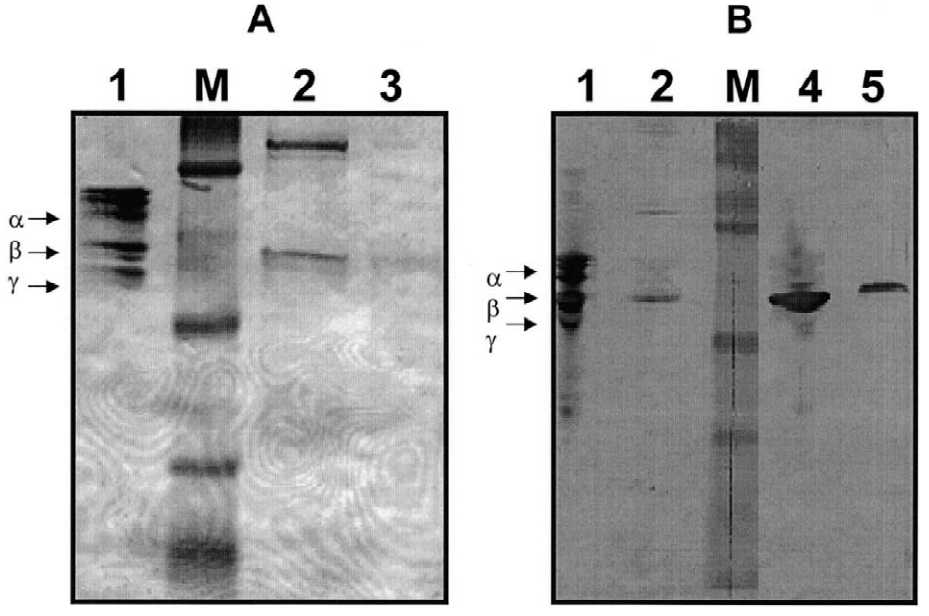

Fig. 4. Extract of PVC and CVC. Immunodetected by anti-Fg–HRP. Lane 1A and 1B, Fg. Lane 2A and 2B, extraction from PVC. Lane 3A, extraction from CVC. Probed by GST–Fbe, followed by rat's anti-GST–Fbe and then rabbit's anti-rat-HRP. Lane 4B, Fg. Lane 5B, extraction from PVC. Lane M, pre-stained marker (83, 62, 47, 32, and 25×10^3, respectively).

4. Conclusion

In summary, we report methods to express and purify a portion of Fbe, a fibrinogen binding protein of *S. epidermidis*, in its fusion form with glutathione S-transferase (GST). A sufficient amount of GST–Fbe has been obtained. The binding properties of GST–Fbe to Fg have been studied. Results on extraction from implant materials revealed that interaction between GST–Fbe and β-chains of Fg absorbed on the implant surfaces may be important for the pathogenesis of foreign body infections caused by *S. epidermidis*.

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