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Purification of PIA and rSesC as Putative Vaccine Candidates Against *Staphylococcus aureus*

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Abstract

Background: *Staphylococcus aureus* is predominant at sites of biomaterial-associated infection (BAI) and frequently infects hospitalized individuals.
Methods: The polysaccharide intercellular adhesin (PIA) and *S. epidermidis* rSesC protein, major macromolecules in biofilm formation, were purified under native conditions and cloned and expressed in a prokaryotic host.
Results: Purification of the macromolecules was confirmed by FTIR and Western blotting.
Conclusions: The *S. epidermidis* SesC protein and PIA were successfully purified. Both are considered as vaccine candidates.

Keywords: Biofilm formation, Polysaccharide Intracellular Adhesin, *Staphylococcus aureus*.

Introduction

Nosocomial infections are known to be a serious threat to healthcare systems in both industrialized and developing countries (1). *Staphylococcus aureus*, as an opportunistic pathogen, is notable for its frequency and severity of infections, with more than 20% of nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA) (2, 3). *Staphylococcus aureus*, one of the most common causes of infections related to implanted medical devices, infects both hospitalized patients and immunocompromised individuals (4). Due to *S. aureus'* antibiotic resistance, proper strategies may be needed to control MRSA infections (5). *Staphylococcus aureus* possesses diverse virulence factors involved infection and antibiotic resistance. These include both capsule and cell wall-bound adhesion molecules, surface proteins, and toxins (6). *Staphylococcus aureus* and *S. epidermidis* are predominant at sites of biomaterial-associated infections (BAIs), causing approximately 66% of these infections. Their ability to form biofilms *in vivo* makes them highly resistant to chemotherapeutics, which may lead to chronic diseases (7). A biofilm is defined as a community of cells encased within a slimy exopolymeric matrix made up of extracellular polymeric substances (EPS) and attached to a surface (8, 9). Biofilms are resistant to antimicrobial therapy and host defenses (7). The *ica* operon of *staphylococcus* spp encodes the production of polysaccharide intercellular adhesin (PIA), also known as poly-N-acetyl glucosamine (PNAG). The PIA-dependent mechanism is the best understood mechanism of biofilm formation (10-12). Cerca et al. demonstrated that rabbit anti-PIA antibodies protect against infections with planktonic cells of PIA-positive *S. aureus* and *S. epidermidis* (13). Based on a study by Maira-Litran et al., anti-dPIA antibodies mediated opsonic killing and protected against *S. aureus* infection (14). Recent studies indicate other proteinaceous mechanisms of biofilm formation may exist. Shahrooei revealed that monoclonal antibodies against *S. epidermidis* surface-exposed

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Ses proteins can significantly reduce the accumulation phase (15). Clumping factor A (ClfA) is a fibrinogen (Fg) -binding microbial surface molecule recognizing adhesive matrix molecules (MSCRAMM) of *S. aureus*; 65.1% similarity has been shown between SesC and a 341-aa fragment of ClfA (16).

Purification of PIA and cloning and expression of rSesC, as putative vaccine candidates against *S. aureus*, were the main goals of this study.

Materials and methods

For DNA manipulation and recombinant protein production, *Escherichia coli* strain BL21 (DE3) was applied. Moreover, we employed two biofilm-forming *S. epidermidis* strains, ATCC 14990 and 35984, also known as 1457 and RP62A, and a transmutant strain, 1457-M10, for PIA purification and related procedures.

Sequences of forward and reverse primers for the 388 bp (sesc) and 1400 bp (*rsesc*) targeted gene PCR product were listed in table 1. (13, 14). Amplification was done on Gene Amp PCR system (Applied Biosystem, USA) by uniplex PCR method in total volume 25 µl (*sesc* and *rsesc*) containing; 12.5 µl master amplicon (Biolab, New England, UK), 1 pmol of each forward and reverse primers, a 1.5 µl of crude DNA as the template and 10 µl distilled water. Amplification of both gene determinants were accomplished using program an initial cycle of denaturation 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 57 °C (*sesc* and *rsesc*) for 30 seconds, 72 °C for 1 min with a terminal extension for 10 min. Resulting PCR were visualized with 1% agarose gel (KBC, Max Pure agarose, Spain). Molecular approach was optimized using S. *epidermidis* 1457, RP62A and *S. epidermidis* strain 10b as the control strains. Fig. 1a.

Table 1. Sequences of utilized primers in current study.

Targeted genes	Sequences of primers
sesc	Forward: gttgataaccgtcaacaagg
	Reverse: catgttgatcttttgatccc
rsesc	Forward: acgtgctagcgcagattcagaaagtacatc
	Reverse: atgcggatcctagtgatggtgatggtgatgatcagctgtagctgttcc

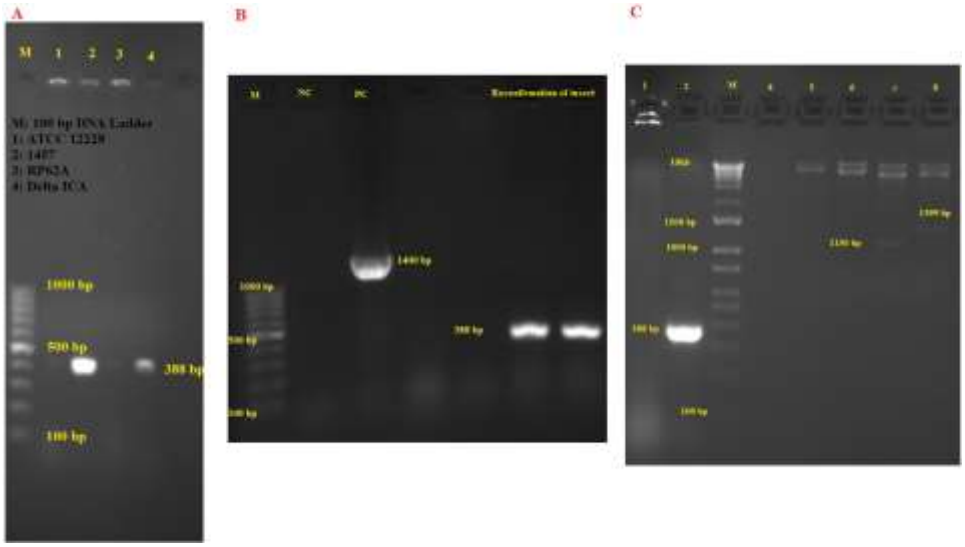

Fig. 1. Agarose gels of PCR products. Gel A) *sesC* amplifications taking advantage of specific primer to sesC gene (sequences of the primer listed methods section) Lane M, DNA ladder; Lanes 1-4: S. *epidermidis* ATTC 1228; S. *epidermidis* 1457; S. *epidermidis* RP62A; and transmutant S. *epidermidis* 1457 Δ *ica*. B) Amplification of 1400 bp insert sequence by colony PCR using specific primer (Sequence of primers and PCR condition has been enlarged in Table 1). NC, Negative control; PC, Positive control (S. *epidermidis* 10b wild), Reconfirmation of insert, *sesC* gene indicator 388 bp. C) Confirmation of presence of the 1400 bp (responsible gene for protein synthesis (1359 bp plus 40 bp restriction sites and hexa-His sequence) insert by restriction enzymes in expression vector *pEt11c* (M, DNA ladder; 5, purified expression vector; 6, single digestion by *BamH1* enzyme: 4, single digested vector by *Nhe1* Enzyme: 7, double digested expression vector by *BamH1* and Nhe1 restriction enzymes, 8, single digestion by *HindIII* restriction enzyme.

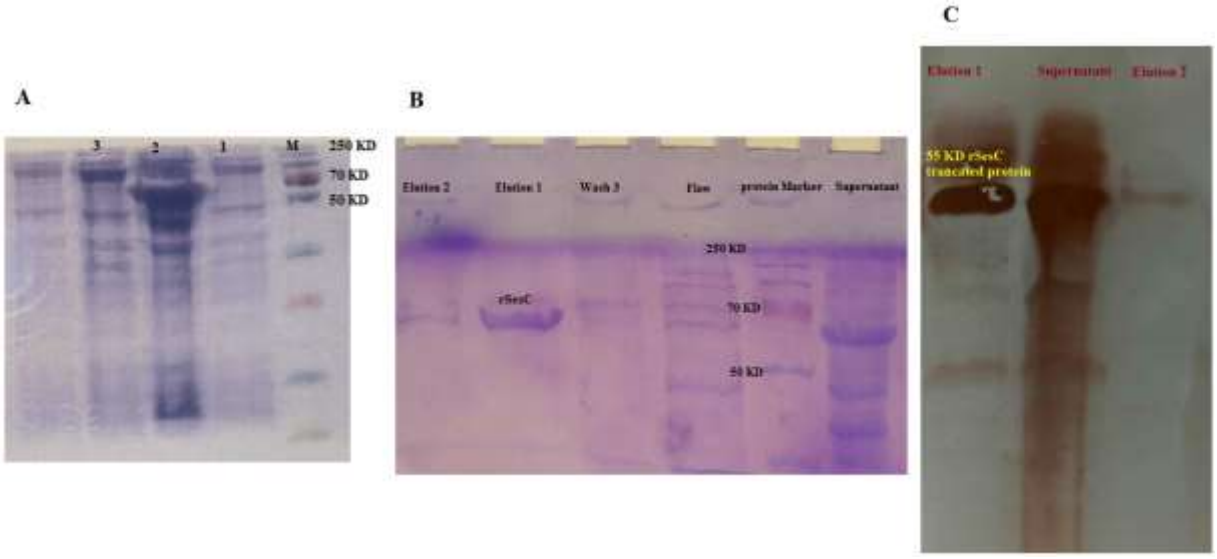

Fig. 2. SDS-PAGE of purified recombinant protein. A) Analysis of expression of rSesC protein (55 KD) by SDS-PAGE. 1, uninduced BL21 strain; 2, induced BL21 by 1 mM IPTG, 3, induced control strain by 1 mM IPTG. B) Analysis of purified rSesC protein (55 KD) by SDS-PAGE. Protein was purified by Ni- affinity procedure in a 12% acrylamide gel; the size and purity of the truncated protein was determined by SDS-PAGE. M, pre-stained protein ladder; W3, wash 3, E1, 2, Elution 1, 2. C) Western blot of the purified rSesC with anti-hex histidine antibody. The purity of the protein was estimated to be about 90%.

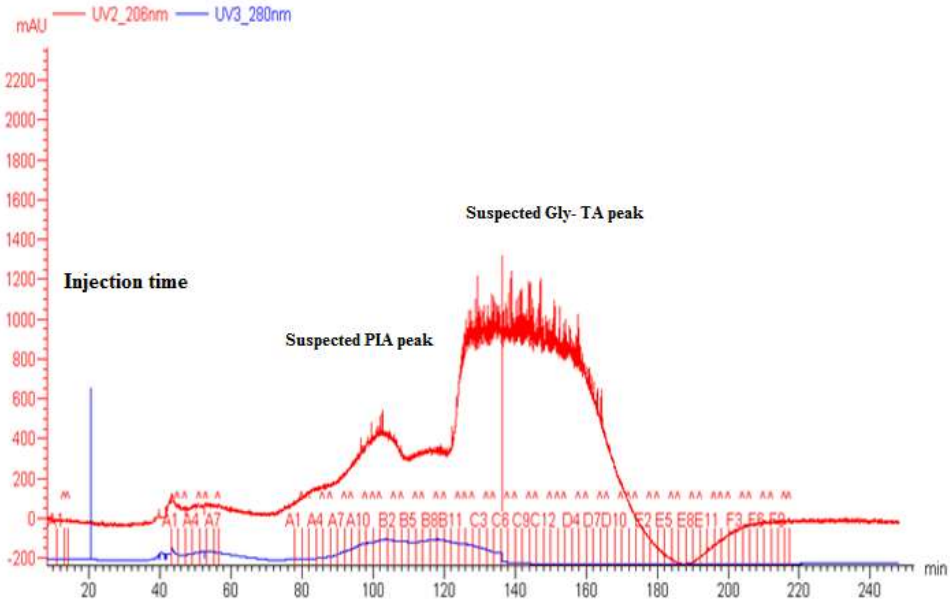

Fig. 3. Representative FPLC chromatograms for S. *epidermidis* strain 1457 (wild type); Gly-TA: glycerol teichoic acid. Purified PIA collected 80 min after injection. The PIA peak (a) was collected and pooled and applied for the coupling process.

Construction and purification of His-tagged rSesC protein

Utilizing a previously reported procedure (16) a 1,359-bp fragment of *sesC* encoding a 459-aa extracellular part of SesC containing a six-His tag at the C-terminus was amplified. In short, A 459 aa extracellular part of SesC containing a six-His tag at the C-terminus was constructed and purified utilizing a known procedure. rSesC protein was purified by expression of the representative sequence in a prokaryotic host. Expression of rSesC (55 KD) was determined by SDS-PAGE and Coomassie Brilliant Blue staining. (Fig. 2). Recombinant protein was expressed in *Eshershia*

coli BL21 DE3 as prokaryotic host following the induction of by 1 mM imidazole in optical density 0.7-0.9 in shaker incubator (150 rpm in 37 ˚C for 3 hrs). Target protein was purified by a commercial affinity chromatography Kit (GE health care, Sweden) according to the manufacture recommendation. The purity of the recombinant protein was determined by Coomassie blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then analysed by a western blotting procedure followed by extraction of soluble protein from induced host using sonication (4 times for 30 s on ice). An additional centrifugation (8000 G for 20 min at 4 °C) was performed in order to clarify (16). *E. coli* BL21 (DE3) cells were transformed with the vector. Cells were grown with shaking at 250 rpm at 37 °C in Luria-Bertani broth with 100 g/ml ampicillin to an optical density at 600 nm of 0.6 to 0.8. Expression was induced by addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG). Following cooling on ice, cells were collected by centrifugation at 8,000 rpm for 5 min at 4 °C, resuspended in imidazole buffer, and frozen at -20 °C. The preparation was sonicated three times for 30 s on ice. rSesC (55 KD) expression was determined by SDS-PAGE and Coomassie Brilliant Blue staining.

Extraction and purification of PIA

PIA was purified as previously described (18, 19). Briefly, 2 L of trypticase soy broth (TSB) were inoculated with PIA^+ *S. epidermidis* 1457 strain colonies at 37 °C for 24 h with shaking at 40–50 rpm/min. Cells were harvested by centrifugation at 1000 G for 20 min at 4 °C. Pellets were resuspended in 20 ml of 50 mM sodium phosphate buffer, pH 7.5, and sonicated four times for 30 S on ice. Extracts were clarified by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was dialyzed in 12 KD dialysis bag; overnight against the same buffer and concentrated using Centriprep 10 (Amicon, Witten, Germany). Following elimination of soluble proteins by with Proteinase-K, the sample was directly loaded onto an equilibrated 1.6 x 100 cm Sephacryl S-100 column (Pharmacia LKB GmbH, Freiburg, Germany) with 50 mM sodium phosphate. (Fig. 3). At the end, purified PIA was stored at -20 °C in Amicon cell (Fig. 3). The concentration of the purified PIA was assessed via the amount of hexosamine, taking advantage of the 3-methyl-2-benzothiazolone hydra- zine hydrochloride method with N-acetyl glucosamine as standard (18).

Biochemical analysis

The protein content of the rSesC was determined using the Bradford assay (17). The constitution and structure of the purified native PIA was analysed using Fourier transform infrared spectroscopy (FTIR) as well.

Results

Construction and purification of candidate protein

Using specific primers (Table 1), a coding fragment of the *sesC* gene of *S. epidermidis* strain 10b was amplified incorporating flanking *BamHI* and *NheI* restriction sites (Fig. 1b). The amplified fragment encoding the truncated recombinant SesC (rSesC) protein was cloned into a *pET11c* expression vector and reconfirmed by double digestion procedure. Fig. 1c. The PCR product was sequenced and compared to the nucleic acid sequence encoding the rSesC recombinant protein from the National Center for Biotechnology Information (NCBI) using BLAST online software. rSesC protein purified by using imidazole in 300 mM concentration as elution buffer. The purity of the recombinant protein was determined by Coomassie blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then the C-terminal hexa-His-tagged residue in the protein was confirmed by Western blotting. Fig. 1c.

Purification of PIA

Suspected PIA fractions were analyzed according to the obtained peaks using a colorimetric assay. This procedure provides analysis of hexosamine present in glycosaminoglycan under conditions of mild acid treatment (pH=5). Results of FPLC were analyzed by the amount of hexose by the phenol sulfuric acid assay with glucose as the standard, hexosamine by the 3-methyl-2-benzothiazolone hydrazine hydrochloride

method with N-acetylglucosamine as the standard, and ketoses by the phenol boric acid-sulfuric acid assay with fructose as the standard (20).

Native PIA, a major component of bacterial biofilm formation was isolated from *S. epidermidis* wild type strain 1457. Contaminating DNA, RNA, and protein were eliminated by enzymatic digestion. The 100 kDa PIA molecule was isolated by size exclusion chromatography using a Sephacryl S-100 column. PIA was purified at a flow rate of 1 ml/min and 2 ml fractions were collected in a fraction collector. Chemical analysis of purified PIA indicated negligible contamination (< 2% protein and < 0.4% nucleic acid) and showed that the purified PIA contained 65% hexosamine (5700 µg/ml), uronic acid (29 µg/ml), and ketose (170 µg/ml). The composition and structure of PIA were determined using FTIR. Using the regularized method of deconvolution, the purified polysaccharide was evaluated by infrared spectroscopy. In short, powdered samples were dispersed in KBr pellets and recorded with a TENSOR 27 Bruker instrument, averaging of 256 scans on the FTIR spectrometer (Fig. 4).

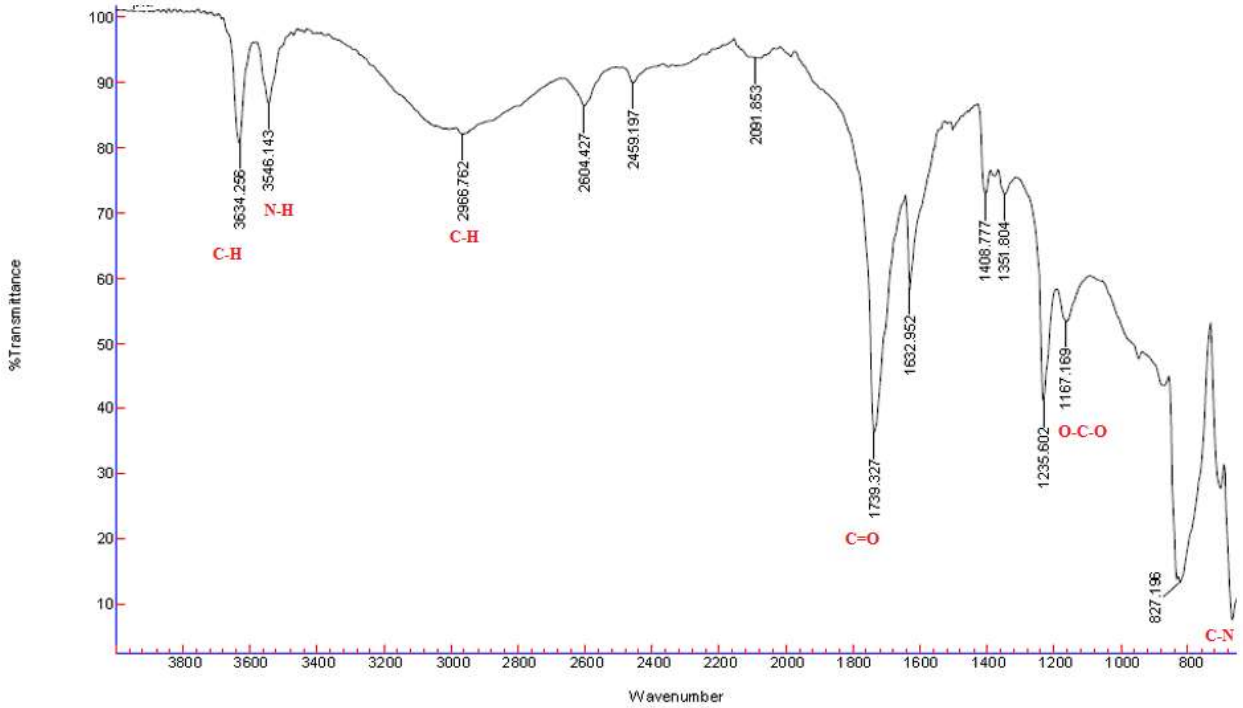

Fig. 4. IR spectra of PIA in the 4000-500 см-1 range and the upshot of this deconvolution. Using of the deconvolution method it is possible to establish spectral characteristics of polysaccharides with glycosidic linkage in 4000-500 см-1 range. Spectral curves in the figure show that in the 3600-850 cm-1 range all the bands are observed. Regions where stretching vibrations ν(CO) C-O-C glycosidic bridge in oligosaccharides manifest in the 1175-1140 cm-1 region.

Discussion

Biofilm formation has been assigned as a main staphylococcal virulence factor. There is yet no agreement on the life cycle of staphylococcal biofilm, however, most researchers believe that it develops in four stages including attachment, accumulation, maturation, and dispersal (16). Bacterial surface proteins such as the cell wall-anchored (CWA) play important roles in microbial adherence to host tissues. Evasion of host defense systems and biofilm formation and are known as important virulence factors among gram-positive bacterial pathogens (15, 16, 18). rSesC and PIA as two relevant macromolecules in biofilm formation process are studied at the recent decades (15, 16). Because of similarity between 341-aa fragment of ClfA and SesC protein (65.1%)

rSesC protein is selected as a putative vaccine candidate in *S. aureus* (16).

PIA has extensively been evaluated as a putative candid for vaccine development. When PIA is the major component of the extracellular matrix, biofilm formation is classified into either PIA-independent or PIA-dependent. PIA confers major functional effects in biofilm formation, which bears an important role in the pathogenicity of *S. epidermidis* (18). Our results show similarity with previously published findings (11, 18). In the current study, rSesC protein and PIA of *S. epidermidis* were purified. The potential of mouse polyclonal antibodies raised against the PIA, rSesC, and a combination of PIA and rSesC, will be measured for eradication of *S. aureus* biofilms *in vitro*.

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