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Functional analysis of ATP-binding cassette transporter of *Streptomyces coelicolor*

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ABSTRACT

ATP- binding cassette (ABC) transporters are characterized as multi drug resistant transporters utilizing ATP hydrolysis. The SCO5113 (GeneID: 1100554) encoding a multidrug resistance ABC transporter in *Streptomyces coelicolor* was expressed in *E.coli BL21 (DE3) plysS,* using pET 21 a(+). The expression of his – tag fused SCO5113 at 65 kDa under 0.1 M IPTG in 6 h was detected on SDS-PAGE after purification using affinity chromatography. The *E. coli BL21 (DE3) plysS* harboring SCO5113 was used to test for the antibiotic resistance, using disc diffussion method. The results showed the resistance of *E. coli* BL21 (DE3) *plysS* harboring SCO5113 to five kinds of tested antibiotics such as cephalothin (1mg/ml), kanamycin (1mg/ml), ampicillin (10mg/ml), erythromycin (10mg/ml), and chloramphenicol (1mg/ml). The study reported the function of the SCO5113 gene of *Streptomyces coelicolor* A3(2).

INTRODUCTION

ATP-binding cassette (ABC) transporters transport their substrates up a concentration gradient in or out of cells using the energy of ATP hydrolysis. Prokaryotic ABC proteins can be both importers and exporters, eukaryotic family members are only exporter (Vasilis *et al.,* 2009). In microorganisms ABC transporters participate in translocation of ions, sugars, amino acids, vitamins, lipids, antibiotics and drugs to larger molecules such as oligosaccharides, oligopeptides and even high molecular weight proteins (Christopher, 2001). In human, ABC transporters are related to many with genetic diseases including cystic fibrosis, obstetric cholestases, and drug resistance of cancers (Borst and Oude, 2002). ABC protein comprisesfour core domain: two membrane-bound domains that form the permeation pathway for transport of substrates, and two nucleotide binding domain that hydrolyze ATP to power (Dean *et al.,* 2001). ABC transporters lead to resistance of cancer cells against drugs used in chemotherapy, lipid disorder and inherited diseases (Hristos, 2004). They are often major players in complex pathways affecting gene expression (e.g., sporulation, competence and virulence development (Esther *et al.,* 2006). Among of the previously studied ABC transporters, LmrAin *Lactococcuslactis* was the first ATP- dependent prokaryotic multi drug resistance transporter (Bolhuis *et al.,* 1996), playing a role in the resistance of lincosamides, aminoglycosides, tetracyclines, macrolides, streptogramins, quinolones, the chloramphenicol broad- spectrum and slightly resistance β-lactam antibiotics (Poelarends *et al.,*2000). Besides, HorA of *Lactobacillus brevis* resisted to iso-α-acids from hop and HorA mediates resistance to the structurally unrelated compounds as novobiocin, ethidium bromide and hoechst 33342 (Sakamoto *et al.,* 2001).

Streptomyces, largest genus of *actinobacteria,* is a heterogeneous group of gram-positive microorganisms. They are used to produce the majority of antibiotics used in human and veterinary medicine and agriculture, as well as anti-parasitic agents, herbicides, pharmacologically active metabolites and several enzymes important in the food and other industries (Euzéby, 2008).

*Streptomyces coelicolor*was known producing at least four kinds of antibiotic; namely actinorhodin (Rudd and Hopwood, 1979), methylenomycin, the calcium-dependent lipopeptide antibiotic (CDA) (Kempter*et al.,* 1997), and a analogue of prodigiosin called undecylprodigiosin (Rudd and Hopwood, 1980).

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However, this strain can survive in the conditions of these products. It meant that there might be a component relating to adapt to these harsh conditions.

As the above stated reasons, the aim of this study was to over-express and evaluate the multi- antibiotic resistance of SCO5113 gene encoding an ABC transporter multidrug in *Streptomyces coelicolor* A3(2) exploiting the *E.coli* host expression system. Since the bacterial ABC transporter has been considered to play roles in nutrient uptake and drug resistance, an ideal candidate for the novel antimicrobial compounds both as target and delivery system to treat diseases, the *Streptomyces coelicolor* ABC transporter should be functional analysis.

MATERIALS AND METHODS

ABC transporter gene isolation

The chromosomal DNA from *Streptomyces coelicolor* was isolated (Jasmina *et al.,* 2003). DNA concentration was determined by recording the absorbance at 260 nm (A_{260}) using a spectrophotometer (Bio-Tek instruments, USA.). The purity of the DNA was determined from the A_{260}/A_{280} ratio. The quality of the isolated DNA was also evaluated by 0.8% agarose gel electrophoresis using 2µl of isolated DNA. The type of band pattern indicates the quality of the DNA. The DNA marker Lambda/Hind III Digest provided by Takara (Japan) was used to estimate the intensity and approximate size of the isolated DNA.

PCR amplification was performed for the isolation of gene from *Streptomyces coelicolor*. Forward primer TT1: CATATGAGCATTCTCCGTAACCGCAC and reverse primer TT2: CTCGAGCTTCTTGAGGAAGACCCGGwere designed in accordance with the published nucleotide sequence of the SCO5113 gene via the NCBI database and Primer3Plus software. Then the primers were ordered from Sigma- Aldrich (USA). PCR reactions were carried out in a total volume of 50 µl with a reaction mixture containing 5 µl of 10 x PCR buffer, 4 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTPs, 0.25 µl of forward primer and reverse primers, 0.25 µl of 5 u/µlTaq DNA polymerase, 2.5 µl of DMSO, 10 µl of genomic DNA and 23.75 µl of sterile distilled water. All the reaction mixtures were obtained from Sigma-Aldrich, USA.

The reaction mixture in micro-centrifuge PCR tube was amplified in a thermocycler (Eppendorf) with optimized conditions as followed: initial denaturation was performed at 96 °C for 2 minutes and the target DNA was amplified in 30 to 40 cycles. Each cycle consisted of denaturation (95°C, 30s), annealing (60 °C, 30s) and extension (72 °C, 60s).

After PCR running, 15µl amplified products were then separated by electrophoresis on a 0.8 % agarose gel and purified using a QIAquick gel extraction kit (QIAGEN, Netherlands). The PCR product was sequenced for checking (Nam Khoa company).

SCO5113 gene over-expression and protein isolation

E. coli BL21(DE3)plysS carrying SCO5113 was cultured in LB media containing ampicillin (100µg/ml) and chloramphenicol (34µg/ml) flask at 37 °C in shaking incubator condition for 2-3 hours until reaching the OD_{600}= 0.5 then induced with isopropyl-β-d-thiogalactopyranoside(IPTG). The optimized induction was checked at 6 h. The culture wascentrifuged at 10000 rpm for 10 minutes at 4 °C. The pellet was collected, dissolved in the buffer including 200 mMTris chloride, 100 mMNaCl, 5 mM imidazole, 5% glycerol, 2 mM EDTA. The suspensions were disrupted by sonication within 15 minutes. Then supernatant was collected after ultracentrifugation at 10000 rpm for 15 minutes. The protein extraction was ready for purification.

Protein purification

The protein extraction was purified using his-tag affinity chromatography to identify the expressed protein.The column washing buffer includes 200 mMTris chloride, 100 mMNaCl, 50mM Imidazole, 5% glycerol, 2 mM EDTA. The elution buffer includes 200 mMTris chloride, 100 mMNaCl, 300 mM Imidazole, 5% glycerol, 2 mM EDTA. After collection of the elution, the SDS – PAGE was done.

Hydrophobic analysis

To characterize the protein, the hydrophobic analysis was done, using Kyte and Doolittle software (1982).

Antibiotic resistance assay for functional analysis of SCO5113

To study the function of SCO5113 gene in antibiotic resistance, *E. coli BL21(DE3)plysS* carrying SCO5113 induced after 6 h was used for disc diffusion method to test antibiotic resistance tofive discs containing ampicillin (AM), cephalothin (CF), erythromycin (E), chloramphenicol (C), kanamycin (KM) at different ten-fold dilution concentrations from each 100mg/ml stock.The tests were done according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS) with Luria broth agar inoculated with transformants collected at every fixed expression time mentioned above (turbidity equivalent to that of 0.5 McFarland Standard which represents 1.5×10^8 bacteria/ml) (NCCLS, 2003) and each experiment was performed in triplicate. Inhibition- zone diameters were measured after aerobic incubation at 37°C for 24 hours and used as an indication for the borderline between sensitive and resistant cultures. *E. coli BL21(DE3)plysS* without SCO5113 was used as a control for the antibiotic resistance comparison and evaluation.

RESULTS

SCO5113 gene overexpression

PCR productwas checked on agarose (0.8 %) after purification (Figure 1). The sequence of the PCR product had 1803 bp and similarities with SCO5113 gene (100%), according to homology search by BLAST.Therefore, the PCR product was the target SCO5113. The SCO5113 gene was digested withNdeI and XhoI. This gene was ligated with pET21a(+) which was digested withNdeI and XhoI. The construction was introduced into *E.coli BL21 (DE3) plysS*. The optimal expression was induced for 6 h.To confirm the expression; the protein was purified from the cell extraction using the affinity chromatography. With the expression construction, the protein fused with his–tag showed high affinity to chromatography. Therefore, the unbound proteins will be washed out of the column. Therefore, the purified protein was the expected protein (figure 2). To determine the function of expressed protein in drug resistance, the *E. coli BL21 (DE3) plysS* containing SCO5113 was harvested at 6 h was established for disc diffusion testing of antibiotic resistance.

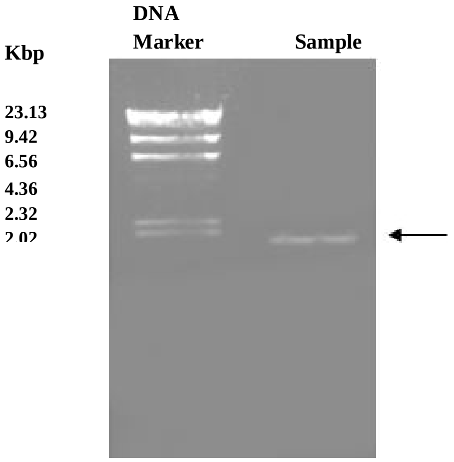

Fig. 1: PCR product analysis on agarose electrophoresis (0.8%). The arrow shows the PCR product.

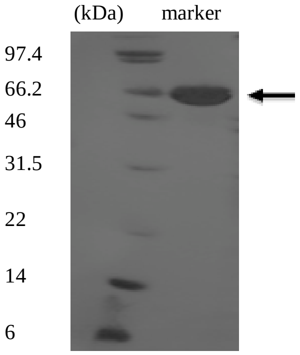

Fig. 2: SDS-PAGE for purified protein purification. Marker and purified protein were run in lane 1 and lane 2, respectively. The arrows show the expressed proteins.

Antibiotic resistance assay

According to table 1, antibiotic resistance tests of the over-expressed *E. coli BL21(DE3)plysS* harboring SCO5113 were done. The high resistance to cephalothin (1mg/ml), erythromycin (10mg/ml), chloramphenicol (1mg/ml), kanamycin (1mg/ml) and ampicillin (1mg/ml) in *E. coli* harboring the SCO5113 while the *E. coli* without SCO5113 is sensitive to the tested antibiotics. Because *E. coli BL21 (DE3)plysS* was used as host with the chloramphenicol resistance gene existence, the chloramphenicol resistance must occur surely. Normally, the chloramphenicol concentration was applied at 34 µg/ml for the bacteria selection. Therefore, *E. coli BL21 (DE3)plysS* was sensitive to chloramphenicol (1 mg/ml). However, in this study, the *E. coliBL21 (DE3)pLysS*-SCO5113 could resistant to chloramphenicol at high concentration (1 mg/ml) . Similarly, the pET 21 a(+) vector carrying ampicillin resistance gene was resistant to ampicillin at 100 µg/ml. However, in this study, the *E.coli BL 21(DE3)pLysS-SCO 5113* could resistant to chloramphenicol at high concentration (10 mg/ml) while the *E. coli BL21(DE3)pLysS* was sensitive at this concentration. Also, the E. *coli BL21(DE3)pLysS-SCO 5113* was resistant to cephalothin (1mg/ml), erythromycin (10 mg/ml), kanamycin (1mg/ml) pointed the function of ABC transporter gene. Obviously, SCO 5113 plays a role in multidrug resistance ABC transporter in *Streptomyces coelicolor*.

Table. 1: Diameter of inhibition zones for control cells in disc diffusion testing of 5 antibiotics.

Antibiotics	*E. coli BL21 (DE3)pLysS*	*E. coli BL21(DE3) pLysS-SCO 5113*
Cephalothin (1mg/ml)	S	R
Kanamycin (1mg/ml)	S	R
Ampicillin (10mg/ml)	S	R
Erythromycin (10mg/ml)	S	R
Chloramphenicol (1mg/ml)	S	R

(S): Sensitive (R): Resistant

Hydrophobic analysis

By hydrophobic analysis according tothe Kyte and Doolittle software (1982), the protein encoding by SCO 5113 has a structure like globular proteins (Figure 3). The window size has been set to 9, showing strong negative peaks indicating possible surface regions of globular proteins.

The horizontal axis is scaled to include only those amino acids for which a windowed hydropathy score is computed. A globular protein can act as transporter of other molecules through membranes, or messenger for transmitting messages to regulate biological processes. In this study, SCO5113 could play a role in multi – drug resistance. The other functions will be performed in further study.

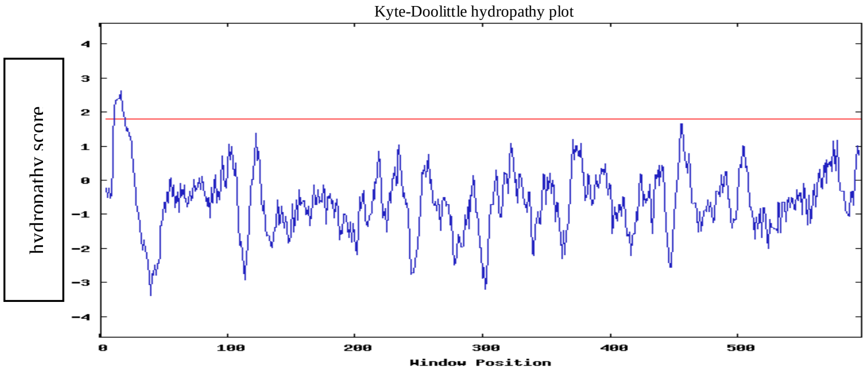

Fig. 3: Hydropathy plot of ABC transporter encoded by SCO5113.

CONCLUSION

The study has expressed successfully the multidrug resistance SCO5113 gene encoding for ABC transporter in *Streptomyces coelicolor*. And this gene showed the resistant activities to five tested antibiotics such as cephalothin (1mg/ml), kanamycin (1mg/ml), ampicillin (10mg/ml), erythromycin (10 mg/ml), and chloramphenicol (1mg/ml). To characterize this protein, further studies should be done.

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