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Identification of a Methyltransferase Encoded by Gene *ste16* and Its Function in Ebosin Biosynthesis of *Streptomyces* sp. 139

Hong-Guan Xie[1†], Yong-Gang Bao[1†], Li-ping Bai[1], Jun-Jie Shan[2], Rong Jiang[1], Yang Zhang[1], Lian-Hong Guo[1], Ren Zhang[1,3], and Yuan Li[1*]

[1]*Key Laboratory of Biotechnology of Antibiotics, Ministry of Health, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, P. R. China*
[2]*Institute of Pharmacology and Toxicology, Beijing 100850, P. R. China*
[3]*School of Biological Sciences, University of Wollongong, NSW 2522, Australia*

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***Streptomyces* sp. 139 generates a novel exopolysaccharide (EPS) designated as Ebosin, which exerts an antagonistic effect on IL-1R *in vitro* and anti-rheumatic arthritis activity *in vivo*. A *ste* gene cluster for Ebosin biosynthesis consisting of 27 ORFs was previously identified in our laboratory. In this paper, *ste16* was expressed in *Escherichia coli* BL21 and the recombinant protein was purified, which has the ability to catalyze the transfer of the methyl group from S-adenosylmethionine (AdoMet) to dTDP-4-keto-6-deoxy-D-glucos, which was thus identified as a methyltransferase. In order to determine the function of *ste16* in Ebosin biosynthesis, the gene was disrupted with a double crossover via homologous recombination. The monosaccharide composition of EPS-m generated by the mutant strain *Streptomyces* sp. 139 (*ste16*⁻) was found to differ from that of Ebosin. The IL-1R antagonist activity of EPS-m was markedly lower than that of Ebosin. These experimental results have shown that the *ste16* gene codes for a methyltransferase which is involved in Ebosin biosynthesis.**

Keywords: methyltransferase, *ste16*, gene disruption, ebosin biosynthesis, *Streptomyces*

Microbial polysaccharides can be present as constituents of cell walls as components of lipopolysaccharides (LPS), which are frequently referred to as O-antigents or capsular polysaccharides (CPS) associated with the cell surface, or they can be secreted as exopolysaccharides (EPS) to the cellular environment. EPSs can be synthesized either extracellularly from exogenous substrates or intracellularly from sugar nucleotide precursors. They harbor repeating units, the biosynthesis of which involves glycosyltransferases which sequentially link sugars from intracellular nucleotide sugars to a lipid carrier (Boels *et al*., 2001).

Streptomyces is a group of Gram-positive bacteria that has been studied extensively, particularly with regard to their secondary metabolites. However, little is currently known regarding exopolysaccharide generation in *Streptomyces.* Recently, a novel EPS, referred to as Ebosin, was isolated from *Streptomyces* sp. 139 and was determined to exert an antagonistic effect against IL-1R *in vitro* and a definite anti-rheumatic arthritis effect *in vivo*. Ebosin is a heteropolysaccharide constructed from multiple copies of oligosaccharides harboring eight different monosaccharides (Wu *et al*., 1999). The biosynthetic gene cluster (*ste*) for Ebosin was identified in our laboratory, and the individual roles of the constituent putative genes were characterized (Wang *et al*., 2003; Zhang *et al*., 2006; Sun *et al*., 2007; Bai *et al*., 2008).

Previous homology searches have indicated that the protein product encoded by the *ste16* gene is highly homologous with some methyltransferases generated by a variety of microorganisms (Wang *et al*., 2003). In an effort to confirm its function, the gene was cloned and expressed in *E. coli* BL21 and the purified recombinant protein proved capable of transferring the methyl group from S-adenosylmethionine to dTDP-4-keto-6-deoxy-D-glucose, thereby identifying it as a methyltransferase.

To further evaluate its role in Ebosin biosynthesis, gene knockout and complementation experiments were carried out followed by monosaccharide composition and bioactivity analyses of the exopolysaccharides generated by the mutants. The results demonstrate its involvement in Ebosin biosynthesis, possibly as a modifier gene.

Materials and Methods

Bacterial strains, plasmids, and cultivation conditions
All strains and plasmids utilized are provided in Table 1. *Streptomyces* sp. 139 was isolated from a soil sample in China and deposited in the China General Microbiology Culture Collection Center (No 0405). The strain was cultured at 28°C with shaking (250 rpm) either in TSB medium (Difco, USA) supplemented with 5 mM $MgCl_2$ and 0.5% glycine, or in fermentation medium (1% glucose, 2% starch, 2% soybean extract, 0.2% tryptone, 0.2% beef extract, 0.4% yeast extract, 0.05% K_2HPO_4, 0.3% $CaCO_3$, pH 7.3). *E. coli* strains

† These authors contributed equally to this work.
* To whom correspondence should be addressed.
(Tel) 86-10-6301-3336; (Fax) 86-10-6301-7303
(E-mail) yuanwli@263.net

Table 1. Bacteria, plasmids, and primers used in this study

Bacterium, plasmid or primer	Description	Reference or source
Streptomyces		
sp. 139	Ebosin producing strain	Our lab
sp. 139 (*ste16*$^-$)	*ste16* knockout mutant of *Streptomyces* sp. 139	This study
Escherichia coli		
DH5α	F^- *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA- argF) U169*λ^-*(φ80dlacZΔM15)*	Sambrook and Russell (2001)
ET12567	methylation-deficient *E. coli*; *dam*$^-$ *dcm*$^-$ *hsdM*	MacNeil *et al.* (1992)
BL21 (DE3)	F^- *ompT hsdSB*(r_B^- m_B^-) *dcm gal λ(DE3)*	Novagen
Plasmid		
pUC18	*E. coli* general cloning vector with multiple cloning site; Amp^r	Sambrook and Russell (2001)
pKC1139	Shuttle plasmid (*E.coli-Streptomyces*); pSG5, pBR322; *aac(3)IV lacZa* $oriT_{RK2}$; Am^r	Bierman *et al.* (1992)
pKC16	pKC1139 derived plasmid carrying F1, F2 and Km^r fragments; Km^r Am^r	This study
pKC16c	pKC1139 derived plasmid carrying 0.45 kb ErmE* promoter fragment and *ste16*; Am^r	This study
pFD666	Resource of kanamycin resistance cassette; Km^r	Denis and Brzezinski (1992)
pGEM-3Zf-ErmE*	Resource of ErmE* promoter; Amp^r	Zhang *et al.* (2006)
pET30a-*ste16*	pET30a derived plasmid carrying *ste16*; Km^r	This study
Primer		
P1	CCAAGCTTCTCCACAACGTCTTCCC (*Hin*dIII)	This study
P2	GCTCTAGATGTCATCTGTTTCCCCCAT (*Xba*I)	This study
P3	GCTCTAGACGGCTGGTCTTCCCCATC (*Xba*I)	This study
P4	GCGAATTCCGGCATCTTGTCCACGGT (*Eco*RI)	This study
P5	GCTCTAGAATGACACGTTGCCGACT (*Xba*I)	This study
P6	CCAAGCTTTCATCGCTCGACCT (*Hin*dIII)	This study
P7	CGGGATCCATGACCCGTTGCC (*Bam*HI)	This study
P8	CGGAATTCTTATCGCTCGACCTCG (*Eco*RI)	This study

Amp^r, ampicillin resistance; Am^r, apramycin resistance, Km^r, kanamycin resistance

were grown in LB medium at 37°C and selected with the appropriate antibiotics.

General DNA manipulation

E. coli plasmid DNA was isolated and standard recombinant DNA techniques were performed in accordance with the protocols developed by Sambrook and Russell (2001). *Streptomyces* plasmids and genomic DNA were isolated as described by Kieser *et al*. (2000). For Southern analysis, a fluorescein labeling and detection kit acquired from Amersham Life Sciences (USA) was used in accordance with the manufacturer's instructions.

Disruption of the *ste16* gene

The primers utilized in this study are shown in Table 1. A 1,035 kb internal fragment (F1, upstream of *ste16*) and another 1.1 kb internal fragment of *ste16* (F2, downstream of *ste16*) were amplified via PCR from the chromosomal DNA of *Streptomyces* sp. 139 using the primer pairs P1, P2, P3, and P4, respectively. PCR amplification was then conducted under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 95°C, 0.75 min at 56°C and 1.5 min at 72°C, followed by a final 10 min extension at 72°C. A 1.23 kb fragment (F3) harboring the Km^r gene was digested with *Xba*I from pFD666IC plasmid (Dennis and Brzezinski, 1992). The F1, F2, and F3 fragments were cloned into the pKC1139 plasmid (Bierman *et al*., 1992) digested with *Hin*dIII-*Eco*RI to construct the pKC16 mutator plasmid. Following propagation in *E. coli* ET12567 (MacNeil *et al.*, 1992), pKC16 was introduced into *Streptomyces* sp. 139 via polyethyleneglycol (PEG)-mediated protoplast transformation (Kieser *et al*., 2000). After 16 to 20 h of incubation at 28°C, the plates were overlaid with soft R2YE (0.7% agar) containing apramycin (80 μg/ml). Plasmid pKC16 harbors a temperature-sensitive *Streptomyces* replication origin (MacNeil *et al.*, 1992) which is incapable of replicating at temperatures in excess of 34°C. Therefore, the transformants were first incubated for 2 days at 28°C until pinpoint-sized colonies became visible, then shifted to 37°C for further incubation. Mutants resulting from a double crossover via homologous recombination grew out of the original pinpoint-size colonies in several days.

Genetic complementation of *ste16* disruptant

A 1.25 kb DNA fragment of *ste16* was PCR amplified using primers P5 and P6 (Table 1), with *Streptomyces* sp. 139 genomic DNA as template. The amplification conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of 1 min at 95°C, 30 sec at 58°C and 2 min at 72°C plus a final 10 min extension at 72°C. A 0.45 kb ErmE* promoter fragment originated from pGEM-3Zf-ErmE* (Zhang *et al*., 2006) was digested by *Xba*I-*Eco*RI and then ligated with the 1.25 kb *ste16* fragment, followed by cloning into the pKC1139 digested by *Eco*RI-*Hin*dIII to create pKC16c. Following propagation in *E. coli* ET12567, pKC16c was transformed into the knockout mutant *Strepto-*

myces sp. 139 (*ste16*⁻). After 16~20 h of incubation at 28°C, the plates were overlaid with soft R2YE (0.7% agar) containing Am (80 μg/ml) and the transformants (Am^r) were selected.

Isolation and composition analysis of EPSs

Ebosin and its derivatives were isolated from the supernatants of fermentation cultures of *Streptomyces* sp. 139 and mutants as described previously (Sun *et al*., 2007). The sugar compositions of these EPSs were analyzed using gas chromatography (GC) (Xu *et al*., 1998), except that galacturonic acid was determined via a method based on the uronic acid carbazole reaction (Bitter and Muir, 1962).

Molecular exclusion chromatography

Molecular exclusion chromatography was performed to determine the molecular weight (Mw) of EPSs. The samples were dissolved in 1 ml of mobile phase (0.1 mM Na_2SO_4)

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Ste16.seq      .......MTRCRLCGSTALESVVDLGATPPCESFLAAEQLDAPEPAYPLH    43
SCO0392.seq    .......MTRCRLCGSAAMESVVDLGATPPCESFLAADQLDRPEPAYPLH    43
CouU.seq       MQDIVRPINECRVCGHNDWLDVVSFGSTPLAGNLLGTEDDPRDETLFPLD    50
NovU.seq       MQDIVRPIDECRVCGHDDWLDVVSFGSTPLAGNLLGDEDDAGGETLFPLD    50

Ste16.seq      LRVCTDCWLAQIPPLITPEDTFSEYAYFSSYSSSWVEHARTFVADTAARL    93
SCO0392.seq    LRVCTDCWLAQIPALITPEETFTQYAYFSSYSSSWVEHARTFVTGAVERL    93
CouU.seq       VGVCRKCWLMTLRHVIEPNALFGHYRYVASDAGSIIQHMRQLVELCIRWV   100
NovU.seq       VVVCRRCWLMTVRHVIEPDVLFGHYRYVASDAGSIIQHMRKLVDLCVERI   100

Ste16.seq      GLGGDSFVVEVASNDGYLLRHVVERGIRCLGVEPSVNVGAAARDAGVPTV   143
SCO0392.seq    GLGPGSFVVEVASNDGYLLRHMVDRGIRCLGVEPSVNVGAAAREAGVPTL   143
CouU.seq       GLTDRDLVVEFGSNTGAHLELFQQAGMRVVGVDPARNLADIANDRGVTTI   150
NovU.seq       GLTDGDLVVEFGSNTGAHLELFRQAGPRVVGVDPARNLAGVANDRGVETI   150

Ste16.seq      TEFLSPDTGAAVRAEHGPADLVVANNVYAHIPDVIGFTKGLRALVADDGW   193
SCO0392.seq    TEFLSPDTGAAVSAEHGPADLVVANNVYAHIPDVVGFTQGLRALVADDGW   193
CouU.seq       PAGFTTEVGQEITTGHGPARLVFGRQCFAHIHDVHEVLDGVSALLAPDGL   200
NovU.seq       PAGFTAEVGEEIATRHGLARLVYGRQCFAHIPDIHEVLNGVSALLAPDGL   200

Ste16.seq      VSIEVSDLLTLIEENQYDTIYHEHFQYYTVASAIRALASGGLTLVDVELL   243
SCO0392.seq    VSIEVQHLLTLIEENQYDTIYHEHFQYYTVASAIRALASGGLALVDVELL   243
CouU.seq       FFVEVPYLVELLANNQFDTIFHEHLSYFSLGALCRLFEAHGLRVVDVHTV   250
NovU.seq       FFVEVPYLVELLKNNQFDTIFHEHFSYFSLGSLCTLFESHGLRVVDVHTA   250

Ste16.seq      PTHGGSLRLWARPDEVAGEPGRRVADVLAREKAAGLQELSGYTEFSARVA   293
SCO0392.seq    PTHGGSIRLWARPAEVAGEPSRRVSDVLDREKAAGLRELSGYTEFSSRVA   293
CouU.seq       DVHGGSIVVFAAPATAGYEVRPAVAEMLAEERSQGIAEESTYQKFAERTE   300
NovU.seq       DVHGGSIVVFAAPAAADHEVRPAVAEMLAEERSQGIAEERTYREFADRTE   300

Ste16.seq      KVRRDLLRFLIEAAEDGRTVVGYGAPGKGNTLLNHCGVRPDLLPYTVDRN   343
SCO0392.seq    KVRRDLLRFLIDAAERGDTVVGYGAPGKGNTLLNHCGVRPDLLAYTVDRN   343
CouU.seq       RVRAQIRELVRSLVADGKTVAGYGAPTKGSALLTACGLGHQEIRFCSDTT   350
NovU.seq       RVRAQIRELVRGVVADGKTVAGYGAPTKGSALLAACGLGHQEIRFCSDTT   350

Ste16.seq      PYKHGRYTPGTRIPILPPDRIAADRPDYVLVLPWNLRAELVEQLSFVHEW   393
SCO0392.seq    PYKHGRFTPGTRIPILSPERIAADRPDYVLVLPWNLREELVEQLSFVHEW   393
CouU.seq       ALKQGKLLPGSRVPIWSPEQASDHVPDYYLLLAWNYASEIITKEKSFLEG   400
NovU.seq       VLKQGKILPGSRIPIWSPEQAAGHVPDYYLLLAWNYAPEIIDNEKEFLEN   400

Ste16.seq      GGRLVFPIPELSIVEVER....                                411
SCO0392.seq    GGRLVFPIPELSVVEAASEKGT                                415
CouU.seq       GGRFIVPIPEPRVISAESAW..                                420
NovU.seq       GGRFIVPIPEPRVISAESTL..                                420
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Fig. 1. Amino sequence alignment of Ste16 from *Streptomyces* sp. 139, *Streptomyces coelicolor* A3 (2) (SCF62.18), Bme21 from *Brucella melitensis*; and Bra0438 from *Brucella suis*.

and kept at room temperature for overnight. Twenty-five microliter of sample solution was injected into a TSK-GEL G-5000W$_{XL}$ column (7.8×300 mm, Japan) with 0.1 mM Na_2SO_4 as the mobile phase and a flow rate of 0.4 ml/min at 30°C (Waters 600, Detector2414, College Park, USA).

Assay for EPS bioactivity

The previously described ELISA method was used to analyze antagonistic activity for IL-1R of isolated EPSs (Sun *et al.*, 2007).

Cloning and expression of the *ste16* gene

With *Streptomyces* sp. 139 genomic DNA as template, the *ste16* gene coding region (1.24 kb) was PCR amplified using P7 and P8 primers (Table 1) under the same conditions as were utilized for the amplification of the *ste16* fragment mentioned above. The amplified DNA fragment was cloned into pET30a plasmid (Invitrogen) digested by *Bam*HI-*Eco*RI to construct pET30a-ste16. The correct nucleotide sequence of *ste16* gene fragment cloned into pET30a-ste16 was verified via sequencing using an ABI PRISM 377XL DNA Sequencer (Applied Biosystems).

Competent *E. coli* BL21 (DE3) cells were transformed with pET30a-ste16 plasmid and cultured overnight at 37°C in LB broth containing kanamycin (100 μg/ml). The culture was diluted to 1:20 with LB broth and subjected to further incubation at 37°C until the absorbance at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.05 mM. After an additional 3 h of incubation at 37°C, the bacterial cells were harvested and resuspended in binding buffer (10 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 8.0). The bacteria were sonicated and the cell debris was removed via centrifugation (12,000×g, 15 min) at 4°C.

Purification of the recombinant protein encoded by *ste16*

After centrifugation, the supernatant was loaded on a 2.0 ml Ni-NTA His-Bind resin column (Novagen) pre-equilibrated with binding buffer. The unbound proteins were then removed via elution with 5 ml of binding buffer and 10 ml of washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 8.0), and the recombinant protein was eluted with 5 ml of eluting buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 8.0). The fractions containing the recombinant Ste16 protein were collected and dialyzed with H_2O at 4°C. The purity of the protein was ascertained by SDS-PAGE and HPLC.

Enzyme assay

The enzyme activity of the purified protein was measured with a Methyltransferase activity kit (Assay Designs, USA) based on a coupled-fluorescence assay described by Collazo *et al.* (2005). The reaction mixture (200 μl) containing 25 μl of 1× Transferase Assay Buffer with varying quantities of the purified Ste16 recombinant protein (0~5 μg/ml), 25 μl 1× Reaction Buffer containing S-adenosylmethionine (AdoMet) and 4 mM dTDP-4-keto-6-deoxy-D-glucose (Genechem, Korea), 100 μl 1× Detection Solution of fluorescent substrate in DMSO. The reactions were performed in black 96-well flat-bottomed plates (Costar). After 30 min of incubation at 24°C with shaking (180 rpm), the reaction was stopped with 50 μl of ice cold isopropyl alcohol. The amount of S-adenosylhomocysteine (AdoHcy) produced over time was assessed by the increase in fluorescence at 380ex/520em using a POLARstar plate reader (BMG).

Results

Homologous analysis of the *ste16* gene

The DNA sequence of *ste16* reported in this study has been deposited in GenBank under accession number AY131229. Database searches revealed that the protein bears 89% sequence identity and 94% similarity over a 408-aa region to SC00392 of *Streptomyces coelicolor* A3(2) (GenBank accession no.: NP_624714). To NovU of *Streptomyces caeruleus* (GenBank accession no.: AF1708880) the identity is 37% and similarity 52% over a 407-aa region. It shows 36% identity and 53% similarity over 413-aa to CouU of *Streptomyces rishiriensis* (GenBank accession no.: AAG29803). According to such searches, the protein encoded by *ste16* is highly homologous to some known microbial methyltransferase (Fig. 1).

Disruption of *ste16* and complementation of the mutant

With the method mentioned above, more than 5 of Km^r Am^s colonies were selected and the chromosomal DNA of the wild and mutant *Streptomyces* sp. 139 were separately digested with *Nco*I, then subjected to agarose gel electrophoresis. The assumed gene replacement of *ste16* is shown in Fig. 2A. Using the 1.1 kb fragment downstream of *ste16* (F2) as a probe, Southern hybridization was performed (Fig. 2B). The hybridization signal appeared at the expected sizes of 7.9 kb (wild-type strain) and 5.2 kb (*ste16*$^-$ mutant). Hence, the colonies with Km^r Am^s had integrated the kanamycin resistance cassette into the *ste16* gene, which has

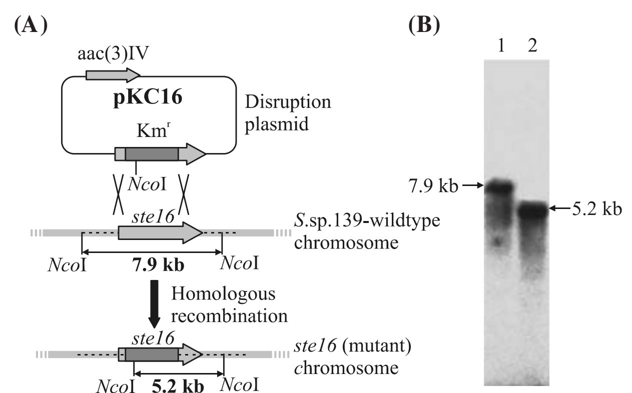

Fig. 2. (A) Gene replacement diagram of *ste16* with a double crossover via homologous recombination. The gray box indicates the location of *ste16*. Restriction maps of the wild-type *Streptomyces* sp. 139 and knock-out mutant (*ste16*$^-$) illustrate the predicted fragment sizes upon *Nco*I digestion. (B) Autoradiograph of the Southern blot analysis of wild and disrupt mutant *Streptomyces* sp. 139 strains using a 1.1 kb fragment downstream of *ste16* (F2) as a probe. Lane 1, DNA of wild type strain; 2, DNA of *ste16*$^-$ mutant.

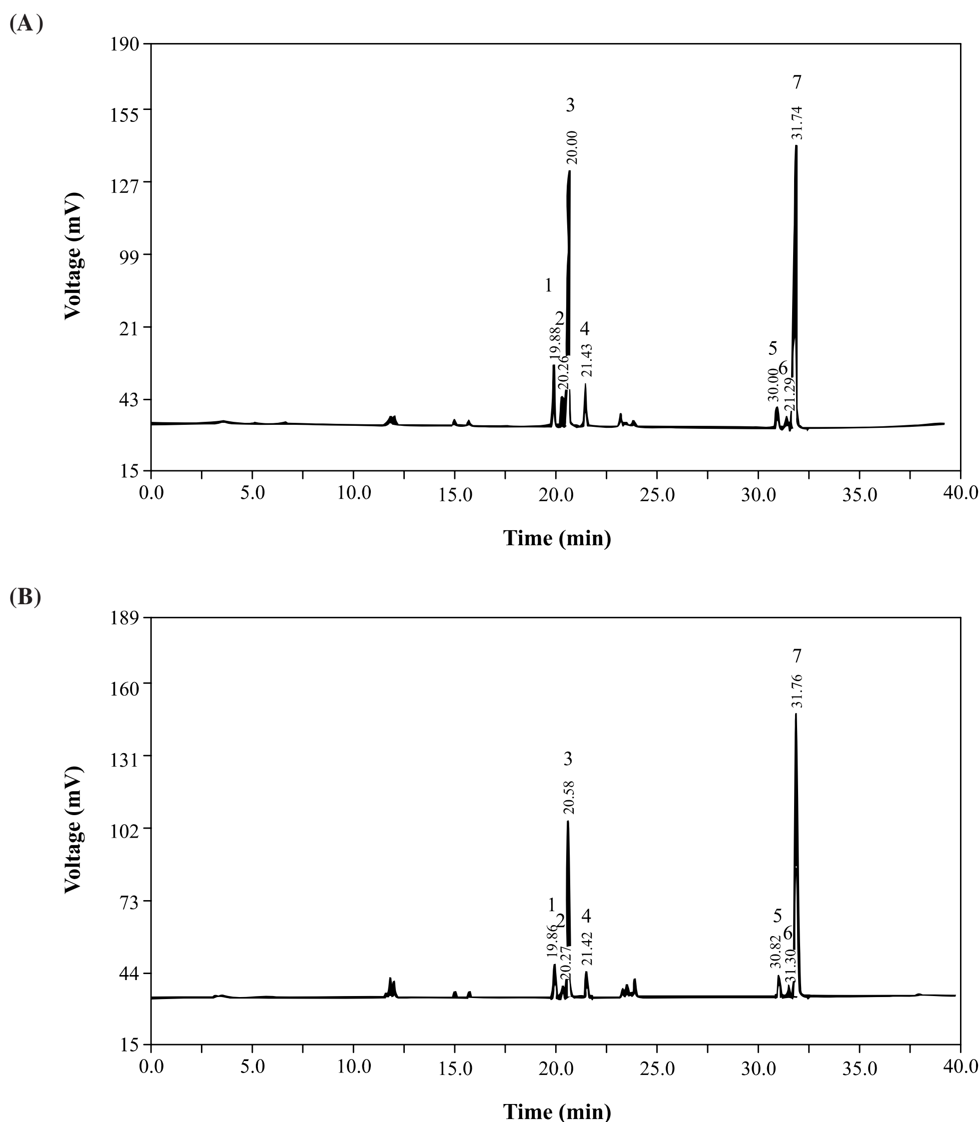

Fig. 3. GC chromatogram of sugar analysis of Ebosin produced by *Streptomyces* sp. 139 (A), and EPS-16m produced by *ste16* knockout mutant (B) separately. 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, glucose; 7, galactose.

therefore been deleted.

Following propagation in *E. coli* ET12567, pKC16c was transformed into the knockout mutant *Streptomyces* sp. 139 (*ste16*⁻). More than 10 apramycin-resistant transformants (Am^r) were selected, and subsequently verified in accordance with the specific restriction digestion (*Eco*RI-*Hin*dIII) patterns of the isolated plasmids.

Sugar composition of EPSs

GC analysis of EPS-16m produced by *Streptomyces* sp. 139 (*ste16*$^-$) showed that it was comprised of glucose, mannose, arabinose, galactose, fucose, xylose, galacturonic acid, and rhamnose (Fig. 3B), and the percentage was similar for most of monosaccharides as compared with Ebosin (Fig. 3A).

The analysis of EPS-16c produced by the *ste16*-complemented mutant strain with GC evidenced no marked changes as compared with EPS-16m.

Molecular weights of EPSs

The molecular weights of EPSs were analyzed using molecular exclusion chromatography. The results showed that the Mw of EPS-16m produced by the knockout strain *Streptomyces* sp. 139 (*ste16*$^-$) was 55.21×10^4 and EPS-16c isolated from the *ste16*-complemented mutant strain was 54.2×10^4, and both are smaller than Ebosin (65.64×10^4).

Antagonist activity of EPSs for IL-1R

The results of the ELISA assay demonstrated that the antagonist activities of EPS-16m produced by the *Streptomyces* sp. 139 (*ste16*$^-$) mutant for IL-1R were 23.2% (at 3.2 ng/μl) and 8.3% (at 0.64 ng/μl), which were significantly lower than those of Ebosin (54.5% and 46.2%, respectively) at the same concentrations. After Following gene *ste16* complementation by plasmid pKC16c transformation, the antagonist activities of EPS-16c increased to 47.1% (at 3.2 ng/μl) and

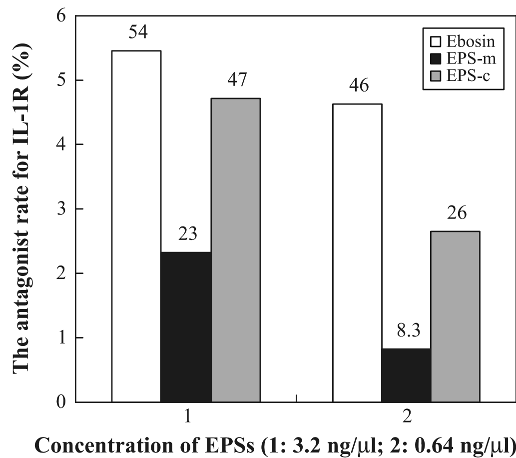

Fig. 4. IL-1R antagonist activities of EPSs produced by *Streptomyces* sp. 139 (Ebosin), its *ste16* knockout mutant (EPS-16m) and complement strain (EPS-16c).

26.6% (at 0.64 ng/μl), respectively, evidencing partial recovery as compared with EPS-16m, but still lower than the original levels of Ebosin (Fig. 4).

Cloning and expression of gene *ste16* in *E. coli*

The gene *ste16* was cloned and expressed under the control of the T7 promoter with pET30a as a vector in *E. coli* BL21 (DE3). The nucleotide sequence of the *ste16* gene fragment cloned in pET30a-ste16 was determined by via sequencing and proved to be identical to the original genomic sequence (GenBank accession no.: AY131229). The recombinant protein promoter was found to be generated mostly produced in principally in soluble form. The SDS-PAGE of the cell lysate samples stained with Coomassie Blue demonstrated that the supernatant contained the recombinant protein with a molecular weight in agreement with the expected size of ~47 kDa (including 2.5 kDa of His-tag originated from pET30a) (Fig. 5).

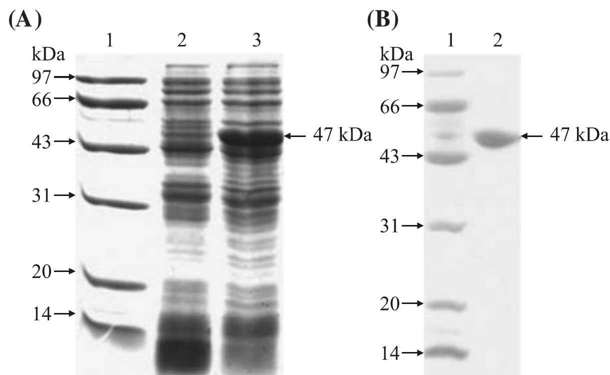

Fig. 5. SDS-PAGE analysis of the recombinant Ste16 protein before (A) and after (B) purification by affinity chromatography. (A) Lane 1, Protein size markers; 2, the supernatant of *E. coli* BL21 (pET30a); 3, the supernatant of *E. coli* BL21 (pET30a-*ste16*). (B) Lane 1, Protein size markers; 2, the purified recombinant protein Ste16.

The recombinant Ste16 protein was purified to homogeneity by the His-Bind resin affinity chromatography, which was examined by SDS-PAGE (Fig. 5). HPLC analysis showed the purity of the protein eluted by the final elution buffer to be about 90%.

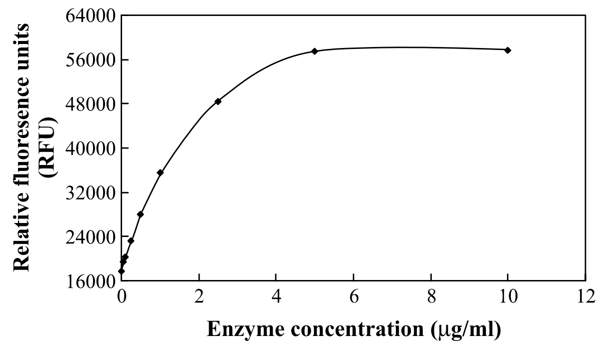

Fig. 6. Effect of protein concentrations on the reaction of S-adenosylhomocysteine formation catalyzed by the purified recombinant protein Ste16.

Methyltransferase activity of the *ste16* gene product

The methyltransferase assay was performed with a Methyltransferase activity kit (Assay Designs, USA) on the basis of a coupled-fluorescence assay as described by Collazo *et al.* (2005). In the reactions, the amount of S-adenosylhomocysteine (AdoHcy) over time increased obviously with increasing concentrations of the Ste16 protein (Fig. 6), thereby showing that the Ste16 protein possesses the methyltransferase activity which catalyzes the transfer of the methyl group from S-adenosylmethionine (AdoMet) to dTDP-4-keto-6-deoxy-D-glucose and then the production of S-adenosylhomocysteine (AdoHcy). When the concentration of the enzyme protein reached 6.0 μg/ml, the activity curve tended to be flat.

With the fluorescent assay, the methyltransferase encoded by *ste16* evidenced Michaelis-Menten kinetics and an apparent *K*m value of 0.43±0.02 mM.

Discussion

In recent years, EPSs originated from various microbes have attracted a great deal of interest because some of them have been recognized as remedies for the treatment of several diseases. They have been shown to possess diverse physiological activities (Welman and Maddox, 2003). EPSs isolated from lactic acid bacteria (LAB) may confer health benefits such as immunostimulatory (Chabot *et al.*, 2001), antitumoral (Kitazawa *et al.*, 1991), and blood cholesterol lowing activities. EPSs produced by *Trichoderma erinaceum* DG-312 had a strong anti-inflammatory activity against inflamed mice (Joo and Yun, 2005). *Latiporus sulphureus* var. *miniatus* produced EPSs with anti-diabetic activity (Hwang *et al.*, 2008).

The novel EPS Ebosin isolated from *Streptomyces* sp. 139 (Wu *et al.*, 1999) represents another example. This heteropolysaccharide, consisting of eight different monosaccharides

has been found to possess antagonist activity for IL-1R *in vitro* and remarkable anti-rheumatic arthritis activity *in vivo*. To elucidate the molecular mechanism controlling its production is of application significance as well as advancing our understanding of bacterial EPS biosynthesis pathways. The focus of this paper is on the function of the *ste16* gene.

After cloning and expression of the *ste16* gene in *E. coli* BL21, the recombinant protein was purified with the affinity chromatography, which was identified to have the ability of transferring the methyl group from S-adenosylmethionine (AdoMet) to dTDP-4-keto-6-deoxy-D-glucose and production of S-adenosylhomocysteine (AdoHcy). Thus, Ste16 has been determined to be a methyltransferase that can use dTDP-4-keto-6-deoxy-D-glucose as a substrate. dTDP-4-keto-6-deoxy-D-glucose has been reported to be a key intermediate of the 6-deoxyhexoses (6DOHs) biosynthesis (Patallo *et al*., 2001). Bearing this in mind, we assume that Ste16 transfers methyl groups to rhamnose or fucose residues (6DOHs) of Ebosin *in vivo*, although the direct evidence has yet to be provided.

Recently, several novel bacterial methyltransferases involving different biosynthetic pathways have been characterized. Novobiocin produced by *Streptomyces spheroids* belongs to the aminocoumarin antibiotics and comprises an unusual deoxysugar moiety named noviose conferring the biological activity to this compound. The *novU* gene was found to code for a C-methyltransferase which catalyzes methylation of the sugar group (Thuy *et al*., 2005). Sequence comparison between the *ste16* gene product and NovU (GenBank accession no.: AF1708880) showed that the identity is 37% and similarity 52% over a 407-aa region. The molecular weight of Ste16 is 47 kDa, lower than NovU (56 kDa). Whether Ste16 is also a C-methyltransferase in *Streptomyces* sp. 139 needs further investigation.

The disruption of *ste16* in our study also influenced the biosynthesis of Ebosin, hence observed reduction of molecular weight and changes in sugar composition of the EPS. In addition, ELISA assay demonstrated that the antagonist activity of EPS-16m for IL-1R is significantly lower than that of Ebosin. The methyltransferase encoded by *ste16* may have its role of methylating 6DOHs (rhamnose or fucose and derivatives) in Ebosin during its biosynthesis, which affects not only the polymerization of the repeating units but also the bioactivity significantly. Therefore, *ste16* may act in the Ebosin biosynthesis pathway as a modificator gene.

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