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Short communication

Identification of protein components and quantitative immunoassay for SEC2 in staphylococcin injection

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ABSTRACT

In China, staphylococcin injection has been commonly used in combined cancer therapy to enhance the systemic immune response and reduce the toxicities associated with chemotherapy or radiation therapy in the last decade. It is claimed that the main effective component is staphylococcal enterotoxin C2 (SEC2). However, no standard method based on the concentration of SEC2 has been established for quality control of the injection products. In this study, a sensitive and reliable biotin–streptavidin–ELISA (BS–ELISA) method was established for detection and quantification of SEC2. In addition, 1-D SDS-PAGE coupled with nano-LC–MS/MS was performed to identify the protein components in the injection products from one manufacturing company. The results of the BS–ELISA showed that SEC2 only accounted for less than 0.1% of the total protein in the injection products, and the nano-LC–MS/MS results showed that fifty-five proteins of *Staphylococcus aureus* were confidently identified in the injection solution. Seventeen out of these proteins, including SEC2, were well-known virulence factors. In addition, eighteen proteins of other Gram-positive bacteria were also confidently identified. Thus, the results indicated that SEC2 is of very low concentration in the injection products and the process of the injection preparation should be improved for health and safety consideration.

1. Introduction

In China, staphylococcin injection prepared from fermentation broth of *Staphylococcus aureus* (Staphylococcaceae) was widely used as a biological response modifier combined with chemotherapy or radiotherapy in cancer therapy for the last decade. Results of many clinical studies demonstrated that the patients treated with the injection showed significant increases of leucocyte count, CD4/CD8 ratio and IL-2 level, when compared with the control group [1–4]. Short-term efficacy of staphylococcin injection combined with chemotherapy or radiation therapy was confirmed in most reported clinical studies. Also, long-term survival benefit of combined tumour therapy with the staphylococcin injection was reported in several clinical studies. For instance, 63 patients with non-small-cell lung carcinoma (NSCLC) were treated with chemotherapy/radiation therapy alone or combined with the injection. The 1-year, 2-year, 3-year survival rates for patients in the combined group were 70.6%, 35.3%, and 29.4%, respectively, with the median survival of 19.4 months. In the chemotherapy/radiation therapy group, the 1-year, 2-year, 3-year survival rates were 44.8%, 17.2% and 13.8%, respectively, with the median survival of 11.4 months. The results suggested that the injection-treated patients had prolonged survival compared with patients that received chemotherapy/radiation therapy alone [5].

However, adverse events were encountered in approximately 30% of the patients treated with the injection. The most frequent side effect was mild-to-moderate fever, the frequency of which was roughly 10–30% higher than that in the control group [2,6,7]. Local side effects at the injection site such as pain, swelling and redness also commonly occurred in the injection-treated patients. Based on the clinical studies, the adverse events were similar among the patients treated with the injection, which indicates that the staphylococcin injection has a similar toxicological profile in a majority of patients with malignant diseases.

Research and development of the injection are limited by a lack of establishment of a quality standard. It is claimed that the main active component is staphylococcal enterotoxin C2 (SEC2) for its potential to enhance the systemic immune response. However, no standard methods have been developed to monitor the batch-to-batch variation based on the amount of this enterotoxin during the process of the injection preparation.

The research and development of the staphylococcin injection have also met with little success due to the lack of comprehensive identification of the complicated components, including both active

Abbreviations: SE, staphylococcal enterotoxin; MHC, major histocompatibility complex; GST, glutathione *S*-transferase.

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components and impurities. Observations from clinical reports suggested that the anti-tumour effect of the injection was correlated with its immunomodulatory properties. However, whether SEC2 is the most important component for the anti-tumour effect of the injection remains controversial. Consequently, the molecular basis for the efficacy of the injection has not been elucidated clearly, and the application of the injection for cancer therapy is, to a certain extent, limited by the toxicities encountered during the treatment. Thus, component analysis of the injection is highly desirable and the development of the next-generation staphylococcin injection would strongly depend on the assessment of both anti-tumour effect and toxicity of the components in the injection.

In this study, a sensitive biotin–streptavidin–ELISA (BS–ELISA) method for detection and quantification of SEC2 was established. Furthermore, 1-D gel electrophoresis coupled with nano-LC–MS/MS analysis was conducted to identify the protein components in the staphylococcin injection from one manufacturing company.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice and male New Zealand rabbits, weighing 20 ± 2 g and 2.2 ± 0.2 kg, respectively, were purchased from the animal research centre in Academy of Medical Science at Zhejiang province, China. The animals were housed in an air-conditioned room, with temperature $23 \pm 2\,^{\circ}C$, relative humidity 50–60%, controlled illumination of a 12 h light–dark cycle. All procedures described in this study were reviewed and approved by the ethics committee for the use of experimental animals at Zhejiang University, China. Thymine (Bio Basic Inc., Markham, Ontario, Canada), hypoxanthine (Bio Basic Inc., Markham, Ontario, Canada) and aminopterin (Sigma–Aldrich, St. Louis, MO, USA) were used in cell fusion. Biotin conjugated affinity purified Goat anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), biotin conjugated affinity purified Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and streptavidin-labelled horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) were used in the BS–ELISA system. Peroxidase conjugated affinity purified Goat anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and peroxidase conjugated affinity purified Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used in indirect ELISA.

2.2. Drugs and recombinant staphylococcal enterotoxins

Staphylococcin injection A (manufacturing company A, China), staphylococcin injection B (manufacturing company B, China) and staphylococcin injection C (manufacturing company C, China) were purchased from the Second Affiliated Hospital, Zhejiang University College of Medicine, Zhejiang Province, China. Recombinant staphylococcal enterotoxins (rSEA, rSEB, rSEC2, rSEE, rSEG, rSEI, rSEK, rSEM, rSEN, rSEO and rSEQ) obtained from thrombin-digested GST-tagged SEs were purified and preserved in our lab. Recombinant His-tagged SEC2 was purified and preserved in our lab.

2.3. Production of monoclonal and polyclonal antibodies against SEC2

Three male New Zealand rabbits were immunized subcutaneously on the lower back with 200 μg of purified His-tagged SEC2 in complete Freund's adjuvant (Bio Basic Inc., Markham, Ontario, Canada). After the first injection, the rabbits were injected subcutaneously with 200 μg of the immunogen in incomplete Freund's adjuvant (Bio Basic Inc., Markham, Ontario, Canada) at 15-day intervals over a period of 8 weeks. Blood samples were taken 6–8 days after each injection and the titre of the antisera against purified recombinant SEC2 was determined by indirect ELISA.

Six male BALB/c mice were injected subcutaneously with 30 μg of purified recombinant SEC2 emulsified in complete Freund's adjuvant. The mice received a booster injection of 30 μg of the antigen in incomplete Freund's adjuvant every 2 weeks. The immune response was monitored by testing the titre of polyclonal antibody in mouse serum using indirect ELISA and Western blot. Three days before cell fusion, the animals were boosted intraperitoneally with 50 μg of recombinant SEC2 in phosphate buffered saline (PBS, pH 7.4). The splenic lymphocytes obtained from the immunized mice were fused with SP2/0-Ag14 mouse myeloma cells at a ratio of 4:1 using 50% (w/v) PEG 4000 (Sigma–Aldrich, St. Louis, MO, USA). Fused cells were suspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY, USA), supplemented with 10% (v/v) foetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA) and hypoxanthine aminopterin thymidine (HAT), and seeded in 96-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany). The plates were incubated at 37 °C in a humidified CO_2 incubator (Model 3111, Thermo Forma, USA) for 10–15 days. The hybridoma cells capable of producing antibodies against purified His-tagged SEC2 were screened by indirect ELISA and cloned by limiting dilution. Positive hybridoma cells were further cultured and expanded in DMEM containing 10% (v/v) FBS and injected intraperitoneally to BALB/c mice pretreated with paraffin oil. Ascite fluid was collected from the mice 10–14 days later and the monoclonal antibodies (MAb) against purified His-tagged SEC2 were purified by Protein A chromatography (Amersham Biosciences, Uppsala, Sweden) from the supernatant of ascites on an ÄKTA purifier system (Amersham Biosciences, Uppsala, Sweden). Subtype of the monoclonal antibodies was determined by Mouse Monoclonal Antibody Isotyping Reagents (Sigma–Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

2.4. Establishment of quantitative BS–ELISA system for SEC2

To establish the BS–ELISA system for the detection and quantification of SEC2, two procedures were investigated: (1) use of plate coated with rabbit polyclonal serum and murine MAb as secondary antibody and (2) use of plate coated with murine MAb and rabbit polyclonal serum as secondary antibody. The optimal conditions for BS–ELISA assay were determined by a series of checkerboard titrations with various dilutions of coating antibody, secondary antibody, biotinylated antibody and streptavidin-labelled enzyme. The time of incubation and wash was also optimized. To set up the standard curve, purified His-tagged SEC2 was serially diluted 2-fold from 80 to 0.078 ng/mL in PBS (pH 7.4) with 0.5% (w/v) BSA (Bio Basic Inc., Markham, Ontario, Canada). The LOD was determined by calculating the mean value and the standard deviation of blank samples ($n = 20$): LOD = mean + 3 SD. The LOQ was estimated as the lowest concentration of His-tagged SEC2 that could be measured with acceptable precision (RSD $\leq$ 15%). Intra-day accuracy and precision were assessed by analysing five replicates of each standard sample of His-tagged SEC2 at six concentrations (30, 20, 15, 10, 5, and 2.5 ng/mL) on three separated days. Inter-day accuracy and precision were assessed by analysing ten replicates of standard samples on one day. To estimate the specificity of the BS–ELISA method for SEC2, several types of purified recombinant staphylococcal enterotoxin including rSEA, rSEB, rSEE, rSEG, rSEI, rSEK, rSEM, rSEN, rSEO and rSEQ at a concentration of 1 μg/mL in blocking buffer (0.5% BSA, 0.02% Tween-20 in PBS, pH 7.4) were tested in the established BS–ELISA system.

2.5. Measurement of SEC2 in staphylococcin injection products

After optimization of the conditions, the BS–ELISA was performed in 96-well polystyrene microtitre plates (Corning Inc., Corning, NY, USA) with the following procedure: All wells were coated with 0.1 μg of purified mouse anti-SEC2 monoclonal antibody in 100 μL of 0.1 mol/L sodium bicarbonate (pH 9.6) overnight at 4 °C. After washing five times for 10 min each with wash buffer (0.02% Tween-20 in PBS, pH 7.4), the plates were incubated with 100 μL of blocking buffer for 1 h at 37 °C. The wells were then emptied and washed five times for 10 min each. 100 μL of staphylococcin injection solution was added to each well and purified His-tagged SEC2 serially diluted in blocking buffer was used as the concentration standard. The plates were incubated at 37 °C for 2 h followed by five washes for 10 min each with wash buffer. 100 μL of rabbit anti-SEC2 serum, diluted 1:1000 in blocking buffer, was added and the plates were incubated at 37 °C for 1 h. After washing, biotin-labelled Goat anti-rabbit IgG, diluted 1:2000 in blocking buffer, was added and the plates were then incubated at 37 °C for 30 min. The wells were washed with wash buffer as above and incubated with streptavidin-labelled horseradish peroxidase, diluted 1:500 in blocking buffer at 37 °C for 30 min, followed by the wash procedure. Freshly prepared 3,3′5,5′-Tetramethylbenzidine (TMB, Bio Basic Inc., Markham, Ontario, Canada) substrate solution was then added to each well and incubated at room temperature for 10–30 min. The reaction was stopped with 200 μL of 0.3 mol/L citric acid. The absorbance was read at 450 nm on an ELISA microplate reader (Model 680, Bio-Rad, USA). The amount of SEC2 in the staphylococcin injection solution was calculated according to the standard curve of purified His-tagged SEC2 as an average of five parallel experiments. The total protein concentration of the injection solution was determined by BCA kit following the manufacturer's instructions (Beyotime, Jiangsu Province, China). Results are presented as the mean ± standard error.

2.6. Nano-LC–MS/MS analysis

2.6.1. Ultrafiltration and SDS-PAGE

The solution of staphylococcal injection from one manufacturing company was concentrated approximately 40-fold by ultrafiltration (Biomax-5K NMWL, Millipore, Bedford, MA, USA). 1-D SDS-PAGE analysis of the concentrated solution was then performed on a precast Novex 12% Tris/glycine mini-gel (Invitrogen, Carlsbad, CA, USA) and stained with colloidal Coomassie blue (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.6.2. In-gel digestion

Following visualization of the gel, the entire gel lane was divided into 14 sections, based on band intensity, and cut into small pieces. All gel pieces were placed into 0.5 mL eppendorf tubes for in-gel digestion and manual extraction following the methods reported by Zhang et al. [8]. For each extraction, the combined extracts were taken to a dry state in a SpeedVac (Thermo Savant, Holbrook, NY, USA).

2.6.3. Nano-LC–MS/MS condition

Samples were reconstituted in 20 μL of 0.5% formic acid with 2% acetonitrile and sonicated for 5 min prior to nano-LC–MS/MS analysis. The nano-LC was performed with an LC Packings Ultimate integrated capillary HPLC system equipped with a Switchos valve switching unit and FAMOS autosampler (Dionex, Sunnyvale, CA, USA). Gel-extracted peptides (6.4 μL) were injected onto a C18 PepMap100 trap column for online desalting and concentration. The peptides were then separated on a PepMap C-18 RP nanocolumn, eluted with a 60-min linear gradient of 5–45% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min. The nano-LC was connected in-line to a hybrid triple quadrupole linear ion trap mass spectrometer (4000 Q Trap ABI/MDS Sciex, Framingham, MA, USA), equipped with a Micro Ion Spray Head ion source for online analysis.

The MS data acquisition on the 4000 Q Trap was performed using the Analyst 1.4.1 software (Applied Biosystems) in the positive ion mode for information-dependent acquisition (IDA) analysis. A 2.0 kV nanospray voltage was used for all the experiments. Nitrogen was used as both the curtain (value of 10) and collision gas (set to high), and the heated interface was on. The declustering potential (DP) was set to 40 eV to minimize in-source fragmentation. In an IDA analysis, after each survey scan (the m/z range from 400 to 1600) and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for MS/MS, with rolling collision energy applied on the basis of different charge states and m/z values.

2.6.4. Protein identification and data interpretation

The MS/MS spectra generated from nano-LC based IDA analyses were submitted to Mascot 1.9 for database searching against the NCBI firmicutes database (downloaded in August 2006). One trypsin miscleavage was allowed. Oxidation of methionine residues and carboxyamidomethylation of cysteine residues were set as variable modifications. Monoisotopic values were used for the database search. Peptide tolerance and MS/MS tolerance was set to 1.5 and 0.6 Da, respectively. Only those peptides with Mascot scores above the significance threshold defined by Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) greater than "identity" were considered to be confidently identified and used for protein identification.

3. Results

3.1. Production of polyclonal and monoclonal antibodies against SEC2

Three male New Zealand rabbits were immunized with purified His-tagged SEC2 and the titres of the antisera reached approximately 1:400,000 against recombinant SEC2. The immunization procedure for the BALB/c mice generated high antibody titre against His-tagged SEC2 in sera, detectable even at a 1:12,800 dilution of the sera when determined by indirect ELISA. The splenocytes from the mice were isolated and fused with murine myeloma cells. Of the 480 wells, culture supernatants from 191 primary hybridomas were positive in indirect ELISA. During the dilution, many of the positive clones either failed to grow or grew but lost antigen reactivity. Finally, one stable hybridoma cell line designed 3F5 was obtained. The hybridoma cell line was expanded using the mouse ascites method and the monoclonal antibodies were purified from the ascites collected using a Protein A column. Two components of apparent molecular weight of 50,000 and 25,000 Da were observed in SDS-PAGE under a reducing condition, and the 3F5 antibody was of the IgG1 subclass.

3.2. Measurement of SEC2 in staphylococcin injection products

Two BS–ELISA configurations were evaluated for the quantification of SEC2. On the basis of linear fit and detection cut-off level, the second procedure coupled with the biotin–streptavidin system was adopted. The optimal dilutions of coating antibody, secondary antibody, biotinylated antibody and streptavidin-labelled enzyme were determined by a series of checkerboard titrations. The optimal time of each step including incubation and wash was also determined. The standard curve for purified His-tagged SEC2 in the BS–ELISA system was almost linear between 0.625 and 40 ng/mL. The linear equation was $y = (0.02127 \pm 0.0006)x + (0.1050 \pm 0.0096)$ with

Table 1
Intra-day precision and accuracy of the BS–ELISA system for detection and quantification of SEC2 ($n = 5$).

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Precision (%)	Accuracy (%)
30			
(Day1)	29.1 ± 0.8	2.7	97.0
(Day2)	32.5 ± 0.9	2.8	108.3
(Day3)	28.5 ± 0.7	2.5	95.0
20			
(Day1)	18.2 ± 0.4	2.2	91.0
(Day2)	21.5 ± 0.6	2.8	107.5
(Day3)	18.9 ± 0.5	2.6	94.5
15			
(Day1)	15.7 ± 0.5	3.2	104.6
(Day2)	15.3 ± 0.7	4.6	102.0
(Day3)	14.0 ± 0.8	5.7	93.3
10			
(Day1)	9.5 ± 0.4	4.2	95.0
(Day2)	9.7 ± 0.3	3.1	97.0
(Day3)	10.4 ± 0.6	5.8	104.0
5			
(Day1)	5.4 ± 0.2	3.7	108.0
(Day2)	4.6 ± 0.4	8.7	92.0
(Day3)	4.7 ± 0.2	4.3	94.0
2.5			
(Day1)	2.2 ± 0.2	9.1	88.0
(Day2)	2.4 ± 0.3	12.5	96.0
(Day3)	2.7 ± 0.2	7.4	108.0

Precision was calculated as (SD/mean) × 100%. Accuracy was calculated as (observed concentration/nominal concentration) × 100%.

$r^2 \geq 0.98$ for all the standard curves (three replicate tests in five separate experiments). The LOD was nearly 0.625 ng/mL determined by the mean value and SD of twenty replicates of blank samples, and the LOQ that was estimated as the lowest concentration of His-tagged SEC2 that could be calculated with acceptable precision (RSD ≤ 15%) was approximately 1.0 ng/mL. Intra-day precision and accuracy were determined by analysing standard samples of His-tagged SEC2 at six concentrations in five replicates on three days. Inter-day precision and accuracy were assessed by analysing standard samples of His-tagged SEC2 at six concentrations in ten replicates on the same day. The intra-day precision and accuracy varied from 2.2% to 12.5% and 88.0% to 108.3%, respectively (Table 1). The inter-day precision and accuracy ranged from 2.1% to 8.7% and 92.0% to 106.0%, respectively (Table 2). The results indicated that the BS–ELISA system was accurate and repeatable. To test the specificity of the BS–ELISA system, purified rSEA, rSEB, rSEE, rSEG, rSEI, rSEK, rSEM, rSEN, rSEO and rSEQ (1 μg/mL) were subjected to the BS–ELISA system and no cross reactivity was observed. The concentration of SEC2 in staphylococcin injection from different manufacturing companies and lot numbers were shown in Table 3. The results demonstrated that the relative amount of SEC2 in the injection products was less than 0.1%. In addition, the concentrations of SEC2 were different in the products from different companies and lot numbers, which indicated that the manufacturing process was not monitored on the basis of SEC2 amount.

Table 2
Inter-day precision and accuracy of the BS–ELISA system for detection and quantification of SEC2 ($n = 10$).

Nominal Concentration (ng/mL)	Observed Concentration (ng/mL)	Precision (%)	Accuracy (%)
30	28.7 ± 0.7	2.4	95.6
20	19.1 ± 0.4	2.1	95.5
15	15.4 ± 0.6	3.9	102.6
10	9.7 ± 0.5	5.2	97.0
5	5.3 ± 0.4	7.5	106.0
2.5	2.3 ± 0.2	8.7	92.0

Precision was calculated as (SD/mean) × 100%. Accuracy was calculated as (observed concentration/nominal concentration) × 100%.

3.3. Nano-LC–MS/MS analysis of the staphylococcin injection

The staphylococcin injection solution from one manufacturing company was approximately 40-fold concentrated by ultrafiltration. The proteins were visualized by 1-D SDS-PAGE followed by colloidal Coomassie blue staining. The gel was divided into 14 sections according to the intensity of the protein bands (Fig. 1). Peptides were extracted from the gel pieces and subjected to nano-LC–MS/MS analysis. A total of fifty-five proteins of *S. aureus* were confidently identified from the gel (Table 4). Enolase, dihydrolipoamide dehydrogenase and alpha-haemolysin were identified with the highest scores, demonstrating that these proteins were of high abundance in the injection. Of those identified staphylococcal proteins, seventeen proteins are known to be virulence determinants which could be classified into three subcategories [9]: (1) proteases and lipases involved in degradation and modification of proteins and lipids, such as serine proteinase, V8 protease and glycerophosphoryl diester phosphodiesterase; (2) important pathogenic factors such as alpha-haemolysin, enterotoxin C2, autolysin and leucocidin; (3) virulence factors involved in the microbe–host interaction such as fibronectin binding protein and enolase. It is known that these virulence factors, including SEC2, are important in several disease states caused by severe staphylococcal infection. For example, alpha-haemolysin, which is dermonecrotic and neurotoxic, may be associated with pulmonary oedema or adult respiratory distress syndrome [10]. Leucocidin is primarily involved in necrotic lesions of the skin and subcutaneous tissues and associated with community-acquired severe necrotizing pneumonia [11]. Fibronectin-binding protein contributes to the

Table 3
Measurement of total protein and SEC2 in the staphylococcin injection products from three manufacturing companies. Each value represents means ± SD ($n = 5$).

Products	Lot number	Total protein concentration (μg/mL)	Concentration of SEC2 (ng/mL)
Staphylococcin injection from manufacturing company A	20050701	49.2 ± 1.5	19.6 ± 0.3
	20060501	47.6 ± 1.9	15.7 ± 0.2
	20060902	46.3 ± 1.2	18.6 ± 0.3
	20080401	45.3 ± 2.1	17.6 ± 0.4
	20080501	47.2 ± 1.3	15.3 ± 0.4
	20080803	44.9 ± 1.5	19.1 ± 0.4
Staphylococcin injection from manufacturing company B	20041207	18.3 ± 0.7	9.7 ± 0.2
	20060501	18.8 ± 0.3	12.3 ± 0.3
	20060502	19.7 ± 0.4	11.6 ± 0.3
	20071110	17.2 ± 0.5	13.5 ± 0.4
	20071212	18.1 ± 0.4	11.3 ± 0.6
	20080705	17.3 ± 0.6	12.4 ± 0.3
Staphylococcin injection from manufacturing company C	20040801	27.5 ± 0.4	3.2 ± 0.2
	20041001	28.7 ± 0.5	3.9 ± 0.1

Table 4
Proteins of *Staphylococcus aureus* identified from the staphylococcin injection from one manufacturing company.

Accession number	Protein name	M.W. (Da)	Mascot scores	Uniq pept
Amino acid/carbohydrate transport and metabolism				
gi|3152725	Enolase	47,088	574	9
gi|48874	Dihydrolipoamide dehydrogenase: subunit E3	49,421	346	6
gi|82750578	Glucose-6-phosphate isomerase A	49,791	245	4
gi|14248380	Fructose–bisphosphate aldolase homologue	33,021	222	4
gi|14247553	Putative transaldolase	25,742	212	4
gi|14246544	2,3-Diphosphoglycerate-independent phosphoglycerate mutase	56,419	115	2
gi|49484632	Putative phosphoglycerate mutase	26,707	96	2
gi|14247910	Deoxyribose–phosphate aldolase	23,327	90	2
gi|49243561	L-lactate dehydrogenase 1	34,548	85	1
gi|581570	Dihydrolipoamide acetyltransferase: subunit E2	46,411	66	1
gi|14246373	Alcohol dehydrogenase I	36,039	61	1
gi|7162049	Triosephosphate isomerase	14,406	57	1
gi|87128023	Phosphopentomutase	43,768	55	1
gi|82751098	Glucose-6-phosphate 1-dehydrogenase	56,943	46	1
gi|49244722	Alanine dehydrogenase 2	40,209	45	1
Virulence/defence mechanisms				
gi|49484059	Serine protease	25,564	308	5
gi|14247584	Serine protease	26,125	135	2
gi|14248276	Fibronectin-binding protein homologue	105,947	98	2
gi|224650	Nuclease	26,774	91	2
gi|14247581	Serine protease	25,625	69	1
gi|49483119	Putative glycerophosphoryl diester Phosphodiesterase	35,325	60	1
gi|9931632	Serine protease-like exoprotein A	25,498	57	1
gi|49483762	Putative peptidase	40,236	56	1
gi|82750421	Probable transmembrane sulfatase	74,352	53	1
gi|265412	V8 protease	29,972	51	1
gi|14247583	Serine protease	26,083	47	1
Toxins and haemolysins				
gi|2914575	Chain G, alpha-haemolysin	33,227	344	6
gi|46609	F component of leucocodin R	36,789	185	3
gi|13549150	Leucocidin LucS component	32,563	175	3
gi|76009542	Enterotoxin C2 precursor	27,567	109	2
gi|21203559	SET26	25,910	67	1
gi|21204103	Autolysin	137,323	48	1
Stress response proteins				
gi|87128174	Alkyl hydroperoxide reductase subunit C	20,963	125	2
gi|16329169	Superoxide dismutase	22,968	58	1
Cell division and maintenance				
gi|87127711	Ferritins family protein	19,576	99	1
gi|49483128	Fumarylacetoacetate (FAA) hydrolase Family protein	33,148	85	1
gi|14246026	Cell division and morphogenesis-related protein	25,452	59	1
gi|57285831	Peptidyl–prolyl *cis*–transisomerase, Cyclophilin-type	21,635	47	1
Protein synthesis				
gi|49484831	Ornithine carbamoyltransferase	37,730	217	4
gi|87127645	50S ribosomal protein L17	13,739	116	2
gi|87126127	Ribosome recycling factor	20,341	92	2
gi|14248373	3-Methyl-2-oxobutanoate hydroxymethyltransferase	29,222	61	1
gi|57286811	Ribosomal protein S6	10,809	50	1
gi|7106008	Ornithine carbamoyltransferase Otc6850	37,511	46	1
gi|57284277	Ribosomal subunit interface protein	22,211	45	1
Nucleotide biosynthesis				
gi|87128196	Translation elongation factor P	20,541	66	1
gi|14247027	Elongation factor TS	32,473	63	1
gi|87126996	Translation elongation factor Tu	43,077	51	1
gi|82751814	DNA-directed RNA polymerase alpha chain	34,980	49	1
Unknown				
gi|87128187	Conserved hypothetical protein	13,059	110	2
gi|49484519	Hypothetical protein SAR2388	16,997	70	1
gi|87126383	Conserved hypothetical protein	26,319	67	1
gi|14246355	Conserved hypothetical protein	29,371	46	1
gi|49482843	Hypothetical protein SAR0622	18,554	46	1
gi|14247490	Conserved hypothetical protein	17,085	46	1

Protein accession number, protein name, M.W., number of unique peptides and Mascot scores are provided. Peptides with ion score >43 were considered to be confidently identified and used for protein identification. Uniq pept: matched distinct peptides. M.W.: theoretical or predicted molecular weight.

bacteria–host cell interactions and may play an important role in the induction of experimental endocarditis [12]. Staphylococcal enterotoxin C2 was detected from the injection solution with two peptides confidently identified. The Mascot score of each peptide was 53 and 58, respectively. No other staphylococcal enterotoxins were identified by the MS methods. A member of a novel family of superantigen-like proteins (SETs), SET26, was detected from the gel. However, it appears that none of the SETs exhibit any of the properties of known superantigen proteins such as MHC class II binding or broad T cell stimulation [13]. In addition, fifteen proteins of *S. aureus* involved in amino acid/carbohydrate transport and metabolism, seven proteins of *S. aureus* associated with protein synthesis, four proteins of *S. aureus* involved in transcription and replication, four proteins of *S. aureus* associated with cell division, two proteins of *S.*

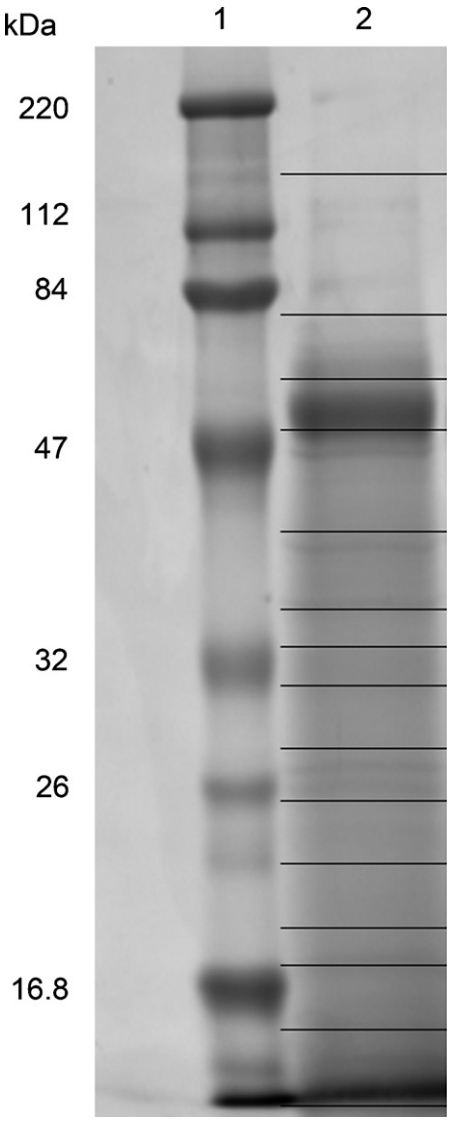

Fig. 1. SDS-PAGE analysis of the staphylococcin injection solution concentrated by ultrafiltration. The gel was stained with colloidal Coomassie blue and then divided into 14 sections according to the band intensity. Lane.1: Molecular mass marker. Lane.2: Concentrated solution of the staphylococcin injection.

aureus involved in the environmental stress responses and several staphylococcal proteins of unknown functions were also identified in the concentrated injection solution. Furthermore, eighteen proteins of other Gram-positive bacteria were identified from the gel pieces (Table 5), such as ornithine carbamoyltransferase of *Bacillus licheniformis* (Bacillaceae), *N*-acetylmuramoyl-L-alanine amidase of *Staphylococcus epidermidis* (Staphylococcaceae) and tryptophan synthase beta chain of *Thermoanaerobacter tengcongensis* (Thermoanaerobacteraceae).

4. Discussion

As the most powerful T cell mitogens, SEs can elicit massive T cell proliferation and cytokine release both *in vivo* and *in vitro* even at concentrations of pg–ng/mL [13,14], which suggests their use in immunotherapy for human malignant diseases. From the results of several pre-clinical studies and early-phase clinical trials, engineered antibody-targeted superantigens seemed to be desirable candidates for anti-tumour agents [15–19]. SEC2 is proposed as the main active component in the staphylococcin injection that is commonly used in the combined treatment of cancer to enhance the systemic immune response and reduce the toxicities associated with chemotherapy or radiation therapy in China. Nevertheless, the results of the BS–ELISA method demonstrated that SEC2 only accounted for less than 0.1% of the total protein in the injection solution. In addition, the results of nano-LC–MS/MS showed that more than seventy proteins of Gram-positive bacteria, including SEC2, were confidently identified in the injection solution from one manufacturing company.

In this study, one sensitive and reliable BS–ELISA system was established for the detection and quantification of SEC2 using a combination of the newly developed murine monoclonal antibody and rabbit polyclonal antibody. The relative amount of SEC2 in the injection solution determined by the BS–ELISA method was less than 0.1%, demonstrating that SEC2 was of very low concentration in the injection products. The amount of SEC2 was remarkably different in the injection products from different companies and lot numbers, suggesting that the preparation of the injection was not monitored on the basis of the concentration of SEC2. The *in vitro* stimulatory effect of injection products from manufacturing company A on the murine splenocytes was higher, although not significantly, than that of the injection products from manufacturing B or C (data not published), which may due to the higher concentration of SEC2. Assessment of the anti tumour effect and toxicity of the toxin are required to ascertain whether SEC2 is the most important component in the injection. Compared to HPLC/MS-based protein quantification methods, although time-consuming, the BS–ELISA method is easy-to-operate and cost-effective, in that no expensive equipments are required and there are no complicated sample preparation procedures prior to analysis. In our previous study, one biotin–avidin–ELISA method for the detection of SEC2 was established [20]. In the study, concentrations of SEC2 in injection products of several lot numbers were preliminarily indicated base on results from one test. For comparison, the BS–ELISA system described in this study was found to be more sensitive and with wider linear range of standard curve and the results seemed to be more accurate. Thus, if it is confirmed that SEC2 is the most critical component in the injection in future studies, the BS–ELISA method could be introduced into the process of the injection preparation as a standard method to assess batch-to-batch variation. In addition, the BS–ELISA results indicated that identification of protein components in the injection solution is highly necessary.

Proteomics-based approaches have been used in several studies to investigate exoproteins or cytoplasmic proteins of *S. aureus* [9,21]. In this study, 1-D SDS-PAGE coupled with nano-LC–MS/MS analysis was performed to identify the proteins in the concentrated solution of staphylococcin injection from one manufacturing company. Fifty-five proteins of *S. aureus* and eighteen proteins of other Gram-positive bacteria were confidently identified. However, according to the gel image, we inferred that a large number of proteins in the solution were still unidentified, which may be due to the interference of contaminants such as small organic molecules. Staphylococcal enterotoxin C2 was detected as expected with two peptides confidently identified by the MS-based method. No other types of staphylococcal enterotoxin were identified from the injection solution. However, we could not rule out the coexistence of other serological types of SE in the injection since it is known that many *S. aureus* strains often carry multiple enterotoxin genes. Approaches with higher selectivity and sensitivity for the detection of different types of SE in the injection need to be developed. It would be of great importance to determine whether other types of SE could also contribute to the anti-tumour effect of the injection. Seventeen of these staphylococcal proteins, including SEC2, were well-known virulence factors. Unexpectedly, eighteen proteins of other Gram-positive bacteria were also confidently identified, which suggested that the injection was contaminated by

Table 5
Proteins of Gram-positive bacteria identified from the staphylococcin injection from one manufacturing company.

Accession number	Protein name	M.W. (Da)	Mascot scores	Uniq pept	Organism
gi|21392833	Spore germination protein XA	55,107	43	1	*Bacillus anthracis* str. A2012
gi|52003439	Protein kinase PKN/PRK1	22,828	48	1	*Bacillus licheniformis* ATCC 14580
gi|52005656	Ornithine carbamoyltransferase	37,635	43	1	*Bacillus licheniformis* ATCC 14580
gi|23507115	Ita22A	20,119	45	1	*Bacillus* sp. CY22
gi|75759054	Hypothetical protein RBTH_07017	63,396	47	1	*Bacillus thuringiensis* serovar *israelensis* ATCC 35646
gi|82745058	Flagellar biosynthetic protein FlhB	68,078	45	1	*Clostridium beijerincki* NCIMB 8052
gi|89896920	Hypothetical protein DSY4174	21,415	44	1	*Desulfitobacterium hafniense* Y51
gi|68056418	Enolase	46,299	119	2	*Exiguobacterium sibiricum* 255–15
gi|31541636	DeoD	26,677	53	1	*Mycoplasma gallisepticum* R
gi|26554146	FKBP-type peptidyl–prolyl *cis*–transisomerase	50,766	43	1	*Mycoplasma penetrans* HF-2
gi|39721879	Enolase	47,052	87	1	*Onion yellows phytoplasma* OY-M
gi|48870028	COG0125: Thymidylate kinase	23,707	43	1	*Pediococcus pentosaceus* ATCC 25745
gi|8050834	Pyruvate dehydrogenase complex subunit E2	46,934	51	1	*Staphylococcus epidermidis*
gi|27316202	Purine nucleoside phosphorylase	25,853	53	1	*Staphylococcus epidermidis* ATCC 12228
gi|27315212	*N*-acetylmuramoyl-L-alanine amidase	148,195	48	1	*Staphylococcus epidermidis* ATCC 12228
gi|68447950	Unnamed protein product	26463	73	1	*Staphylococcus haemolyticus* JCSC1435
gi|20517265	DNA-directed RNA polymerase alpha subunit/40 kDa subunit	35,130	49	1	*Thermoanaerobacter tengcongensis* MB4
gi|20516583	Tryptophan synthase beta chain	42,945	45	1	*Thermoanaerobacter tengcongensis* MB4

Protein accession number, protein name, M.W., Mascot score, number of unique peptides and name of the organism are provided. Peptides with ion score >43 were considered to be confidently identified and used for protein identification. Uniq Pept: matched distinct peptides. M.W.: theoretical or predicted molecular weight.

those Gram-positive bacteria during the process of preparation. The obvious question raised here is, other than SEC2, whether these identified proteins are correlated with the therapeutic benefit provided by the injection. Based on the biological properties, it seems that most proteins identified by MS analysis are not contributing to the immunomodulatory capacities of the injection. It is more likely that the identified proteins, especially those virulence factors, are associated with the local side effects of the injection observed in the clinical reports, such as pain, swelling and redness [1,2]. For the next generation of staphylococcin injection, those impurity proteins that are identified in future studies should be removed and the manufacturing process should be improved to prevent unanticipated contamination.

The present work sheds insight into the research and development of the second-generation staphylococcin injection. Both the BS–ELISA results and the nano-LC–MS/MS results demonstrated that SEC2 accounted for a low percentage of the total protein in the injection products. Since several pre-clinical studies and clinical trials have shown that mutated tumour-targeted superantigen products are promising immunomodulatory candidates for cancer therapy [15–19], the genetic engineering approach has been suggested as an alternative approach to the traditional manufacturing process of the staphylococcal injection. In addition, clinical trials of superantigen-based immunotherapy revealed that: (1) there was a correlation between the maximum tolerated dose (MTD) and the pretreatment anti-superantigen sera antibody concentrations; (2) the ratio of anti-superantigen sera antibody level to the administered drug dose correlated with cytokine release and the grade of clinical toxicities [18,19,22]. Accordingly, individual therapeutic strategies of the next-generation staphylococcin injection encompassing dose and timing of administration should be determined to optimize efficacy and reduce toxicity in future cancer therapy.

5. Conclusions

The results in this study demonstrated that at least seventy-three proteins were present in the staphylococcin injection solution, and SEC2 was of very low concentration. Further studies are required to evaluate the anti-tumour effect and the toxicities of the proteins in the injection, and the manufacturing process of the injection should be improved to remove the impurity proteins. The established BS–ELISA system, which was sensitive and reliable for the detection of SEC2, could be applied to the preparation of the injection products.

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