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Keywords
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Biochemical Characteristics and Function of a Fucosyltransferase Encoded by *ste7* in Ebosin Biosynthesis of *Streptomyces* sp. 139

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A novel exopolysaccharide named Ebosin was produced by *Streptomyces* sp. 139, with medicinal activity. Its biosynthesis gene cluster (*ste*) has been previously identified. For the functional study of the *ste7* gene in Ebosin biosynthesis, it was disrupted with a double crossover *via* homologous recombination. The monosaccharide composition of EPS-7m produced by the mutant strain *Streptomyces* sp. 139 (*ste7*$^-$) was found altered from that of Ebosin, with fucose decreasing remarkably. For biochemical characterization of Ste7, the *ste7* gene was cloned and expressed in *Escherichia coli* BL21. With a continuous coupled spectrophotometric assay, Ste7 was demonstrated to have the ability of catalyzing the transfer of fucose specifically from GDP-β-L-fucose to a fucose acceptor, the lipid carrier located in the cytoplasmic membrane of *Streptomyces* sp. 139 (*ste7*). Therefore, the *ste7* gene has been identified to code for a fucosyltransferase, which plays an essential role in the formation of repeating sugars units during Ebosin biosynthesis.

Keywords: *ste7* gene, fucosyltransferase, exopolysaccharide, Ebosin biosynthesis, gene disruption, *Streptomyces*

Glycosyltransferases (GTFs) constitute a large family of enzymes involved in biosynthesis of oligosaccharides and polysaccharides. They act through the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. Particularly abundant are a group of such enzymes, present in both prokaryotes and eukaryotes, that utilize activated nucleotide sugars as donor and play significant roles in important biological processes [6].

Many bacteria are known to produce exopolysaccharides (EPSs), which are excreted into the environment. The biosynthesis of EPSs that consist of repeating units includes their assembly on a lipid carrier by sequential transfer of monosaccharides from nucleotide sugars by glycosyltransferases (GTFs) and the subsequent polymerization and export of the repeating units [24]. Although numerous bacterial gene clusters involved in EPS biosyntheses have been described, only a few have been analyzed for the function of their GTF genes.

Streptomyces are a group of Gram-positive bacteria that have been intensively studied for their secondary metabolites, particularly antibiotics. However, little is known of the EPSs production in *Streptomyces*. Recently characterized by our laboratory is a novel EPS produced by *Streptomyces* sp. 139, named Ebosin, with antagonistic activity for IL-1R *in vitro* and remarkable antirheumatoid arthritis activity *in vivo* [26]. With the *ste* gene cluster for Ebosin biosynthesis identified [25], characterization of individual roles of the 27 *ste* genes has been in progress [23, 28]. In this study, gene knockout and complementation experiments were performed followed by the analysis of monosaccharide composition and bioactivities of the exopolysaccharides produced by the *ste7*-disrupted and subsequently complemented strains of *Streptomyces* sp. 139. The *ste7* gene was cloned and expressed in *E. coli* BL21. With a continuous coupled spectrophotometric assay, the recombinant Ste7 was found capable of catalyzing the transfer of fucose specifically from GDP–fucose to a fucose acceptor, the lipid carrier located in the cytoplasmic membrane of the *ste7* gene disrupted mutant *Streptomyces* sp. 139 (*ste7*$^-$). Our results indicated that the *ste7* gene encodes a fucosyltransferase and functions in Ebosin biosynthesis.

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MATERIALS AND METHODS

Bacterial Strains and Cultural Conditions

Streptomyces sp. 139 was isolated from a soil sample in China and kept in the China General Microbiology Culture Collection Center (No. 0405). The strains of *Streptomyces* sp. 139, *Streptomyces* sp. 139 (*ste7*$^-$), *Streptomyces* sp. 139 (*ste15*$^-$), and *Streptomyces* sp. 139 (*ste22*$^-$) were grown at 28°C with shaking (250 rpm), either in TSB medium 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_2HPO4, 0.3% $CaCO_3$, pH 7.3). *Escherichia coli* strains were grown in LB medium at 37°C and selected with appropriate antibiotics.

Construction of the *ste7* Gene Disrupted Strain

A 1.03-kb fragment (F1) upstream of *ste7* was PCR amplified using primers P1 (5'-AGCAAGCTTTGTTCGGGCTGTCGTTCAAG-3' HindIII adaptor restriction enzyme site underlined) and P2 (5'-GCA TCTAGACCATCACGCCCAGGTAGC-3'; XbaI), whereas a 0.899-kb fragment (F2) downstream of *ste7* was amplified using primers P3 (5'-ACGTCTAGATGGCGGAGCAGGTGGAGTTC-3'; XbaI) and P4 (5'-CTGGAATTCACCGTCTGCCGCAGTTCG-3'; EcoRI) with the genomic DNA of *Streptomyces* sp. 139 as template. A Km^r gene fragment (F3, 1.2 kb) was isolated from plasmid pFD666 [5] digested by XbaI. After ligation of the three DNA fragments (F1 and F2, then F3), the 3.13-kb fragment was inserted into pKC1139 [2] at EcoRI–HindIII sites to create the *ste7* disruption vector pKC7. Propagated in the methylation-deficient *E. coli* 12567 [17], pKC7 was isolated and introduced into *Streptomyces* sp. 139 by polyethylene glycol (PEG)-mediated protoplast transformation [13]. The protocol resulting mutants from a double crossover *via* homologous recombination was performed as before [23]. The gene *ste7* disruptants (*ste7*$^-$) were selected by both apramycin sensitivity (Am^s, 80 μg/ml) and kanamycin resistance (Km^r, 100 μg/ml).

Complementation of *ste7* Gene Disruption Mutant

With *Streptomyces* sp. 139 genomic DNA as template, the *ste7* gene (1.215 kb) was amplified with PCR using primers P5 (5'-GCTCTAG AGTGGAGAACCTGTCGGTG-3'; XbaI) and P6 (5'-GCAAGCTTT CAAGGGACG-3'; HindIII). The 0.45-kb ermE* promoter fragment was isolation from plasmid pGEM-3zf-ermE* digested by EcoRI and XbaI. After ligation, the joined ermE*–*ste7* fragment (1.66 kb) was inserted into plasmid pKC1139 digested with EcoRI and HindIII to yield pKC7C. After propagation in *E. coli* ET12567, pKC7C was isolated and transformed into the protoplasts of *Streptomyces* sp. 139 (*ste7*$^-$).

Isolation and Sugar Composition Analysis of EPSs

Ebosin and its derivatives were isolated from the supernatant of fermentation culture of *Streptomyces* sp. 139 and mutants as described before [10]. The sugar compositions of these EPSs were analyzed using gas chromatography (GC) [27], except that galacturonic acid was determined using a method based on the uronic acid carbazole reaction [3].

ELISA Assay for EPSs

This method reported previously was used to analyze the antagonistic activity for IL-1R of isolated EPSs [23].

Cloning of the *ste7* Gene

With *Streptomyces* sp. 139 genomic DNA as template, the *ste7* gene was amplified by PCR using primers P7 (5'-GACCATGGAGAACC TGTCGGTGCCGT-3') and P8 (5'-GCAAGCTTTCATCATCGCTCA AGGGACG-3'; NcoI and HindIII underlined) under the following conditions: an initial denaturation at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 59°C, and 3 min at 72°C; and finally 10 min at 72°C. The amplified DNA fragment was cloned into plasmid pET30a (Invitrogen) digested with NcoI and HindIII to construct pET30a (*ste7*). The correct nucleotide sequence of *ste7* gene fragment cloned in pET30a (*ste7*) was verified by sequencing using an ABI PRISM 377XL DNA Sequencer (Applied Biosystems).

After transformation, an overnight culture of *E. coli* BL21 (pET30a-*ste7*) was subjected to further incubation at 37°C until the absorbance at 600 nm reached ~0.5. Isopropyl-D-thiogalactoside (IPTG) was added to the culture at a final concentration of 0.08 mM. After further incubation at 37°C for 6 h, the bacterial cells were harvested by centrifugation (10,000 ×*g*, 10 min) and suspended in the cell lysis buffer (50 mM Tris-HCl, pH 8.5, 2 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1 mg/ml lysozyme). The cells were lysed by sonication.

Purification of the Recombinant Ste7 Proteins

After sonication and centrifugation (10,000 ×*g*, 10 min), the inclusion bodies were collected at 4°C and were treated with lysis buffer (50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 150 mM NaCl, 6 M guanidine hydrochloride, 200 mM β-mercaptoethanol, 5 mM DTT) overnight at 4°C. Then, the sample was dialyzed against renaturation buffer (20 mM Tris-HCl, pH 8.5, 1 M guanidine hydrochloride, 0.5 mM GSH, 0.5 mM GSSG, 0.5 M arginine, 2.0 mM EDTA, 80 mM NaCl, 8 mM β-mercaptoethanol) at 4°C for 36 h and finally with the binding buffer (10 mM imidazole, 0.3 M NaCl, 50 mM Na_2HPO_4, pH 8.0). One ml of the sample solution was loaded on a 2.0 ml Ni–NTA His-bind resin column (Novagen) pre-equilibrated with the binding buffer. Unbound proteins were removed with 8 ml of washing buffer (80 mM imidazole, 0.3 M NaCl, 50 mM Na_2HPO_4, pH 8.0) and the recombinant protein was eluted with 6 ml of eluting buffer (250 mM imidazole, 0.3 M NaCl, 50 mM Na_2HPO_4, pH 8.0). The fractions containing the recombinant Ste7 protein were collected and dialyzed with H_2O at 4°C and freeze-dried.

Isolation of the Cytoplasmic Membranes from *Streptomyces* sp. 139 (*ste7*$^-$)

With sucrose density gradient ultrocentrifugation [19], the cytoplasmic membranes of the mutant strain was isolated. Mutant strain culture (1 l) grown at 28°C was harvested by centrifugation (3,000 ×*g*, 15 min). The protoplasts were prepared with the protocol described by Kieser *et al* [13] and suspended in 10 ml of PTC buffer (sucrose 10.3%, $CaCl_2 \cdot 2H_2O$ 0.368%, $MgCl_2 \cdot 6H_2O$ 0.202%, K_2SO_4 0.025%, 0.25 M TES buffer 8%, trace element solution 0.2%). The protoplasts were frozen in liquid nitrogen and thawed for 5 times at 60°C, and the supernatant was collected by centrifugation (3,300 ×*g*, 10 min), which then was centrifuged for 30 min at 103,000 ×*g* in a Hitachi RPS-50-2-410 rotor. The pellets were resuspended in 2.5 ml of 20 mM potassium phosphate (pH 7.8) containing 68% sucrose, and the upper phase in 2 ml of 20 mM potassium phosphate (pH 7.8) containing 42% sucrose was gently added and centrifuged for 90 min at 197,000 ×*g*. Between the two phases, about 10 μl of sample with light brown color was collected, diluted with 20 mM potassium phosphate (pH 7.8) to a final sucrose concentration of about 10%, and centrifuged again

for 30 min at 125,000 ×*g*. The pellet cytoplasmic membranes from *Streptomyces* sp. 139 (*ste7*$^-$) was homogenized in 100 μl of 0.25 M sucrose and 5 mM potassium phosphate (pH 7.8). The isolation protocol of the cytoplasmic membranes from *Streptomyces* sp. 139 (*ste15*$^-$) and *Streptomyces* sp.139 (*ste22*$^-$) was the same as above.

Enzyme Assays for Fucosyltransferase

With a continuous coupled spectrophotometric assay for glycosyltransferase [7, 20], the enzymatic activities of Ste7 was identified. UDP-glucose, UDP-galactose, and GDP-fucose were purchased from Sigma (U.S.A.) and TDP-L-rhamnose is the product of GeneChem (Korea). In the assay, the GDP produced by the transferases were coupled to NADH oxidation by pyruvate kinase (PK) and lactate dehydrogenase (LDH) as shown in Eqs. (1)–(3):

$$\text{GDP-fucose+acceptor} \xrightarrow{\text{Fucosyltransferase}} \text{fuco-acceptor+GDP} \quad (1)$$

$$\text{GDP+phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{pyruvate+GTP} \quad (2)$$

$$\text{Pyruvate+NADH} \xrightarrow{\text{Lactate dehydrogenase}} \text{lactate+NAD}^+ \quad (3)$$

The decrease in absorbance at 340 nm is proportional to the amount of fucosyltransferase [Eqs. (1)–(3)]. The reaction mixtures (300 μl) containing 7.5 U of pyruvate kinase (PK), 15 U of lactate dehydrogenase (LDH), 0.7 mM phosphoenolpyruvate, 0.6 mM NADH, 50 mM KCl, 13 mM Hepes (PH 7.0), 13 mM $MnCl_2$, 0.13% BSA, no or varying amounts of the purified Ste7 recombinant protein (5–25 μg), GDP-fucose (10–80 μM), and the cytoplasmic membranes from *Streptomyces* sp. 139 (*ste7*$^-$) (0.5–5.0 μg) were incubated at 37°C for 10 min. The amount of NADH decrease over time was assessed for absorbance at 340 nm.

Results

Disruption of *ste7* Gene and Complementation of the Knockout Mutant

After the double crossover gene knockout process (Fig. 1A), a number of colonies (Km^r Am^s) were selected randomly and genomic DNAs were isolated. With the F2 fragment (0.899 kb XbaI-EcoRI fragment downstream of the *ste7* gene) as probe, Southern hybridization was carried out on BamHI digested genomic DNAs to confirm the gene disruption (Fig. 1B). The results revealed specific hybridization of the probe with expected sizes of 3.1 kb (*ste7*$^-$ mutant) and 2.0 kb (wild-type strain). Hence, the kanamycin resistance cassette was confirmed to have been integrated into *ste7* in the strains with Km^{r}Am^s, which therefore lost the gene function of *ste7*.

After pKC7C containing *ste7* was introduced into the knockout mutant strain *Streptomyces* sp. 139 (*ste7*$^-$), more than 40 transformants (Am^r Km^r) were obtained and the existence of plasmid pKC7C was confirmed by the expected restriction digestion pattern. One such clone was stocked, designated as *Streptomyces* sp. 139 (pKC7C).

Sugar Compositions of the EPSs

GC analysis of EPS-7m produced by *Streptomyces* sp. 139 (*ste7*$^-$) showed that fucose decreased from 2.85% (Ebosin) to 1.21%. The *ste7*- complementation by pKC7C enabled its recovery to 2.2% in EPS-7c produced by the mutant strain.

Bioactivity of EPS-7m and EPS-7c *In Vitro*

With the ELISA assay mentioned above, the antagonist activities of EPS-7m and EPS-7c for IL-1R *in vitro* were determined. The results showed that the antagonist activities of EPS-7m produced by *Streptomyces* sp. 139 (*ste7*$^-$) were 22.9% (at 0.0225 μg/μl) and 20.5% (at 0.0115 μg/μl), respectively, whereas those of EPS-7c produced by *Streptomyces* sp. 139 (pKC7C) were 38.6% (at 0.0225 μg/μl) and 31.1% (at 0.0115 μg/μl), respectively. They were compared with Ebosin's activities of 42.1% and 36.5% at the same concentrations, respectively. According to these results, the biological activity of EPS-7m reduced very

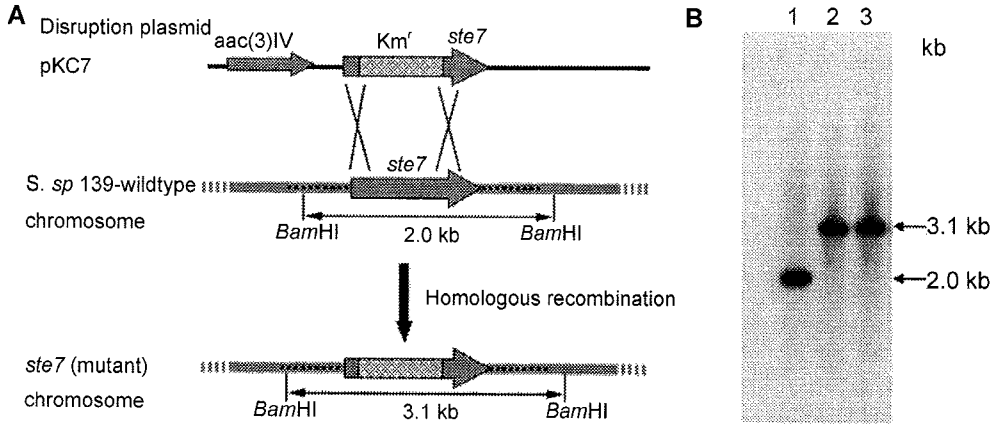

Fig. 1. A. Gene replacement of *ste7* with a double crossover *via* homologous recombination. The grey box indicates the location of *ste7*. Restriction maps of the wild-type *Streptomyces* sp. 139 and the mutant (*ste7*$^-$) show the predicted fragment sizes upon BamHI digestion. **B**. Southern blot autoradiograph of wild and two knockout mutant (*ste7*$^-$) *Streptomyces* sp. 139 strains. 1: Chromosome DNA of *Streptomyces* sp. 139 digested with BamHI; 2 and 3: chromosome DNA of knockout mutant clones (*ste7*$^-$) digested with BamHI.

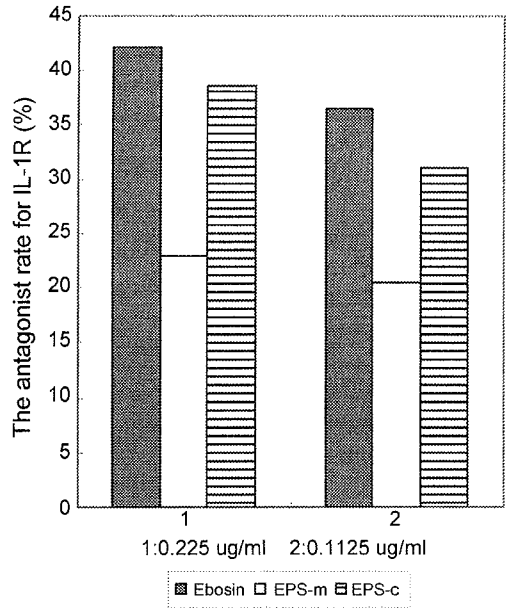

Fig. 2. The antagonist rate of Ebosin, EPS-7m, and EPS-7c for IL-1R at 0.0225 μg/μl and 0.01125 μg/μl, respectively, by ELISA assay.

significantly but the activity of EPS-7c was remarkably recovered (Fig. 2).

Expression of *ste7* in *E. coli* and Protein Purification

After cultivation of the strain *E. coli* BL21 (pET30a-*ste7*), the cell lysate was analyzed by SDS–PAGE with Coomassie blue staining. The result showed that there was an intensive protein band on the gel with molecular mass in agreement with the expected size of 49.6 kDa for Ste7 (Fig. 3). The protein was then purified with Ni–NTA affinity column chromatography. Elution fractions containing Ste7 were collected and dialyzed against H_2O at 4°C before being freeze-dried. The purity of the protein was ascertained by SDS–PAGE (Fig. 3) and HPLC, which demonstrated it to be about 90% (not shown).

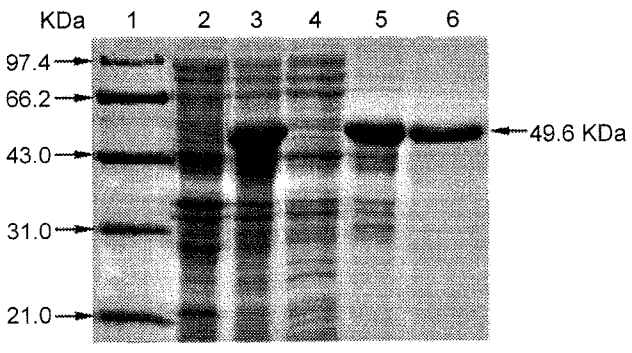

Fig. 3. SDS–PAGE analysis of the recombinant Ste7 protein before and after purification by affinity chromatography.
Lane 1: Protein size markers; lane 2: the lysate of *E. coli* BL21 (pET30a); Lane3: the lysate of *E. coli* BL21 (pET30a-*ste7*); lane 4: the supernatant of *E. coli* BL21 (pET30a-*ste7*) lysate; lane5: the pellet of *E. coli* (pET30a-*ste7*) lysate; lane6: the purified recombinant Ste7.

Fucosyltranferase Activity of Ste7

Using a continuous coupled spectrophotometric assay for glycosyltransferase, the enzymatic reaction of Ste7 in 300 μl was carried out with 2.5 μg of the cytoplasmic membranes of *Streptomyces* sp. 139 (*ste7*$^-$) as fucose acceptors. The result showed that Ste7 has the enzymatic activity of fucosyltransferase catalyzing GDP–fucose and the fucose acceptor to produce GDP, which were coupled to NADH oxidation by pyruvate kinase (PK) and lactate dehydrogenase (LDH). The decrease in absorbance at 340 nm was proportional to the amount of Ste7 (5–25 μg) (Fig. 4A).

After varying the amounts of the cytoplasmic membranes from *Streptomyces* sp. 139 (*ste7*$^-$) (0.5–5.0 μg) in the reaction mixture containing fixed amount of Ste7 (25 μg), the decrease in absorbance at 340 nm was also proportional to the concentration of cytoplasmic membranes from *Streptomyces* sp. 139 (*ste7*$^-$) (Fig. 4B).

In addition, under the same enzymatic reaction as above with fixed amounts of Ste7 (25 μg) and the cytoplasmic membranes from *Streptomyces* sp. 139 (*ste7*$^-$) (2.5 μg), a linear relationship between GDP–fucose concentration (10–80 μM) and the decrease in absorbance at 340 nm was observed (Fig. 4C).

According to the Lineweaver–Burk equation, the *K*m of fucosyltransferase encoded by *ste7* was determined to be 36.87±3.04 μM for GDP–fucose.

Substrate Specificity of Ste7

To test the substrate specificity of fucosyltransferase encoded by *ste7*, other sugar nucleotides including GDP–mannose, UDP–glucose, TDP–rhamnose, and UDP–galactose were separately reacted with the cytoplasmic membranes isolated from *Streptomyces* sp. 139 (*ste7*$^-$), but none produced catalyzed nucleotide. It was also true when GDP–fucose was reacted with cell membranes from other mutant strains including *Streptomyces* sp. 139 (*ste15*$^-$) and *Streptomyces* sp. 139 (*ste22*$^-$). These results, therefore, showed that Ste7 can only catalyze GDP–fucose and the *Streptomyces* sp. 139 (*ste7*$^-$) cytoplasmic membranes to produce GDP.

DISCUSSION

Much interest has been generated in recent years in microbial EPSs because some of them have been recognized as remedies for treating some diseases. EPSs isolated from lactic acid bacteria (LAB) may confer health benefits such as immunostimulatory [4], antitumoral, and blood cholesterol lowing activities [12, 14, 18,]. EPSs produced by *Trichoderma erinaceum* DG-312 showed a strong antiinflammatory

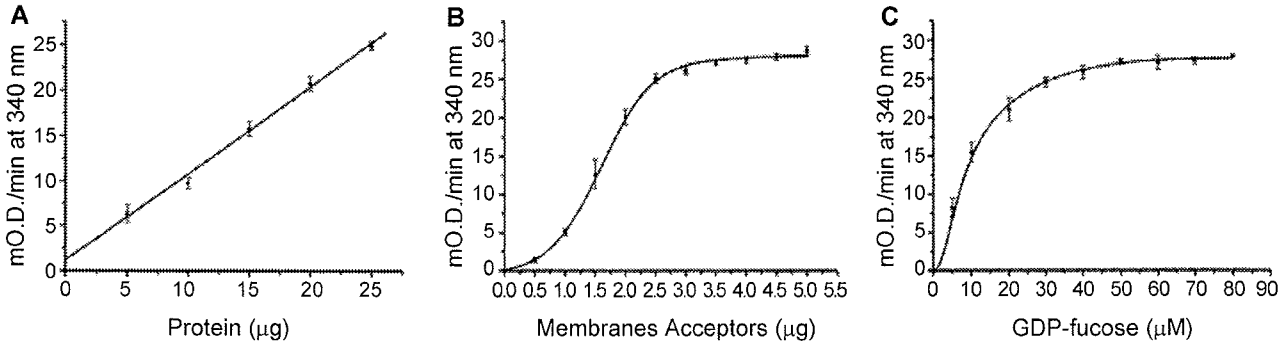

Fig. 4. Effects of varying amounts of Ste7 (**A**), acceptor [*Streptomyces* sp. 139 (*ste7*$^-$) cytoplasmic memberane] (**B**), and GDP-fucose (**C**) present in the continuous glucosyltransferase assay mixtures.

activity against inflamed mice [11]. *Latiporus sulphureus* var. *miniatus* also produced EPSs with antidiabetic activity [9].

Most EPSs consist of polymerized oligosaccharide repeating units and each such unit is assembled as glycoside-P-P-polyisoprenyl in a sequential series of reactions performed by specific glycosyltranferases (GTFs). These enzymes catalyze the transfer of sugar residues from an activated donor substrate to an acceptor molecule. The donor molecule is often a nucleotide diphosphosugar, but it can also be a sugar phosphate or a disaccharide. The acceptor molecule in most cases is a growing carbohydrate chain, but it can also be a lipid carrier or any of a variety of compounds [8, 21].

To study the role of the *ste7* gene located on the Ebosin biosynthesis cluster, a gene disruption approach was taken. The EPS-7m produced by the gene knockout mutant has been shown to affect mostly on the proportions of fucose, about 60% reductions (2.85% to 1.21%) compared with Ebosin. The complementation by expressing *ste7* on plasmid in the knockout mutant resulted in a recovery of the fucose's proportion (1.21% to 2.2%).

The antagonist activity of EPS-7m for IL-1R *in vitro* was significantly lower than Ebosin at both 0.0225 μg/μl and 0.0115 μg/μl. When the gene *ste7* disruption mutant was complemented, the reduction of this bioactivity was significantly reverted at the same concentrations. Based on such results, gene *ste7* significantly affects the bioactivity of Ebosin. This has led to our further experiments to investigate the enzyme specificity of Ste7.

Using pure recombinant protein and employing a continuous coupled spectrophotometric assay, the Ste7 protein has been confirmed to have the ability of catalyzing the transfer of fucose specifically from GDP–fucose to a fucose acceptor, the lipid carrier located in the cytoplasmic membrane of the mutant *Streptomyces* sp. 139 (*ste7*$^-$), hence it is a fucosyltransferase. Lu *et al.* [16] described that in *Streptomyces galilaeus*, AknK was demonstrated as an L-2-deoxyfucosyltransferase in the biosynthesis of the anthracycline aclacinomycin A. Recently, from *E. coli* O86:B7, Li *et al.* [15] also identified a α-1,2-fucosyltransferase involved in o-antigen biosynthesis. By sequence comparison, Ste7 shares very little homology with these already identified fucosyltransferases (not shown).

A variety of methods for the analysis of GTFs activities have been developed in recent years, as summarized by Palcic and Sujino [20]. These include radiochemical, spectrophotometric, immunological, and chromatographic types. Radiochemical assays are among the most frequently used. With such method, some studies of GTFs involved in EPSs biosynthesis have been performed [1, 22, 24]. However, their use is limited owing to the restricted availability of many substrates of radiochemicals. In our experiments, the continuous spectrophotometric assay for GTFs was used by coupling product formation to other enzymatic reactions that give a measurable change in UV/visible absorption or fluorescence. Although, owing to the limited sensitivity of the spectrophotometer, more enzymes are required than those needed for the radiochemical assays, results of the enzymatic reactions can give a measurable change in absorptions at 340 nm.

In conclusion, this study has demonstrated the *ste7* gene to specify a fucosyltransferase that is undoubtedly involved in the formation of repeating units consisting of monosaccharides during Ebosin biosynthesis.

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