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RESEARCH ARTICLE

Heterologous expression of staphylococcal enterotoxin B (*seb*) gene for antibody production

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Abbreviations:
BCA: Bicinchoninic acid
BW: biological warfare
DAB: diaminobenzidine
FCA: Freund's Complete Adjuvant
FIA: Freund's Incomplete Adjuvant
MHC: major histocompatability complex
OD: optical density
OPD: o- phenylenediamine
SEs: staphylococcal enterotoxins
SFP: staphylococcal food poisoning

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Staphylococcal food poisoning (SFP) is caused by the members of superantigen family called staphylococcal enterotoxins (SEs). About 20 different types of SEs are produced by *Staphylococcus aureus* out of which type A (SEA), B (SEB), C (SEC) and D (SED) are commonly implicated in SFP. Among these, SEB is the most potent toxin and has also gained the status of biological warfare (BW) agent. Therefore, detection of SEB is of utmost importance. Any immunological detection system for SEB requires specific and sensitive antibodies which inturn depends on the purity of the SEB. In the present investigation, *seb* gene of *S. aureus* was cloned and expressed in *E. coli* along with biotin as fusion partner to facilitate the purification process. The yield of purified recombinant SEB was 13.1 mg/L of culture broth. Biotin tag from the biotinylated toxin was removed by protease cleavage, and both biotinylated and non-biotinylated toxin types were used for raising hyperimmune antiserum. Antisera were also specific for SEB amongst different kinds of food poisoning agents tested by indirect plate ELISA and western blot analysis. The quality of the antisera raised in this study was found superior to the commercially available antiserum. The investigation suggests that construction of recombinant staphylococcal enterotoxin B is a good alternative for production of pure enterotoxin to be used in antibody generation.

Staphylococcal food poisoning (SFP), a form of enteritis, is intoxication rather than a disease resulting from ingestion of food contaminated with preformed staphylococcal enterotoxins (Bergdoll et al. 1974). Symptoms of SFP usually occur within 1-6 hrs after the food intake and are characterized by nausea, vomiting, abdominal cramps and diarrhoea. These symptoms usually subside in 1-3 days but the patient remains sick for 7-10 days due to the result of toxic shock (Jett et al. 1994; Do Carmo et al. 2004). Toxic shock causing SEs are single chain polypeptides having a molecular weight ranging from 27-29 kDa. SEs can withstand boiling temperature for several minutes, extremes of pH (3-11) and protease digestion by gastric enzymes (Soriano et al. 2002). Twenty different types of SEs, *viz.*, SEA through SEE, SEG through SER and SEU have already been discovered, however, only a few of the toxin serotypes are frequently associated with food poisoning outbreaks (Martin et al. 2004; Fernández et al. 2006). Toxin serotypes frequently associated with food poisoning outbreaks are SEA, SEB, SEC and SED (Balaban and Rasooly, 2000; Smyth et al. 2005).

Staphylococcal enterotoxins are 'Superantigens' and are able to bind to the major histocompatability complex (MHC) class II antigen, causing massive T cell stimulation coupled with the release of cytokines. The release of cytokines results in stimulation of neuro-receptors in the intestinal tract, and triggers vomiting center in the brain (Komisar et al. 1994; Kaempfer et al. 2002; Hemalatha et al. 2004). This effect is more severe in case of intoxication involving SEB which is also a listed biological warfare (BW) agent (Franz et al. 1997; Baker et al. 2002; Pettersson and Forsberg, 2002; Boles et al. 2003). Therefore, it is of utmost importance to develop immunological detection system for SEB, the success of which is dependent on the purity of SEB used for generating anti SEB antibodies.

Conventionally, molecular sieving and ion exchange chromatography have been used either independently or in tandem for purification of Staphylococcal enterotoxins (Tranter and Brehm, 1990; Coffman et al. 2002; Dainiak et al. 2005). Isoelectric focusing has been proved useful for purification of small amounts of staphylococcal enterotoxins but on a preparative scale the procedure has generally been used in combination with ion exchange chromatography and gel filtration (Tranter and Brehm, 1990). Use of these techniques in tandem is tedious, time consuming, expensive and sometimes does not result in purification of staphylococcal enterotoxins to the level of homogeneity. A slight contamination of toxin with other bacterial proteins results in antibodies that are less specific for the enterotoxin and hence have poor detection potential leading to false positive results. Therefore, in the present investigation, the staphylococcal enterotoxin B (*seb*) gene was cloned and co-expressed with biotin as fusion partner facilitating single step affinity purification. The suitability of the purified recombinant SEB was also studied for raising anti SEB antibodies.

MATERIALS AND METHODS

Staphylococcal enterotoxin B (*seb*) gene amplification

SEB producing *S. aureus* ATCC14458 was used to amplify the toxin gene for cloning and expression. Primers for amplification of *seb* gene were designed in our laboratory using the Primer Select 5.07 software of Lasergene version 5.07/5.52 (DNA STAR Inc., USA) and were custom synthesized by M/s Metabion, Germany. The nucleotide sequences of forward and reverse primers were: 5'-AGAGAGTCAACCAGATCCTAA-3' (21 NT) and 5'-TCACTTTTTCTTTGTCGTAACAT-3' (23 NT), respectively. The optimized PCR conditions were: initial denaturation at 94ºC for 2 min followed by 25 cycles of denaturation (94ºC/30 sec), annealing (52ºC/60 sec), extension (72ºC/90 sec); and final extension at 72ºC for 5 min. The *seb* amplicon was checked by electrophoresis on agarose (1.5%) containing 0.5 µg/ml ethidium bromide and visualized on Gel Documentation System (Bio-Rad, USA).

Ligation and transformation of *seb* into *E. coli*

The *seb* amplicon was purified using Montage Gel Extraction Kit (Millipore, USA) and ligated at 16ºC for 4 hrs with fusion vector, PinPoint Xa-1 T, containing biotin tag as per manufacturer's instructions (Promega Corp., USA). The ligated product was analysed on agarose gel for its integrity and was transformed into the expression host *E. coli* JM109 following the protocol reported earlier (Devvrat

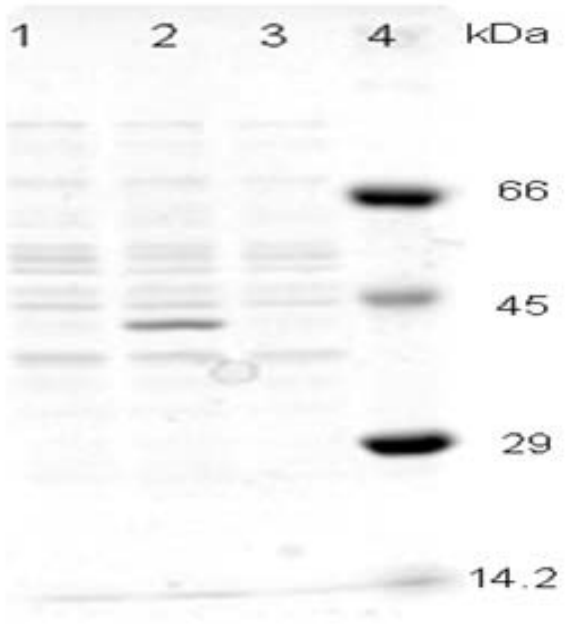

Figure 1. SDS-PAGE profile showing uninduced recombinant clone (lane 1) and induced clone showing r-SEB band at 41.4kDa (lane 2), *E. coli* host JM109 (lane 3) and molecular weight markers (lane 4).

et al. 1994). Transformants were plated on LB agar containing ampicillin (100 µg/ml) for the selection of recombinant clones. PCR was performed to screen the clones for the presence of toxin gene. The toxin gene in the vector was sequenced to check the possible point mutation. The sequencing was carried out by automated capillary sequencer ABI 3100 of Applied Biosystems, USA.

Expression in recombinant clones

Clones, found positive by PCR for the presence of *seb* gene, were checked for the expression after IPTG (isopropyl-β-D-thiogalactopyranoside) induction, lysis and SDS-PAGE (Krishnanchettiar et al. 2003). The presence of enterotoxin protein was confirmed by western blotting (Zhou and Singh, 2004) using both streptavidin alkaline phosphatase (Promega Corp., USA) and antiserum against natural SEB raised earlier in the laboratory.

Optimization of expression conditions for the production of recombinant SEB

One of the SEB positive recombinant clones, 1SEBR4, was used to standardize the growth and induction conditions for expression of recombinant SEB (r-SEB). Induction point, duration of induction and concentration of inducer, *i.e.*, IPTG were optimized by growing the clone in LB medium containing ampicillin. Briefly, a 100 ml flask containing 20 ml of LB medium, biotin (2 µM) and ampicillin (100 µg/ml) was inoculated with 200 µl of overnight grown broth culture obtained from a single colony of 1SEBR4. Different OD values (0.2-0.7) were optimized for the time of adding inducer to the culture followed by induction duration (1-6 hrs). The concentration of inducer (IPTG) was also standardized to obtain maximum yield of recombinant toxin. Cells were harvested by centrifugation at 8000 x g for 10 min and frozen at -80ºC till use.

Recombinant SEB purification

Recombinant SEB was purified from cell lysate using SoftLink™ Soft Release Avidin Resin (Promega Corp., USA). Batch mode affinity purification was done as per the manufacturer's instructions and recombinant toxin was digested with Factor Xa-Protease for removal of biotin tag. Conditions for complete digestion of fusion protein were standardized with regard to the time of incubation (4-16 hrs) and concentration of enzyme (2-10%). Yields were calculated by estimating the total protein and the purified r-SEB using Bicinchoninic acid (BCA) method (Koenig et al. 2004). The N-terminal sequencing of the Factor Xa digested r-SEB was carried out to check the N-terminal site of the digested r-SEB by Automated Edman Degradation method using Applied Biosystems 494 Procise Protein Sequencing System. Further peptide sequencing was performed to check the amino acid sequence homology of digested r-SEB with the known natural SEB protein. The sequencing was carried out by electrospray time-of-flight mass spectrometry (LC/MS/TOF) using Q Star Pulsar Applied Biosystems, USA) and spectra were analysed by Mascot Sequence Matching Software (Matrix Science).

Immunization of mice

Female Balb/c mice (20-25 g) were immunized intraperitonially for 60 days at an interval of 15 days. Undigested and enzyme digested r-SEB (500 µl) emulsified with Freund's Complete Adjuvant (FCA) in 1:1 ratio were used to prime the mice followed by booster doses with Freund's Incomplete Adjuvant (FIA). Doses of 50 ng, 1 µg, 50 µg, 100 µg and 150 µg were used for immunization.

Evaluation of antibodies raised against r-SEB

Mice were bled through retro-orbital route after 14 days of final booster, serum was separated and its titre was determined using western blot. The antiserum was also tested for its cross reactivity with SEA, SEC and SED enterotoxins, *E. coli* JM109 host, non-enterotoxigenic *S. aureus* strain ATCC6538P and other microorganisms that can gain access in the food, *viz.*, *Bacillus subitils, Enterococcus faecalis, Bacillus anthracis, Shigella dysenteriae, Salmonella typhi, Clostridium perfringens* type A, *Clostridium botulinum* types A and E*, and Staphylococcus epidermidis* by indirect ELISA (Agarwal et al. 2002) and western blot. SEA, SEC, SED and supernatants of overnight grown cultures of test organisms were run on polyacrylamide gel for western blot, and

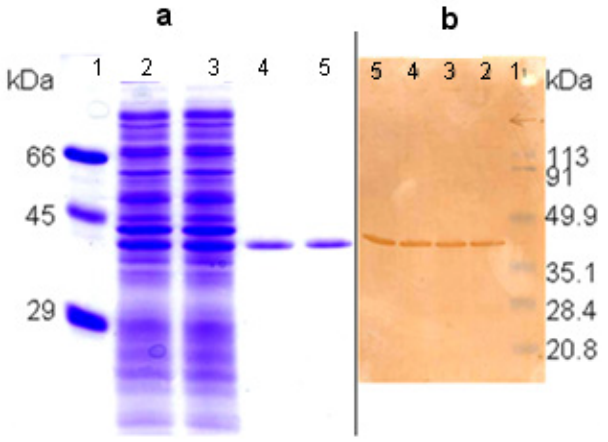

Figure 2. SDS-PAGE and western blot profiles showing affinity purified r-SEB. Panel a shows SDS-PAGE profile of 1SEBR4 cell lysate (lanes 2 and 3), and affinity purified r-SEB (41.4 kDa) (lanes 4 and 5), lane 1 shows molecular weight markers. Panel b shows western blot profile of 1SEBR4 cell lysate showing r-SEB (lanes 2 and 3), and affinity purified r-SEB bands at 41.4 kDa (lanes 4 and 5), lane 1 shows molecular weight markers.

coated onto the 96 wells polystyrene plate (Nunc, Denmark) for ELISA. Mouse anti r-SEB serum as source of primary antibodies and anti mouse immunoglobulins-HRP conjugate (Dako, Denmark) as revealing antibodies were used at 1:2000 dilutions. The 3, 3'- diaminobenzidine (DAB) for western blot, and the *o*- phenylenediamine (OPD) for ELISA were used as substrates to develop the reaction colour. The ELISA reaction was read at 492 nm. Performance of anti r-SEB serum was also compared with the commercially available antiserum from M/s Toxin Technology, USA. Both the antibodies were tested at 1:2000, 1:5000 and 1:10000 dilutions for specificity against culture supernatants of SEB producing *S. aureus* strain ATCC14458 and non-enterotoxigenic strain ATCC6538P using the western blot test format as described above.

RESULTS AND DISCUSSION

SEs are the second most common agents of food poisoning all over the world (Balaban and Rasooly, 2000; Greenfield et al. 2002; Di Pinto et al. 2004; Smyth et al. 2005). Among these, SEB is the most potent and a listed BW agent (Baker et al. 2002; Kaempfer et al. 2002; Pettersson and Forsberg, 2002; Boles et al. 2003). Therefore, it is imperative to have an immunological system for detection of SEB. The major problem encountered in the development of an immunological detection system is the limitation of high affinity and specific antibodies against SEB because of its superantigenic nature. Slight contamination of other proteins leads to low affinity and non-specific antibody response against the target super-antigen. Therefore, SEB should be available in highly pure form. Purification of SEB is tedious and time consuming by conventional protein purification methods like size exclusion and ion-exchange chromatography (Tranter and Brehm, 1990; Coffman et al. 2002; Dainiak et al. 2005). An alternative approach to achieve high level of protein purity is heterologous expression of desired gene into a suitable vector containing a fusion partner that can be used as affinity tag for single step purification (Nilsson et al. 1997; Constans, 2002; Mukherjee et al. 2003).

In the present study, *seb* gene of *S. aureus* ATCC14458 was amplified by PCR that resulted in 721 bp amplicon. The PCR amplified gene was ligated to PinPoint Xa-1 T-vector having biotin as fusion partner. The recombinant vector containing the *seb* gene was transformed into *E. coli* JM109. Eight transformants were analysed for the presence of *seb* gene by PCR and all were found to contain the insert. The orientation of *seb* gene was determined by sequencing and the gene sequence was found identical with that of *seb* gene of *S. aureus* strain ATCC14458 already submitted by the authors to the NCBI GenBank (Accession number AY852244). The gene sequence was also identical with the earlier reported *seb* genes from *S. aureus* strain S6 and the partial *seb* gene sequence from strain ATCC14458 (Jones and Khan, 1986; Sergeev et al. 2004).

Seven out of eight transformants showed inducible expression of fusion protein in JM109. A thick band of r-SEB toxin was observed at approximately 41.4 kDa position upon induction of recombinant clones by IPTG (Figure 1). The position of the r-SEB toxin was also confirmed by western blots developed with streptavidin-alkaline phosphatase conjugate and rabbit anti SEB antibodies (data not shown) raised earlier in the laboratory against natural SEB toxin (Nema et al. 2004). Among the inducible clones, 1SEBR4 was selected for further studies of gene expression and protein purification. Induction conditions, *viz*., induction point, induction duration and inducer concentration were optimized for the clone, 1SEBR4. Optimum optical density (OD) for adding the inducer, *i.e.*, IPTG was 0.3-0.4 which was achieved within 2-3 hrs of culture inoculation. The maximum yield of r-SEB was obtained when 1SEBR4 was induced by IPTG at a concentration of 75 µM for four hours. Cells from induced clone were sonicated and the cell lysate was used for single step affinity purification of r-SEB by using SoftLink™ Soft Release Avidin Resin. The purified r-SEB showed single band on SDS-PAGE and western blot developed by rabbit anti SEB antibodies raised earlier in our laboratory (Figure 2). The advantage of using biotin as purification tag was reflected in the purity of the r-SEB as revealed by the presence of single band on SDS-PAGE. On the other hand use of HIS tag, sometimes, results in copurification of non-specific metal binding proteins in addition to heterologously expressed protein (Finzi et al. 2003; Mukherjee et al. 2003). The yield of purified r-SEB was 6.6% (w/w) of total protein and 13.1 mg/L of culture broth. The estimated yield of r-SEB obtained by Ignatov et al. (1993) was 1.7% only whereas Yang et al. (2002) reported a yield of 33.3% in JM109. The major limitation of both these investigations was that they did not use

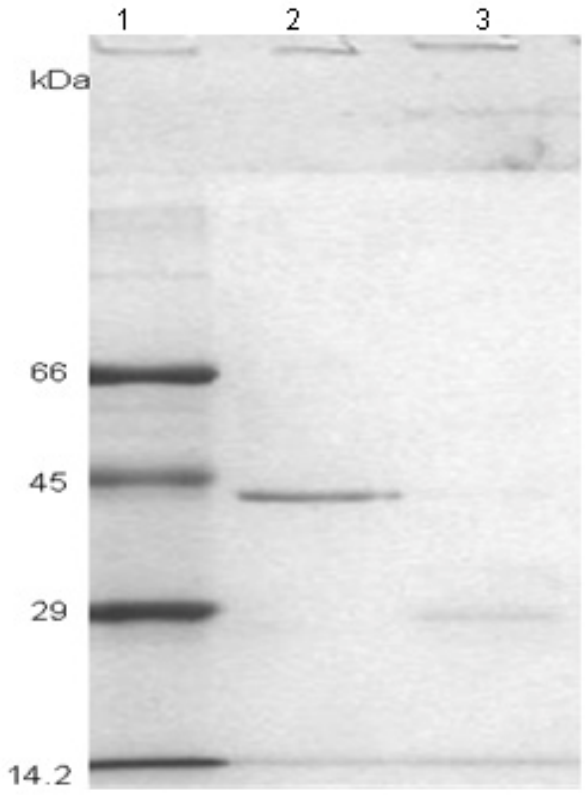

Figure 3. SDS-PAGE profile showing undigested r-SEB at 41.4 kDa (lane 2) and Factor Xa digested r-SEB at 29.5 kDa (lane 3). Lane 1 shows molecular weight markers.

affinity tag for purification of the toxin. Moreover, none of these workers indicated the specific applications of r-SEB.

In the present investigation, r-SEB fusion protein was further used for raising the hyper immune serum in mice to study its suitability for detection of SEB. The r-SEB was subjected to Factor Xa protease digestion to remove the biotin affinity tag so that its possible interference with specific and high titre antibody generation against r-SEB can be avoided. Factor Xa protease concentration of 8% (w/w) was found optimum for complete removal of biotin tag from r-SEB when incubated for 16-18 hrs (Figure 3). The N-terminal sequencing of the protease digested r-SEB confirmed its proper cleavage by Factor Xa protease. The LC-MS profile of the protease digested r-SEB showed that the amino acid sequence of r-SEB was matching with the natural SEB toxin. Size of the protease digested r-SEB was estimated to be 29.5 kDa by LC-MS. The protease digested r-SEB (29.5 kDa) and undigested r-SEB (41.4 kDa) were used for immunizing mice. Serum titres obtained in both the cases were 1:32000 as revealed by western blot. These sera were further evaluated for their cross reactivity with SEA, SEC and SED enterotoxins, non-enterotoxigenic *S. aurues* strain ATCC6538P, *E. coli* host JM109, *Bacillus subitils, Enterococcus faecalis, Bacillus anthracis, Shigella dysenteriae, Salmonella typhi, Clostridium perfringens* type A, *Clostridium botulinum* types A and E, and *Staphylococcus epidermidis*. No cross reactivity of the sera was observed either with other non-SEB enterotoxins of *S. aureus* or with the tested strains by the test formats used, *i.e.*, western blot and indirect plate ELISA. Staphylococcal enterotoxins and bacterial species used in this investigation for evaluation of the cross reactivity of the anti r-SEB serum are the ones which are either commonly present in the food or are important BW agents. Further, the antiserum raised in this study was found more specific for SEB detection than the other commercial antiserum available from M/s Toxin Technology, USA. During comparative evaluation of sera using western blot technique, commercial antiserum showed multiple non-specific bands even at 1:10000 dilution with culture supernatant of SEB producing *S. aureus* strain ATCC14458 and non-enterotoxigenic *S. aureus* (Figure 4). Both the antibodies were used at 1:2000, 1:5000 and 1:10000 dilutions which are commonly employed for ELISA and western blot analysis. Antiserum raised in the present investigation did not show cross reactivity even at 1:2000 dilution. This demonstrates the superiority of the antiserum raised against purified r-SEB in this study and the importance of toxin purity for polyclonal

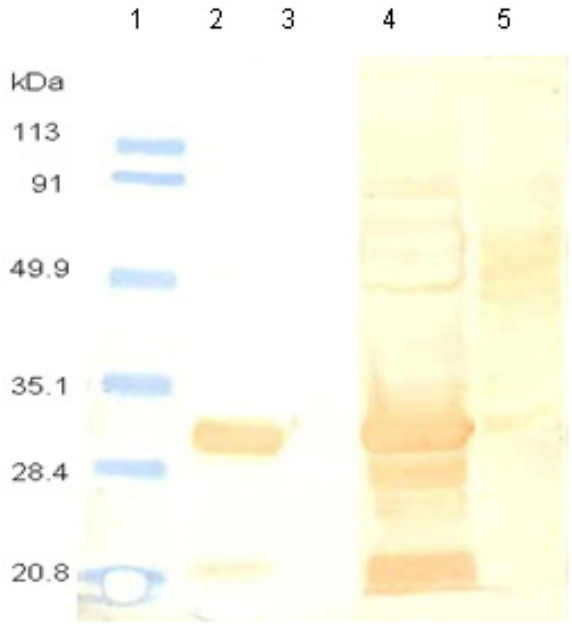

Figure 4. Western blot profile comparing anti SEB antisera from different sources. Lanes 2 and 4 contain culture supernatants of SEB (~28.5kDa) producing *S. aureus* ATCC14458, and lanes 3 and 5 contain culture supernatants of non-enterotoxigenic *S. aureus* ATCC6538P. Lanes 2 and 3 were developed with anti r-SEB serum (present study); lanes 4 and 5 were developed with polyclonal antibodies (M/s Toxin Tech, USA). Lane 1 shows pre-stained molecular weight markers.

antibody production.

Expression of r-SEB with biotin as fusion partner facilitates the single step affinity purification of r-SEB for generation of specific antibodies against it. Removal of biotin tag from the expressed protein is a costly and time consuming process. In this study, r-SEB without biotin fusion did not show any advantages over r-SEB fused with biotin for raising antiserum in terms of titre and cross-reactivity. Therefore, r-SEB along with biotin tag can be used for polyclonal antibody generation saving a lot of downstream processing time and cost. The anti r-SEB serum developed during the present study is specific and highlights the need of pure SEB toxin for generation of specific polyclonal antibodies. This antiserum can be used in a suitable format for development of an immunological system for detection of SEB. This can also be used for quality control of food products as well as for retrospective detection of SEB in cases of sabotage and/ or covert bioterrorism activities.

AY852244. Enterotoxin B gene (*seb*) sequence of *Staphylococcus aureus* strain ATCC14458. NCBI Genbank.

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