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Bacteriocin Release Protein-Mediated Secretory Expression of Recombinant Chalcone Synthase in *Escherichia coli*

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Abstract Flavonoids are secondary metabolites synthesized by plants shown to exhibit health benefits such as anti-inflammatory, antioxidant, and anti-tumor effects. Thus, due to the importance of this compound, several enzymes involved in the flavonoid pathway have been cloned and characterized in *Escherichia coli*. However, the formation of inclusion bodies has become a major disadvantage of this approach. As an alternative, chalcone synthase from *Physcomitrella patens* was secreted into the medium using a bacteriocin release protein expression vector. Secretion of *P. patens* chalcone synthase into the culture media was achieved by co-expression with a psW1 plasmid encoding bacteriocin release protein in *E. coli* Tuner (DE3) plysS. The optimized conditions, which include the incubation of cells for 20 h with 40 ng/ml mitomycin C at OD_{600} induction time of 0.5 was found to be the best condition for chalcone synthase secretion.

Keywords Bacteriocin release protein · Chalcone synthase · Extracellular expression

Introduction

Chalcone synthase catalyzes the formation of naringenin chalcone from *p*-coumaroyl CoA with three acetate units of malonyl-CoA. Several chalcone synthase genes have been cloned and expressed mainly in *Escherichia coli* using various expression systems. However, recombinant proteins, especially eukaryotic genes produced in *E. coli*, are usually associated with the formation of inclusion bodies since they require posttranslational

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modification to be in their active form [1]. As an alternative, several methods have been developed to overcome these problems. One way to overcome the problem of incorrect folding is to secrete the recombinant proteins into the medium.

Co-expression of bacteriocin release protein (BRP) is an alternative strategy for the secretory expression of recombinant proteins. BRP is a small lipoprotein with 28 amino acid residues that is produced as a precursor with a signal peptide and secreted across the cytoplasmic membrane, where it is *N*-acetylated and inserted into the outer membrane. In the outer membrane, BRP can activate the detergent-resistant phospholipase A, resulting in the formation of permeable zones in the cell envelope through which proteins can pass and be released into the culture medium [5]. Currently, there has been no report on the secretory expression of chalcone synthase. Thus, the aim of this work was to express chalcone synthase extracellularly with the use of the BRP vector using *E. coli*.

The main objective of this work was to investigate the best condition of chalcone synthase secretory expression into the culture medium. Therefore, with the aim to increase the production of *Physcomitrella patens* chalcone synthase secretory expression, the effect of inducer concentration, OD induction time, and incubation time on the expression and secretion into the medium was investigated. Aside from providing an oxidized environment to allow proper folding of protein, the BRP-mediated release of chalcone synthase into the culture medium results in an easier purification procedure [1].

Materials and Methods

Bacterial Strains and Plasmids

The *E. coli* strains used for expression were *E. coli* Top10, Tuner (DE3) plysS and BL21 (DE3) plysS. Recombinant plasmid PET32(a)/CHS harboring an open reading frame of *P. patens* chalcone synthase gene was used along with psW1 plasmid (Mobitech, Germany) for secretory expression.

DNA Manipulation

The extraction of the recombinant plasmid DNA (PET32(a)/*P. patens* chalcone synthase (PpCHS)) was carried out using a QIAprep Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The PCR product and digested fragments were purified with GeneClean Kit (Qbiogene, Carlsbad, CA, USA) and gel extraction kit (Qiagen), respectively. Competent cells of *E. coli* strains were prepared by conventional $CaCl_2$ method.

Secretory Expression of *P. patens* Chalcone Synthase

The *P. patens* chalcone synthase gene, which had been previously cloned in PET32 (a)/PpCHS was transformed into both Tuner (DE3) plysS and BL21 (DE3) plysS and was screened with Luria–Bertani (LB) agar plates containing 50 μg/ml ampicillin. The psW1 vector that is resistant toward tetracycline was then co-transformed into the competent recombinant cells for secretory expression. Finally, Tuner (DE3) plysS were cultured using 50 μg/ml ampicillin and 20 μg/ml tetracycline for the selection of positive clones harboring the PET32(a)/CHS and psW1 vectors. Meanwhile, BL21 (DE3) plysS were cultured using 50 μg/ml ampicillin and 35 μg/ml chloramphenicol for the selection of positive clones harboring the PET32(a)/CHS and psW1 vectors.

Optimization of Secretory Expression of *P. patens* Chalcone Synthase

The optimization study was carried out by inoculating recombinant cultures harboring PET32 (a)/PpCHS and psw1 plasmids in 50 ml LB broth in a 250-ml scotch bottle. The culture (20 ml) was centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was used to analyze extracellular chalcone synthase activity. The pellet was resuspended with 20 ml of 20 mM potassium phosphate buffer (pH 7.2) prior to sonication (Branson 250 sonifier: output 2, duty cycle 30 for 2 min). The culture was then centrifuged at 14,000×*g* for 30 min and was used to analyze intracellular chalcone synthase activity, whereas the supernatant was used to analyze the extracellular chalcone synthase activity. The optimization studies on the effect of inducer, OD_{600} induction time, and incubation time of the extracellular expression of *P. patens* chalcone synthase were repeated twice to confirm the validity of the results.

Effect of Inducer Concentration on Secretory Expression of *P. patens* Chalcone Synthase

The recombinant *E. coli* Tuner (DE3) plysS cells were first optimized for extracellular protein expression using different concentrations of (isopropyl-beta-D-thiogalactopyranoside (IPTG; 0, 20, 40, 60, 80, or 100 μM) added at $OD_{600\ nm}$~0.5 for 12 h. The extracellular chalcone synthase activity of the cell lysate was then measured. After obtaining the optimum concentration of IPTG, different concentrations of mitomycin C (0, 20, 40, 60, 80, or 100 ng/ml) were added to test the extracellular expression of chalcone synthase activity.

Effect of Induction Time $OD_{600\ nm}$ on Secretory Expression of *P. patens* Chalcone Synthase

E. coli BL21 Tuner (DE3) harboring recombinant plasmid PET32(a)/CHS was induced at different OD600 nm ranging from 0.25 to 1.25 at 0.25 intervals. The culture was induced with 40 μM of IPTG for 12- and 24-h induction periods. The culture medium was used to determine extracellular chalcone synthase activity. After 12 h of incubation time, 20 ml of the expression culture was transferred to a microcentrifuge tube and was centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant which contains the soluble chalcone synthase was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to measure protein levels.

Time–Course Analysis of Secretory Expression of *P. patens* Chalcone Synthase

The time–course analysis was done by collecting the recombinant cells at different times after 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 h of growth at 37 °C with shaking at 150 rpm. The cell lysate was then used to determine extracellular chalcone synthase activity.

Enzyme Assay and Product Analysis

Enzyme assay was performed according to a modified method [6]. The reaction mixture (500 μl) contained 25 μl 0.8 mM malonyl-CoA, 25 μl 0.4 mM hexanoyl-CoA, and 100 μl enzyme in 20 mM phosphate buffer, pH 7, and was incubated at 30 °C for 2 h. The reaction products were extracted for 2 min with ethyl acetate and the upper layer subjected to high-performance liquid chromatography (HPLC) analysis. The reaction products were identified with HPLC (Nucleosil C18; flow rate, 0.5 ml/min; UV, 280 nm). The chromatography was

run using a gradient of Acetonitrile (30–60% in 30 min) in water. Under these conditions, 4-hydroxy-6-metyhl-2-pyrone and naringenin were used as an internal standard.

For liquid chromatography–tandem mass spectrometry (LC-MS/MS) detection of unknown compounds at a retention time of ~10 min, as shown in Fig. 7, the chalcone synthase assay was scaled up and the extracted product obtained was subjected to the mass spectrometer using naringenin as internal standard. The positive ionization mode was chosen as the detection modewith the collision gas off and was set at 370 °C. The mass spectrometer was operated using electrospray ionization (ESI) with an ion spray voltage of +4,500 V and using nitrogen gas as the collision gas, nebulizer gas, and curtain gas [2].

Results and Discussions

Secretory Expression of *P. patens* Chalcone Synthase

The recombinant plasmid (PET32(a)/CHS) which harbors mature chalcone synthase gene was co-transformed with the psW1 vector in Tuner (DE3) plysS and BL21 (DE3) plysS to express bacteriocin release protein and assist in chalcone synthase secretion. The positive clones were confirmed through both selections on antibiotic-containing agar plate and plasmid extraction, as shown in Fig. 1. Both types of strains harboring the PET32(a)/PpCHS and psW1 plasmids were induced using IPTG and mitomycin C to express the *P. patens* chalcone synthase and secrete it into the medium.

However, based on the SDS-PAGE analysis of the amount of *P. patens* chalcone synthase secreted into the media, *E. coli* Tuner (DE3) plysS shows higher secretion of chalcone synthase in the media compared with BL21 (DE3) plysS and thus was chosen for further studies. In addition, as shown in Fig. 2, Western blot analysis was carried out using anti-His antibody to prove that the protein expressed and secreted into the culture media is *P. patens* chalcone synthase.

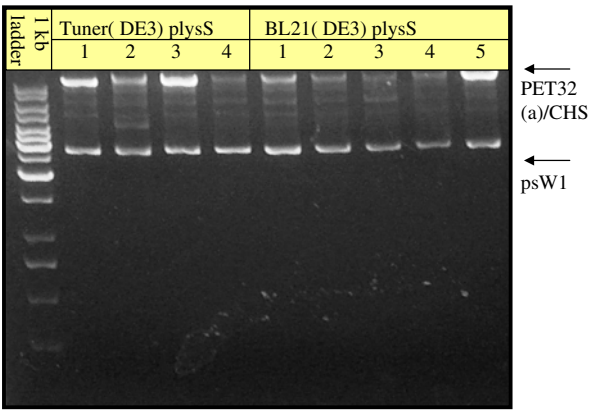

Fig. 1 Plasmid DNA extraction of the recombinant PET32(a)/CHS gene. The plasmid DNA of PET32(a)/CHS gene was electrophoresed on 1.0% agarose gel and viewed under UV light. Two *E. coli* strains harboring PET32(a)/CHS were used in the co-transformation of psW1 (BRP vector), which are BL21 (DE3) plysS and Tuner (DE3) plysS. Random colonies (1, 2, 3, and 4 for Tuner (DE3) plyss; 1, 2, 3, 4, and 5 for BL21 (DE3) plysS) were picked and analyzed to check for positive co-transformation. *Arrows* indicate the extracted plasmid of the recombinant PET32(a)/CHS gene and psW1 plasmid

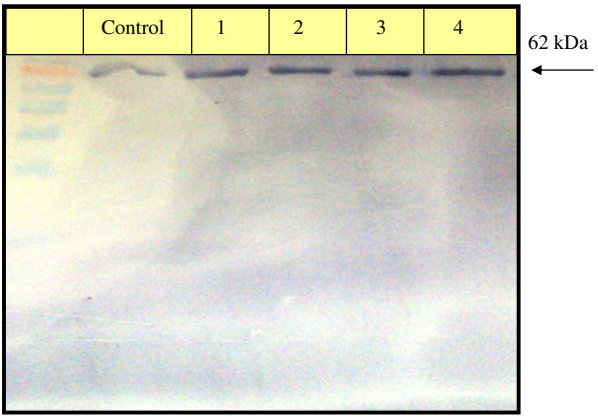

Fig. 2 Western blot of recombinant *P. patens* chalcone synthase. Western blot analysis was carried out on chalcone synthase expressed and secreted by Tuner (DE3) plysS using anti-His Tag monoclonal antibody. Recombinant chalcone synthase (intracellular) was used as a control to determine the size of extracellular chalcone synthase. *Lanes 1–4* show the extracellular expression of chalcone synthase by positive clones picked from Tuner (DE3) plysS. *Arrow* indicates the size of recombinant PpCHS which is 42 kDa

Optimization of Secretory Expression of *P. patens* Chalcone Synthase

A quasi-lysis test to find the suitable concentration of inducer (IPTG) for continuous secretion of chalcone synthase into the media was carried out to maintain cell viability. The quasi-lysis test measures the decline in culture turbidity due to cell lysis. Cell lysis is associated with undergraded BRP signal peptide accumulated in the cytoplasmic membrane caused by a high-level expression of BRP [9]. The recombinant Tuner (DE3) plysS harboring both PET32(a) and psW1 plasmids was incubated at 37 °C until an OD_{600} of 0.5 was reached. The cells were then induced with different concentrations of IPTG plus 20 ng/ml mitomycin C, and the OD_{600} was taken hourly as illustrated in Fig. 3. Based on the results shown in Fig. 3, it was determined that cell lysis occurred 4 h after induction with either 80 or 100 mM IPTG,

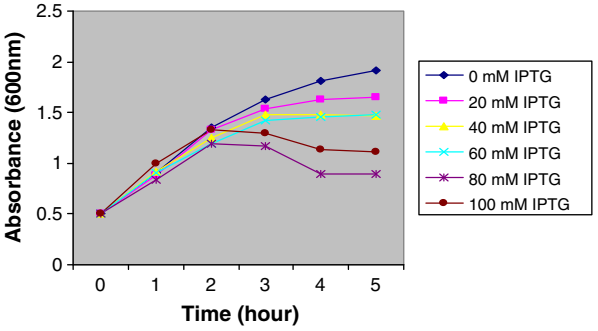

Fig. 3 Quasi-lysis test. The quasi-lysis test was carried out to determine the capability of Tuner (DE3) plysS in the continuous secretion of chalcone synthase without causing cell lysis. This test measures the decline in culture turbidity due to cell lysis. Tuner (DE3) plysS harboring recombinant plasmid PET32(a)/CHS and psW1 plasmid were induced with different concentrations of IPTG—20, 40, 60, 80, and 100 mM—along with 20 ng/ml of mitomycin C. Samples were taken every hour to measure the absorbance (600 nm) until a decline in the culture turbidity was reached

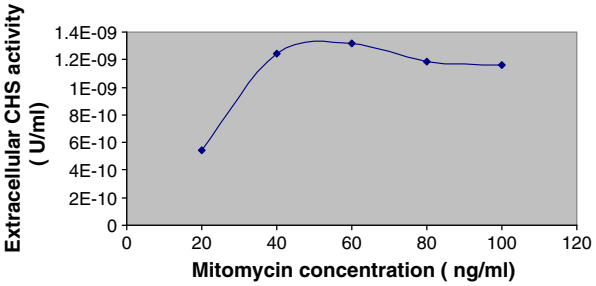

Fig. 4 Effect of inducer concentration on extracellular PpCHS activity. Tuner (DE3) plysS harboring recombinant plasmid PET32(a)/CHS and psW1 plasmid was induced with different concentrations of inducers: 20, 40, 60, 80, and 100 ng/ml of mitomycin C along with 40 μM IPTG at $A_{600\ nm}$~0.5. Samples were taken after 12 h of induction and assayed with 0.4 mM hexanoyl-CoA and 0.8 mM malonyl-CoA as substrates. One unit of chalcone synthase activity was defined as the amount of enzyme producing products (micrograms per milliliter) per minute

whereas the control (without addition of mitomycin C) showed a linear increase in cell growth. Thus, the use of a lower concentration of IPTG should be considered in subsequent experiments.

The Effect of Inducer Concentration on Secretory Expression of P. patens Chalcone Synthase

Co-transformation of psW1 vector into Tuner (DE3) plysS harboring the PET32(a)/CHS plasmid produced clones which expressed chalcone synthase into the culture medium. The PET32(a)/CHS vector contains tandemly the *E. coli* lac promoter–operator system which is regulated by *E. coli* lac repressor. On the other hand, the psW1 vector consists of the BRP gene under the control of mitomycin C-inducible pCloDF13 promoter. Therefore, the effectiveness of BRP co-expression in facilitating the release of chalcone synthase by Tuner (DE3) plysS was determined by varying the inducer concentration (0–100 μM).

As shown in Fig. 4, the optimum secretion of *P. patens* chalcone synthase into the media is achieved using 40 ng/ml of mitomycin C. From the result, increased secretion of chalcone synthase was observed as the concentration of mitomycin increased; however, the

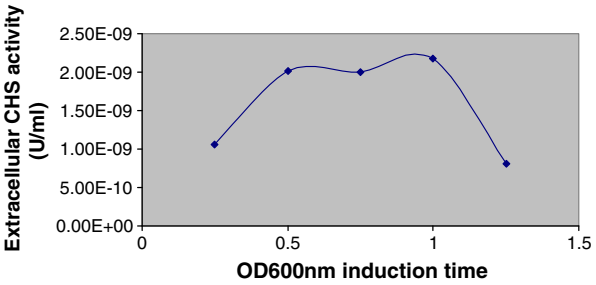

Fig. 5 Effect of OD_{600} induction time on extracellular PpcHS activity. Tuner (DE3) plysS harboring recombinant plasmid PET32(a)/CHS and psW1 plasmid was induced with 40 μM IPTG and 40 ng/ml mitomycin C at several induction $A_{600\ nm}$ times as follows: 0.25, 0.5, 0.75, 1.0, and 1.25. Samples were taken after 12 h of induction and assayed with 0.4 mM hexanoyl-CoA and 0.8 mM malonyl-CoA as substrates. One unit of chalcone synthase activity was defined as the amount of enzyme producing products (micrograms per milliliter) per minute

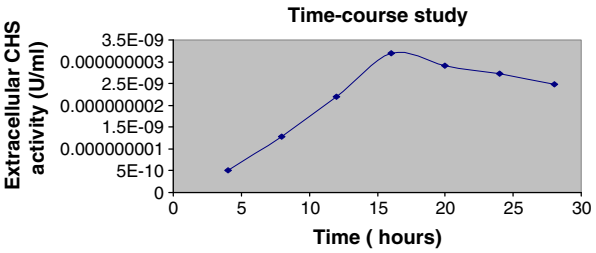

Fig. 6 Time course study on extracellular PpCHS activity. Tuner (DE3) plysS harboring recombinant plasmid PET32(a)/CHS and psW1 plasmid was induced with 40 μM IPTG and 40 ng/ml mitomycin C at $A_{600\ nm}$. The samples were taken at hourly intervals and assayed with 0.4 mM hexanoyl-CoA and 0.8 mM malonyl-CoA as substrates. One unit of chalcone synthase activity was defined as the amount of enzyme producing products (micrograms per milliliter) per minute

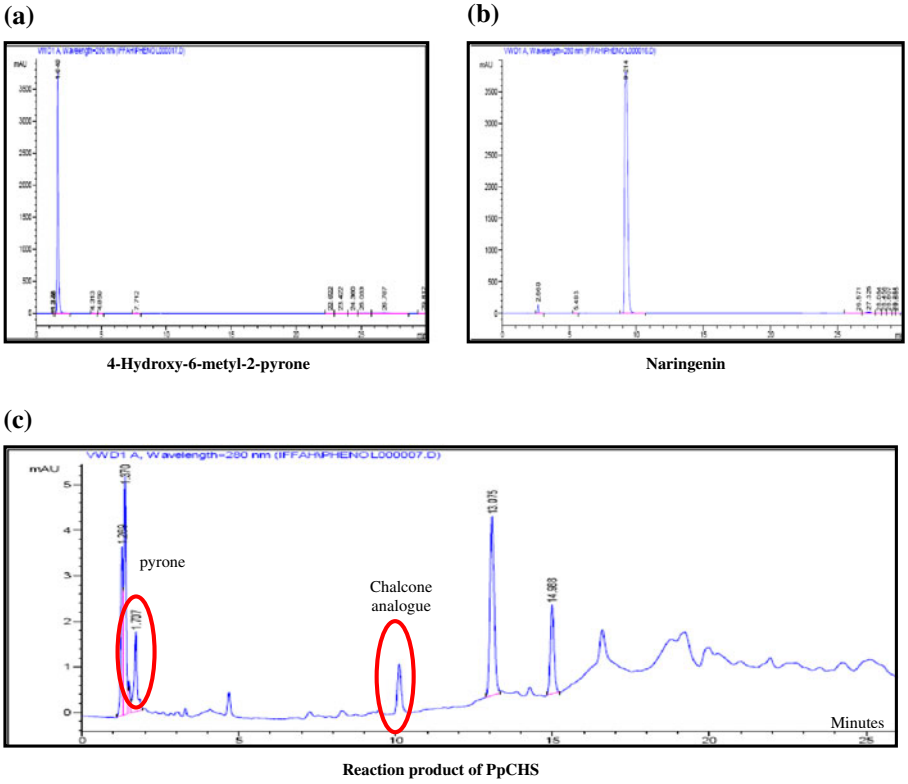

Fig. 7 HPLC elution profiles of the products formed by PpCHS. Two standards were run with HPLC (Nucleosil C18; flow rate, 0.5 ml/min; UV, 280 nm) using a gradient of acetonitrile (30–60%) in water for 30 min. Under these conditions, 1 mg/ml 4-hydroxy-6-metyl-2-pyrone (**a**) was eluted at a retention time of 1.65 min, while 1 mg/ml naringenin (**b**) was eluted at a retention time of 9.214 min. The chalcone synthase assay was carried out using 0.4 mM hexanoyl-CoA and 0.8 mM malonyl-CoA and was run under the same conditions. The reaction product of PpCHS (**c**) showed the formation of pyrone and chalcone analogues as by-products at retention times of 1.707 and 10 min, respectively

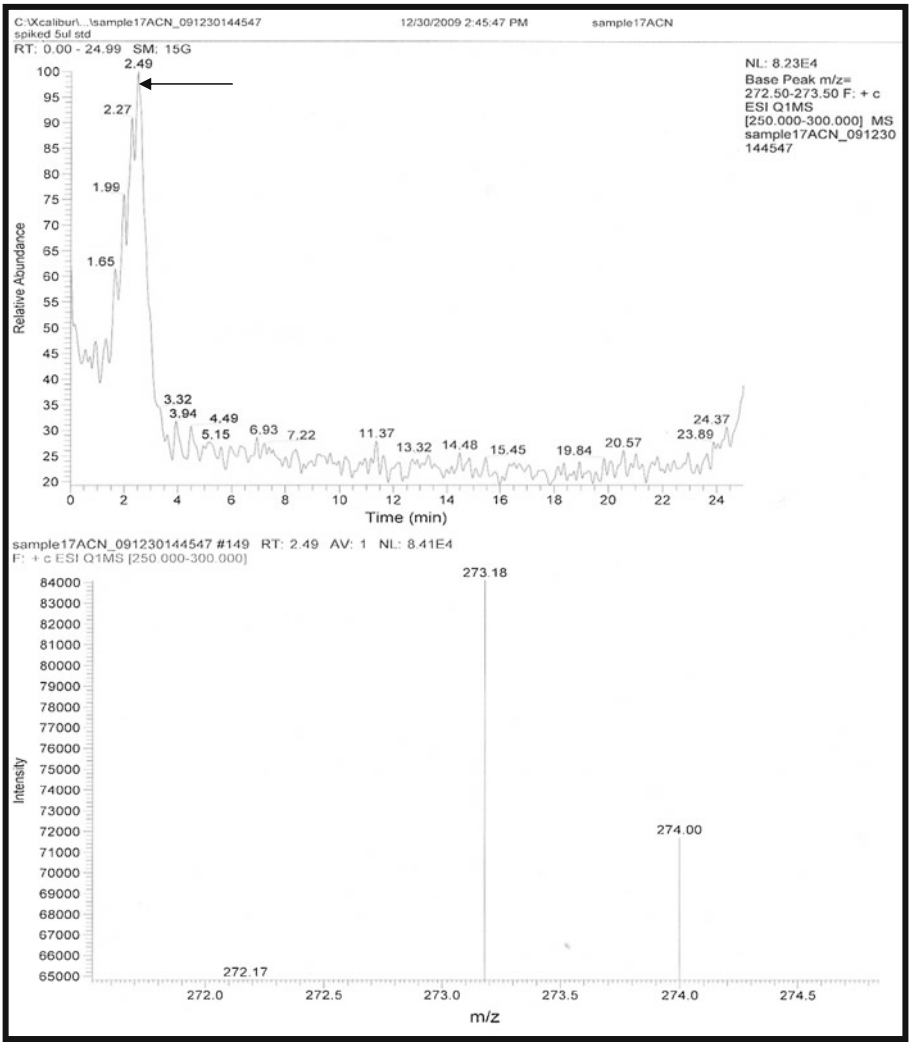

Fig. 8 LC/MS/MS Detection of chalcone synthase reaction product. The positive ion MRM chromatograms of chalcone synthase reaction product at *m/z* 272.50–273.50 were obtained from chalcone synthase reaction product containing 100 ng/ml naringenin (*arrow* indicates the position of the naringenin peak)

formation of precipitate was observed at a higher concentration of mitomycin C. A high inducer concentration causes a retardation of culture growth and lowers chalcone synthase secretion due to the formation of inclusion bodies [3].

The Effect of OD Induction Time on Secretory Expression of P. patens Chalcone Synthase

Upon induction of Tuner (DE3) plysS harboring the PET32(a)/PpCHS and psW1 plasmids with 40 μM IPTG and 40 ng/ml mitomycin C, the cell cultures were induced at various OD_{600} values (0.25, 0.5, 0.75, 1.0, or 1.25) to determine the effect of post-induction growth time on BRP-mediated chalcone synthase secretion. Generally, a higher cell density tends to

produce a higher secretion of protein when induced until it reaches the stationary phase where depletion of nutrients causes a reduction in metabolic activity. As shown in Fig. 5, increased secretion of chalcone synthase was observed as the OD_{600} induction time increases. However, further induction at OD_{600} of 1.25 significantly decreased the secretion of chalcone synthase into the culture media. The decrease in the BRP-mediated secretion of chalcone synthase at higher OD_{600} induction time was due to the deficiency of chemical uptake to fully drive the lac and pCloDF13 promoter system [7]. Apart from that, OD_{600} induction times of 0.75 and 1.0 showed an increase in extracellular chalcone synthase activity. However, cell lysis was observed at OD_{600} induction times of 0.75 and 1.0, which causes cell death. Thus, an OD_{600} induction time of 0.5 was chosen as the best condition for the continuous secretion of chalcone synthase into the culture media.

The Effect of Incubation Time on Secretory Expression of P. patens Chalcone Synthase

Apparently, another crucial factor in optimizing the applicability of the BRP co-expression of heterologous proteins from the *E. coli* periplasm into the culture medium is to maintain cell viability. Therefore, after determining the compatibility of the PET32(a)/CHS with psW1 (BRP vector) in regulating both the expression and secretion of chalcone synthase into the culture medium, the effect of induction time on chalcone synthase production by Tuner (DE3) plysS was investigated. As shown in Fig. 6, longer incubation time resulted in a higher level of *P. patens* chalcone synthase secretion. However, a moderate incubation time of 20 h helped maintain cell viability, allowing for the continuous production of recombinant *P. patens* chalcone synthase into the culture medium without cell lysis. In the case of prolonged induction time, disintegration of the outer membrane rapidly stops cell division [7].

In this work, Tuner (DE3) plyss harboring PET32(a)/CHS induced with 40 μM IPTG was shown to exhibit 0.079 U/ml activity, while Tuner (DE3)plyss harboring PET32(a)/CHS and psW1 (BRP vectors) induced with 40 μM IPTG and 40 ng/ml mitomycin C was shown to exhibit 0.157 U/ml activity. These data prove that chalcone synthase expressed into the culture media produces more functional active protein than when being expressed intracellularly. In conclusion, after performing duplicates on the optimization studies, the best conditions for the extracellular expression of *P. patens* chalcone synthase are as follows: induction of cells at 0.5 OD_{600} with 40 ng/ml mitomycin C followed by incubation for 20 h.

Product Identification of Chalcone Synthase Assay

p-Coumaroyl-CoA was known as the natural starter molecule for chalcone synthase reactions producing naringenin chalcone as the major product. However, previous studies showed that the recombinant *P. patens* chalcone synthase was capable of utilizing various aliphatic CoAs as an alternative producing various novel unnatural polyketides [4]. The ability of chalcone synthase in utilizing different aliphatic CoAs as a starter molecule is associated with the size of the cavity in the active site which determines the substrate selectivity and the chain length of the products formed. However, when incubated with butryl-CoA and hexanoyl-CoA as substrates, phlorobutyrophenone and phlorophenone were produced, respectively [8].

Thus, in this work, hexanoyl-CoA and butryl-CoA were used as substrates to confirm the functionality of the expressed extracellular chalcone synthase. Based on the HPLC analysis of the reaction products as shown in Fig. 7, several peaks were detected under chalcone synthase assay conditions. Therefore, in comparison with authentic standard under the same conditions, compounds eluted at retention times of ~1.7 and 10 min were assumed to be

pyrone and chalcone analogues, respectively. The formation of pyrone compounds using hexanoyl-CoA as a substrate was likely to be expected from the reaction due to the premature termination after only two condensation reactions [10].

In addition, an ESI LC-MS/MS method was developed to identify the compound eluted at a retention time of ~10. Under ESI conditions, the extracted reaction product gave MH^+ at *m*/*z* 273.18 as the base ion, shown in Fig. 8, identified as naringenin (according to the internal standard). Based on the result obtained, it was shown that the enzyme reaction synthesized a flavonoid compound having approximately the same molecular weight as naringenin, most likely due to small differences only in its number and orientation of the hydroxyl group such as lactones. However, since the aim of the work was to determine the functionality of the expressed recombinant protein, thus, it could be concluded that the chalcone synthase is expressed in its active form through the formation of these products.

Concluding Remarks

In this study, we used a bacteriocin release protein system to promote the secretion of recombinant *P. patens* chalcone synthase into the culture media. The best conditions for chalcone synthase secretion were: induction of the culture of Tuner (DE3) plysS with 40 μM IPTG and 40 ng/ml mitomycin C, an $OD_{600\ nm}$ of 0.5, followed by 20 h of growth at 37 °C. Based on the HPLC elution profiles, it was shown that recombinant chalcone synthase expressed extracellularly into the culture medium produced chalcone-like products using hexanoyl-CoA as a starter CoA, which further confirms the functionality of chalcone synthase.

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References

1. Choi, I. H., & Lee, S. Y. (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. *Applied Microbiology and Biotechnology, 64*, 625–635.
2. Fang, T., Wang, Y., Ma, Y., Su, W., Bai, Y., & Zhao, P. (2006). A rapid LC/MS/MS quantification assay for naringin and its two metabolites in rats plasma. *Journal of Pharmaceutical and Biomedical Analysis, 40*, 454–459.
3. Frangioni, J. V., & Neel, B. G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Analytical Biochemistry, 210*, 179–187.
4. Jiang, C., Schommer, C. K., Kim, S. Y., & Suh, D.-Y. (2006). Cloning and characterization of chalcone synthase from the moss, *Physcomitrella patens*. *Phytochemistry, 67*, 2531–2540.
5. Luirink, J., Sande, C. V. D., Tommassen, J., Veltkamp, E., Graff, F. K. D., & Oudega, B. (1986). Effects of divalent cations and phospholipase A activity on excretion of cloacin DF13 and lysis of host cells. *Journal of General Microbiology, 132*, 825–834.
6. Raharjo, T. J., Chang, W. T., Choi, Y. H., Peltenburg-Looman, A. M. G., & Verpoorte, R. (2004). Olivetol as product of a polyketide synthase in *Cannabis sativa* L. *Plant Science, 166*, 381–385.
7. Rahman, R. N. Z. R. A., Leow, T. C., Basri, M., & Salleh, A. B. (2005). Secretory expression of thermostable T1 lipase through bacteriocin release protein. *Protein Expression and Purification, 40*, 411–416.
8. Schuz, R., Heller, W., & Hahlbrock, K. (1983). Substrate specificity of chalcone synthase from *Petroselinum hortense*. *The Journal of Biological Chemistry, 258*, 6730–6734.

9. Van der wal, F. J., Koningstein, G., Ten Hagen, C. M., Oudega, B., & Luirink, J. (1998). Optimization of bacteriocin release protein (BRP)-Mediated protein release by *Escherichia coli*: random mutagenesis of the pCloDF13-derived BRP gene to uncouple lethality and quasi-lysis from protein release. *Applied and Environmental Microbiology, 64*, 392–398.
10. Zuurbier, K. W. M., Leser, J., Berger, T., Hofte, A. J. P., Schroder, G., Verpoorte, R., et al. (1998). 4-Hyroxy-2-pyrone formation by chalcone synthase and stilbene synthase with nonphysiological substrates. *Phytochemistry, 49*, 1945–1951.