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Expression and bioactivity analysis of *Staphylococcal enterotoxin* M and N

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Abstract

Staphylococcal enterotoxins (SEs) are powerful superantigens that stimulate non-specific T-cell proliferation produced by *Staphylococcus aureus* and draw considerable attention as ideal drugs for cancer therapy. The filtrate of *S. aureus* culture has been used as ampul named *Staphylococcal enterotoxin C injection* in clinic for 10 years in China and proved to be effective. The superantigen SEC claimed to be the only active component without certifiable evidences. For further investigations of the active components of this injection and establishment of foundations for the development of novel anti-cancer drugs, in this research we extracted total DNA from *S. aureus* (FRI 1230), cloned, expressed and purified recombinant proteins of *Staphylococcal enterotoxin* M and N (rSEM and rSEN). The MTT assay of the purified rSEM and rSEN demonstrated that their abilities of stimulating T cells and inhibiting the proliferation of K562–ADM cells and B16 cells were equivalent to that of purified SEC2 *in vitro*. These findings suggested that SEC was not the only active component of *Staphylococcal enterotoxin C injection* and the effective procedure of expression and purification may be useful for mass productions of these therapeutically important proteins.
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Keywords: Enterotoxins; Superantigen; *Staphylococcal enterotoxin C injection*; Expression; MTT

Staphylococcal enterotoxins (SEs)[1] are a family of 13 major serological types of heat stable enterotoxins (SEA through SEE, SEG through SEQ, TTST-1, TTST-2 and SEC including subtypes of SEC1, SEC2 and SEC3) produced by *Staphylococcus aureus* [1–3] and share a number of genetic and biochemical characteristics, with similar functions [3]. SEs are powerful superantigens that stimulate non-specific T-cell proliferation based on their V_β receptor usage. Their ability to stimulate lymphocyte proliferation and lymphokine production at concentrations as low as 10^{-13}–10^{-16} M makes them among the most potent T-cell activators known and their ability of inducing apoptotic death of tumor cells is of particular interest [3–5]. The filtrate of *S. aureus* culture has been used as ampul named *Staphylococcal enterotoxin C Injection* for cancer therapy in clinic for 10 years in China and proved to be effective [6], but its active components have not been confirmed yet.

For further investigations of the active components of the injection and establishment of foundations for development of novel anti-cancer drugs, in this research we will extract total DNA from *S. aureus* (FRI 1230), clone the genes of *Staphylococcal enterotoxin* M and N, obtain recombinant proteins (rSEM and rSEN), and compare their abilities of stimulating T cells and inhibiting the proliferation of K562–ADM cells and B16 cells with SEC2 by MTT assay.

Materials and methods

Cell lines and cultures

Anti-adriamycin (ADM) human chronic myelogenous leukemia cell line: K562–ADM, K562 gradually induced

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[1] *Abbreviations used:* SEs, *Staphylococcal enterotoxins;* ADM, anti-adriamycin; FBS, fetal bovine serum; IPTG, isopropyl thiogalactoside.

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with 1 μg/ml ADM. Murine B16 melanoma cell line: B16. These cell lines were provided and conserved by our lab and cultivated with RMPI1640 (Gibco, Eggenstein, Germany) supplemented with 10% FBS (fetal bovine serum) at 37 °C in an atmosphere with 5% (v/v) CO_2, 0.01% ampicillin and streptomycin.

Animals

ICR mouse, outbred stock, produced in 1947 by Hauschka at the Institute of Cancer Research, derived from Swiss mice of the Rockefeller Institute and now widely distributed [7]. Weighting 20 ± 2 g, provided by animal research center, Academy of Medical Science, Zhejiang province, China.

Bacterial strains, plasmids, enzymes, reagents and media

Staphylococcus aureus (FRI 1230) was used for the extraction of the total DNA. *Escherichia coli* (*E. coli*) DH5α was used for the transformation of the vectors. *E. coli* strain BL21 (DE3) was applied as host strain for expression. The plasmid pGEM-T (Promega, USA) was used to sequence the genes of interest. The plasmid pGEX-4T-1 (Promega, USA) was used for the expression of the GST-SEM and GST-SEN genes in *E. coli*. The restriction endonucleases BamHI and XhoI, *Taq* polymerase, T4 DNA ligase were purchased from NEB (USA). IPTG and low melting-point agarose were purchased from BBI (USA). DNA sequencings were completed by Shanghai Association-gene (China); glutathione Sepharose 4B was from Amersham Phamacia Biotech (Sweden); thrombin was from Changzhou Qianhong Co. (China); BCA kit was from Beyotime (China); other biochemical reagent MTT, ConA and DNA gel extraction kit were from Shanghai Sangon (China). *S. aureus* C2 (SEC2) was prepared and conserved by our lab [8]. The culture media LB (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) and 2× YT (tryptone 16 g/L, yeast extract 10 g/L and NaCl 5 g/L) were prepared as described.

DNA extraction and PCR amplification

Total DNA was extracted from *S. aureus* (FRI 1230) with protein K and SDS–Tris solution and purified with saturate phenol by centrifugation extracting to collect the aqueous phase. Mixed the collected aqueous phase with NaAc (pH 5.5) and ice-cold dehydrated alcohol at −20 °C for 1 h. Then purified DNA was separated by centrifugation, dissolved with ion-free water and conserved at −20 °C.

PCR amplification [9] was performed on a Mastercycler gradient (Effendorf, Germany). Two microliters plasmid template was subjected to amplification in 100 μl mixture containing 1 U of *Taq* polymerase, 10 μl 10× PCR buffer, 6 μl 25 mM $MgCl_2$, 4 μl 10 mM each of dATP, dCTP, dGTP and dTTP, 4 μl 20 μM primer P1 and P2 or P3 and P4 (see below) and fill water to 100 μl, and then divided the 100 μl mixture to 4×25 μl for use. PCR conditions were initial denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min.

The mature peptide of SEM coding region was amplified with following primer pair according to the literature [10] and the estimation of SignalP 3.0 Server: forward primer P1: 5′-CAG GAT CCT TTT GCT ATT CGC AAA ATC ATA TCG CA-3′ (the scribed part is an introduced BamHI recognition sequence); and reverse primer P2: 5′-GCC TCG AGT CAA CTT TCG TCC TTA TAA GAT ATT TCT AC (the scribed part is an introduced XhoI recognition sequence).

The mature peptide of SEN coding region was amplified with following primer pair according to the estimation of SignalP 3.0 Server: forward primer P3: 5′-GGA TCC GAA GTA GAC AAA AAA GA-3′ (the scribed part is an introduced BamHI recognition sequence); and reverse primer P4: 5′-CTC GAG ATA ATC ATC AAT CAC TTA (the scribed part is an introduced XhoI recognition sequence). All primers used in the paper were synthesized in Shanghai Sangon (China).

Construction and sequencing of pGEM-SEM and pGEM-SEN

After recovery of the amplified PCR products, the products were cloned into pGEM-T vectors with T4 DNA ligase and the resultant plasmids were transformed to DH5α competent cells. The transformants were cultured in LB plate with IPTG/X-gal and ampicillin, and white clones were selected. The recombinant plasmids with SEM or SEN genes were identified with restriction endonucleases cleavage and confirmed by sequencing in Shanghai Sangon (China). The sequences of the inserts were compared to DNA sequences of the estimations of SignalP 3.0 Server and confirmed to be right.

Construction and verification of GST-SEM and GST-SEN expression vectors

The recombinant plasmids pGEM-SEM and pGEX-4T-1 were cleavaged with restriction endonucleases BamHI and XhoI. The cleavaged products of mature peptide of SEM with sites of restriction endonucleases and the products of the fragment of plasmid pGEX-4T-1 were recovered and connected to construct the expression vector of GST-SEM, named pGEX-4T-SEM. The resultant expression vector plasmids were transformed to DH5α competent cells and the amplification products were identified with restriction endonucleases BamHI and XhoI.

In addition, the expression vector of GST-SEN, named pGEX-4T-SEN was constructed and verificated in the same way.

Expression and purification of GST-SEM and GST-SEN in E. coli

Extracted plasmids pGEX-4T-SEM from *E. coli* DH5α, which contained the plasmids, and transformed them into *E. coli* BL21 (DE3). The transformants were cultured in LB plate with IPTG/X-gal and ampicillin, and white clones were selected. The transformants bearing plasmid pGEX-4T-SEM were inoculated in 5 ml LB culture medium containing ampicillin and shaking cultured for 6 h. Then inoculated the expression strains in 2× YT culture medium containing ampicillin and shaking cultured at 37 °C. When grown to $OD_{600} \approx 0.8$, induced the expression strains with 0.1 M 0.01–0.1% (v/v) isopropyl thiogalactoside (IPTG) at 30 °C. The cells were harvested after inducting for 5 h by centrifugation at 10,000 rpm for 10 min at 4 °C. Suspended each millilitre culture with 50 μl ice-cold phosphate buffer (PBS, pH 6.5) containing 0.5% Triton X-100, quassated cells with FRENCH press (Thermo Spectronic, USA) and transonic, collected supernatant after centrifugation (12,000 rpm, 30 min) and preserved at 4 °C for further purification with glutathione Sepharose 4B (GS-4B) gel column. The supernatant and sediment were detected directly by SDS–PAGE followed by Coomassie blue staining to observe the water solubility of GST-SEM.

The GS-4B gel column was equilibrated with PBS (pH 6.5). Elution was performed using the reduced glutathione buffer (0.05 M Tris–HCl, 0.05 M glutathione, pH 8.0). Use Gene Quant pro to detect the elution of GST-SEM to $OD_{280} < 0.1$. The purity and concentration of collected compositions of proteins were detected by SDS–PAGE.

In addition, GST-SEN was expressed, purified and detected by the same way.

Thrombin digestion and purification of rSEM and rSEN

Fusion proteins GST-SEM and GST-SEN were digested by thrombin and purified with GS-4B gel column. The wanted proteins rSEM and rSEN were harvested in effluent and detected by SDS–PAGE to observe the purity and concentration of the targeted proteins. The purified protein products rSEM and rSEN were freeze dried after desalination for preservation.

Bioactivity analysis of rSEM and rSEN in vitro [11–14]

The abilities of purified rSEM and rSEN to stimulate T cells were observed as follows. Our method involves isolating lymphocytes from ICR mouse's spleen, after getting rid of the red blood cells, placing 5×10^5 of the cells in RPMI1640 with 10% FCS in each well of a 96-well plate with or without various stimuli with four parallel wells each as Table 1 shown. Allow the cells to proliferate for 2 days at 37 °C with 5% CO_2 and 95% humidity. The concentrations of various stimuli, such as ConA, GST, SEC2, rSEM and rSEN, were detected with BCA kit. The amount of proliferation is detected on the 44 h by adding 15 μl PBS containing 5 mg/ml MTT to each well and incubated for another 4 h. The formazan crystals formed were dissolved in 120 μl DMSO and the absorbance was measured at A_{570nm}–A_{630nm} (570 nm as test wavelength and 630 nm as reference wavelength) with an ELISA reader. The values of the absorbance are proportional to the number of proliferating cells, which in turn is a function of the number of lymphocytes that were stimulated by a given antigen to enter the proliferative response. Absorbance values for blank group were subtracted from the results of the other wells. The values of multiplication exponential of lymphocytes were calculated (average absorbance of supplied group/average absorbance of blank group, considered the proliferation index ⩾ 1.5 as positive group) and the effects of detected proteins on lymphocytes were analyzed with pictures. These data were presented as means of SD from five independent experiments and statistically analyzed by Student's *t*-test.

Table 1
Summary of bioactivity analysis on abilities of stimulating T cells of purified rSEM and rSEN compared with rSEC2 *in vitro*

Groups	Compositions (total volume 150 μl)
Zero adjustment	Culture solution
Blank	Culture solution, splenic lymphocyte
Positive control	Culture solution, splenic lymphocyte, 10 μg/ml ConA
Negative control	Culture solution, splenic lymphocyte, 10 μg/ml GST
Test	Culture solution, splenic lymphocyte, three different proteins (rSEM, rSEN or rSEC2) at different test concentrations (1, 0.1, 0.01 μg/ml)

Then, the abilities of purified rSEM and rSEN of inhibiting the proliferation of tumor cells were observed. Splenic lymphocytes were used as effector cells and K562–ADM cells and B16 cells were used as target cells. Four different groups were added in triplicate to each well of 96-well culture plates, respectively, as summarized in Table 2. The cell population of splenic lymphocyte was 5×10^6/well and K562–ADM/B16 was 2.5×10^5/well. Evaluated the inhibition of proliferation of K562–ADM/B16 cells as a consequence of treatment with the target recombinant proteins by MTT assay described previously. Then calculated the inhibition of K562–ADM/B16 (%) with the equation, 100-[(growth inhibition of K562–ADM/B16-lympholeukocyte background discharge)/tumour cell control] × 100, and analyzed the data with pictures. These data were presented as means of SD from five independent experiments and statistically analyzed by Student's *t*-test.

Results and discussion

Cloning and sequencing of SEM and SEN genes

PCR products showed a specific fragment about 700 bp of SEM coincidence with expectant size of 703 bp and

Table 2
Summary of bioactivity analysis on inhibiting the proliferation of K562–ADM/B16 of purified rSEM and rSEN compared with rSEC2 *in vitro*

Groups		Compositions (total volume 150 μl)
Zero adjustment		Culture solution
Tumour cell control		Culture solution, K562–ADM/B16
Lymphocytes background discharge	Blank	Culture solution, splenic lymphocytes
	Positive control	Culture solution, splenic lymphocytes, 10 μg/ml ConA
	Negative control	Culture solution, splenic lymphocytes, 10 μg/ml GST
	SEC2 contrast control	Culture solution, splenic lymphocytes, SEC2 at different concentrations, 1, 0.1, 0.01 μg/ml
	Test of rSEM/rSEN	Culture solution, splenic lymphocytes, rSEM/rSEN at different concentrations, 1, 0.1, 0.01 μg/ml
Growth inhibition of K562–ADM/B16	Blank	Culture solution, splenic lymphocytes, K562–ADM/B16
	Positive control	Culture solution, splenic lymphocytes, 10 μg/ml ConA, K562–ADM/B16
	Negative control	Culture solution, splenic lymphocytes, 10 μg/ml GST, K562–ADM/B16
	SEC2 contrast control	Culture solution, splenic lymphocytes, SEC2 at different test concentrations, 1,0.1, 0.01 μg/ml, K562–ADM/B16
	Test of rSEM/rSEN	Culture solution, splenic lymphocyte, rSEM/rSEN at different test concentrations, 1, 0.1, 0.01 μg/ml, K562–ADM/B16

about 700 bp of SEN coincidence with expectant size of 684 bp as Fig. 1a shown. After clean-up and ligation reaction, pGEM-T with the amplified fragment was sequenced. Sequencing analysis of single clone showed that the main band of 700 bp was complete encoding sequence of SEM or SEN gene, coincidence with the estimated gene sequences by SignalP 3.0 Server completely. Two additional restriction endonuclease sites, BamHI and XhoI, had been introduced at the 5′ and 3′ ends of the DNA encoding of SEM and SEN, respectively.

Construction and verification of fusion expression vectors

Identifications of recombinant expression plasmids pGEX-4T-SEM and pGEX-4T-SEN by BamHI and XhoI were indicated in Fig. 1b and c and the verifications were in accordance with results as expected. DNA sequencing also showed that the gene sequences of interest were authentic and kept inframe. The recombinant expression vectors were transformed into *E. coli* BL21 (DE3) for expressing fusion proteins, after they had been constructed and proved to be correct experimentally.

Expression and solubility of recombinant protein GST-SEM and GST-SEN

Escherichia coli BL21 (DE3) strains harboring pGEX-4T-SEM and pGEX-4T-SEN expressed high-level of target fusion proteins. Molecular weights of the dominant bands were accordance with both of the expected fusion proteins about 53 kDa, while the dominant bands were absent in the untransformed control *E. coli* BL21 (DE3) strain as shown in Fig. 2a. The yield of supernatant after spallation contained 15–25% of GST-SEM or GST-SEN, respectively, which had satisfactory water solubility without observed fold proteins, and 40 mg GST-SEM or GST-SEN was collected from 200 ml of culture, respectively. Then purified target fusion proteins by GS-4B gel column as Fig. 2b shown. After purification, the yield of purified GST-SEM or GST-SEN was about 75–80%.

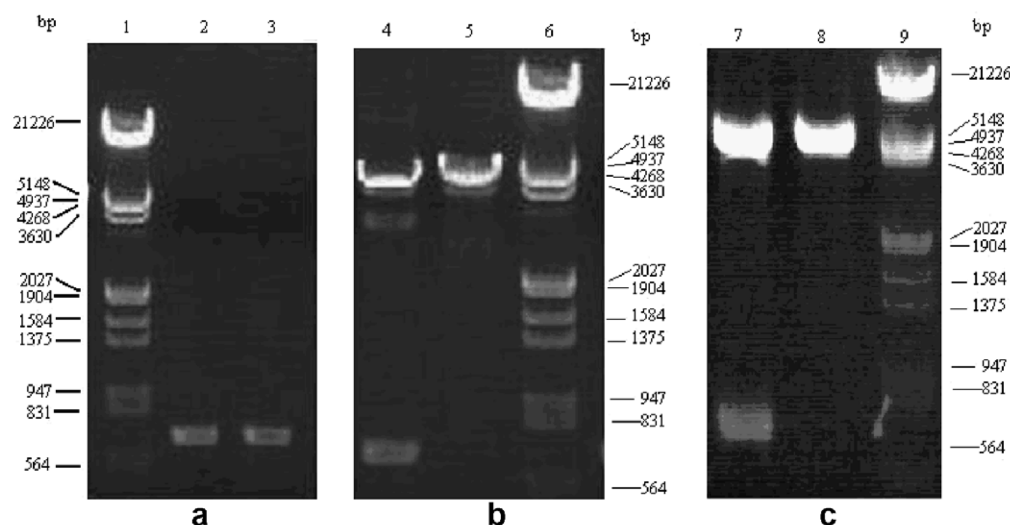

Fig. 1. Agarose-gel electrophoresis of PCR products (a) and identifications of restriction endonucleases stained with ethidium bromide (b and c). Lanes 1, 6 and 9, DNA Marker; lane 2, PCR products of SEM; lane 3, PCR products of SEN; lane 4, pGEX-4T-SEM digested with BamHI and XhoI; lane 5, pGEX-4T-SEM; lane 7, pGEX-4T-SEN digested with BamHI and XhoI; lane 8, pGEX-4T-SEN.

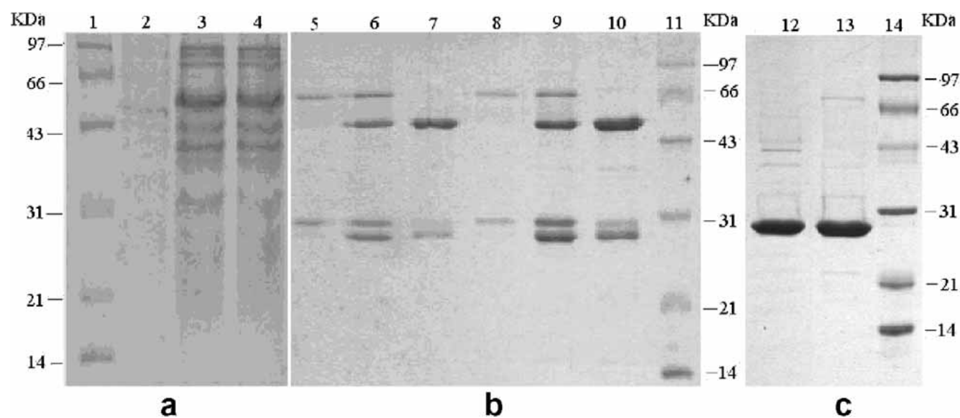

Fig. 2. Analysis of soluble expression (a) and digestion of fusion proteins and purification of target proteins (b and c) by 12% SDS–PAGE. Lane 1, 11 and 14, protein molecular mass marker; lane 2, total protein expression of non-induced BL21 with plasmid pGEX-4T-SEM and plasmid pGEX-4T-SEN; lane 3, supernatant of the induced BL21 with plasmid pGEX-4T-SEM after disruption by sonicator; lane 4, supernatant of the induced BL21 with plasmid pGEX-4T-SEN after disruption by sonicator; lane 5, SEM with thrombin; lane 6, GST-SEM digested by thrombin; lane 7, GST-SEM with GST and rSEM; lane 8, SEN with thrombin; lane 9, GST-SEN digested by thrombin; lane 10, GST-SEN with GST and rSEN; lane 12, purified rSEM; lane 13, purified rSEN.

Digestion and purification of rSEM and rSEN

There is a digestion site of thrombin in two integrity structural domains of GST and SEM in fusion protein GST-SEM, as well as GST-SEN. The fusion proteins digested by thrombin and purified by GS-4B gel column after size-exclusion chromatography taking off glutathione. After digestion and purification, 6–10 mg of the wanted proteins were obtained from 30 mg of fusion proteins, respectively, and the yield of purified protein was about 20–33%. The wanted proteins rSEM and rSEN were purified to the purity about 90% and the SDS–PAGE results were shown in Fig. 2b and c. Given the high yield, relatively low cost and high purity of these isolated proteins, those methods above offered significant advantages for purification of recombinant proteins SEM and SEN. Because of the expression vector, the expressed rSEM and rSEN both have two more amino acids at the end of N compared with wild types. The analysis of software Swiss-Pdb Viewer showed those two amino acids will not influence the bioactivities of rSEM and rSEN.

Bioactivity analysis of rSEM and rSEN in vitro

The bioactivities of rSEM and rSEN were evaluated by MTT assay. Compared with negative control, splenic lymphocyte of ICR mice had exceeding predominance increases ($P < 0.001$) after 48 h cultured with ConA, 1 μg/ml SEM or 1 μg/ml SEN, very predominance increases ($P < 0.01$) after 48 h cultured with 0.1 μg/ml rSEM or 0.1, 0.01 μg/ml SEN and predominance increases ($P < 0.05$) after 48 h cultured with 0.01 μg/ml rSEM. The effects of rSEM and rSEN, which were directly correlated with the concentration of the proteins were showed in Fig. 3. Within the concentration range of this experiment,

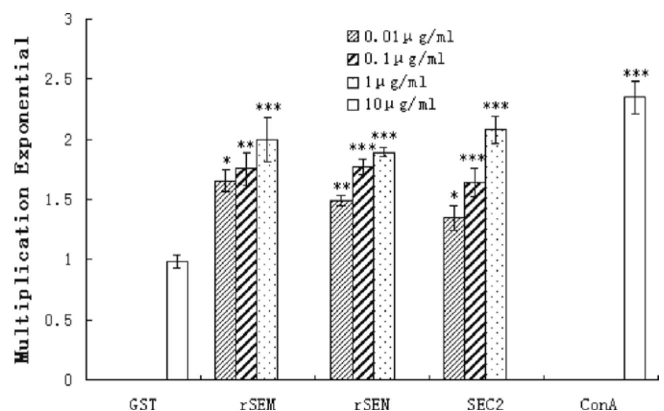

Fig. 3. Effects of rSEM, rSEN and SEC2 on multiplication exponential of mouse lymphocytes by MTT assay, $n = 5$, $\bar{x} \pm s$. Compared with the negative control group: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

the effects of rSEM and rSEN were both equivalent to SEC2.

The effects of inhibition of proliferation of K562–ADM/B16 cells induced by rSEM and rSEN with splenic lymphocyte of ICR mice were examined by MTT assay as well. Compared with negative control, the growth inhibition of K562–ADM cells/B16 cells had exceeding predominance increase ($P < 0.001$) after treating with ConA, 0.01–1 μg/ml SEM or 0.01–1 μg/ml SEN and the effects of rSEM and rSEN were directly correlated with the concentration as showed in Figs. 4 and 5. In the concentration range of these experiments, the effects of rSEM and rSEN were both equivalent to SEC2.

Conclusion

As members of SAg, SEs deserved growing attention as ideal drugs for cancer therapy due to their ability to stimulate T cells at a high frequency, thereby giving rise to

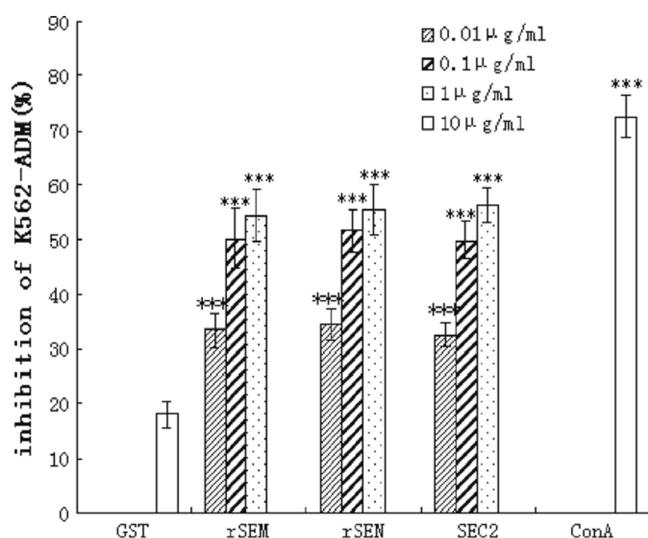

Fig. 4. Effects of rSEM, rSEN and SEC2 on inhibition of proliferation of K562–ADM cells by MTT assay, $n = 5$, $\bar{x} \pm s$. Compared with the negative control group: $^{***}P < 0.001$.

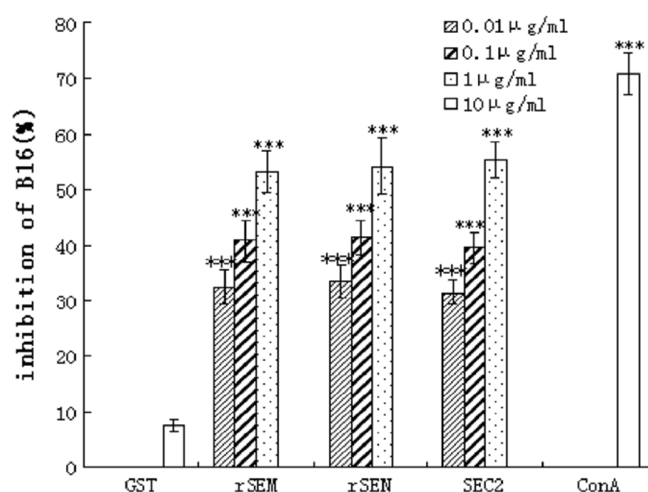

Fig. 5. Effects of rSEM, rSEN and SEC2 on inhibition of proliferation of B16 cells by MTT assay, $n = 5$, $\bar{x} \pm s$. Compared with the negative control group: $^{***}P < 0.001$.

potent cell-mediated immunological responses and producing a large variety of cytokines inducing the final result of apoptotic death of tumor cells.

Gaojushen, prepared and processed from the filtrate of *S. aureus* culture, was licensed for marketing by 1996 after finishing the phase III clinical trial in China and the active component contained in it claimed to be a superantigen SEC, which is a metabolite of the culture [15], without certifiable evidences. There are several other *Staphylococcal Enterotoxin C Injections* like Engefei and Sifusheng used for the treatment of tumor in clinic with the same claimed active component in China and all of these injections use the dosage of SEC as an index of quality control. Though these drugs have proved to be safe and effective and there has no evidence showing organ damages or decreases in white cell counts over the years of clinical treatment [15], their active components had not been confirmed yet and the complicated components make it difficult to control the inevitable side effects and quality of the drugs. It is difficult to point out the real active components of the *Staphylococcal Enterotoxin C Injection*, considered complicated components in the filtrate of *S. aureus* culture, containing other SEs and other components of the culture medium.

There are several SEs genes in *S. aureus* (FRI 1230) including SEC, SEM, SEN and other SEs. In this research, we cloned, expressed and purified the recombination proteins SEM and SEN, which have not been observed much before. In addition, we investigated and evaluated their bioactivities by MTT assay, compared with SEC2. The findings demonstrate great abilities of rSEM and rSEN equivalent to SEC2 *in vitro* as estimated. From the results, we can tentatively put forward that SEC was not the only active component. We can also estimated that SEC cooperated with other components, such as SEM, SEN and other SEs produced by *S. aureus*, working as one of the active components in the treatment of tumor. However, it should be noted that this study has only examined the bioactivities of rSEM and rSEN by MTT assay and we need more researches to find out the real active components of the filtrate of *S. aureus* culture used in clinic. It is possible to prepare one or several pure SEs and use the ideal combination which has the lowest side effects and best treatments in clinic, and that will be helpful to the improvements of effects, controllable side effects and quality of these drugs.

These efficient procedures of expression and purification may be useful for the mass productions of these proteins, which are foundations for researches on mechanism of actions about the drugs from the filtrate of *S. aureus* culture and development of novel potentially therapeutically important protein drugs.

Acknowledgments

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