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The Optimization of Soluble Cephalosporin C Acylase Expression in *E. coli*

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----------***ABSTRACT***----------
ABSTRACT:*7-aminocephalosporanic acid (7-ACA) is an important intermediate compound for producing of most cephalosporin derivatives. It could be synthetized by conversion of cephalosporin C (CPC) in single step using cephalosporin acylase. The application enzymatic conversion is hampered by low activity of enzyme using CPC as the main substrate. Modification of AcyII genes from Pseudomonas SE83 produced S12 variant that could expressed CPC acylase with high-activityon CPC. Based on S12 variant sequence, a synthetic gene was constructed and subsequently transformed to E. coli. The expression of target proteins were performed using LB medium supplemented with ampicillin. Preliminary experiments showed that the target protein (CPC acylase) partially remained as the inclusion bodies. Furthermore to obtain high yield of the target proteins, we explored the factors influenced on protein expression, i.e. isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, induction temperature, and induction time in a series of experiments. The result showed that the optimal condition of soluble cephalosporin acylase expression was induction with 0.1 mM IPTG, incubation temperature 20^{o}C and incubated for 12 h*
KEY WORDS – *7-aminocephalosporanic acid, cephalosporin acylase, cephalosporin, expression, optimization*

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I. INTRODUCTION

7-aminocephalosporanic acid (7-ACA) is an important intermediate compound for producing of most cephalosporin derivatives. This compound is obtained by deacylation of cephalosporin C (CPC), a natural compound produced by fermentation using *Cephalosporium acremonium* fungi. Approximately, the annual worldwide demand is around 100 tons [1], therefore 7-ACA becomes important intermediate for antibiotics manufacturing in pharmaceutical industry. Enzymatic conversion involves two enzymes has been adopted into industrial scale, comprising D-amino acid oxidase (DAAO, EC 1.4.3.3), used to convert CPC into glutaryl-7-aminocephalosporanic acid (Gl-7-ACA), and glutaryl-7-aminocephalosporanic acid acylase (GLA, EC 3.5.1.93), to hydrolyze Gl-7-ACA into 7-ACA. However, the production cost is still high. Recently, enzymatic conversion involves one step process, using cephalosporin C acylase (CPC acylase), is in great demand because it gives efficient and environment friendly process [2]. Unfortunately, this process is hampered by low productivity and high cost associated with production of CPC acylase.

Only few cephalosporin acylase have been discovered capable to use CPC as a substrate. Most of these natural enzymes are more active on Gl-7-ACA and very low activity on CPC, probably because of the unusual nature of the D-aminoadipyl side chain of CPC [3]. Enzyme engineering is a hope to obtain recombinant protein to meet specific purpose. The expression system for the production of recombinant proteins usually involves a combination of plasmids and *E. coli* strains. One of the major limitations for the production of recombinant protein in *E. coli* is getting a large amount of soluble and functional protein. Under overexpression conditions, parts of proteins frequently accumulate as insoluble aggregates (inclusion bodies) within producing bacteria. In general misfolding protein is the main cause of the formation of aggregate proteins which are biologically inactive.

There are two strategies to obtain soluble cytoplasmic fraction, i.e. refolding from inclusion bodies and procedures in which the expression strategy is modified to obtain soluble expression. Refolding from inclusion bodies is in many cases considered as unfavorable method. In vitro refolding, by modified the expression procedure for maximizing the production of recombinant proteins in a soluble form is therefore an attractive alternative. In this study, in order to increase soluble active enzyme yield, the effects of concentration of inducer, different temperatures, and induction periods on the recombinant CPC acylase expression levels by *E. coli* were evaluated.

II. MATERIALS AND METHOD

2.1 Construction of *E. coli* pET21a-S12 expression plasmid

The synthetic gene encoding cephalosporin acylase was synthesized according to the amino acid sequence of S12 as described by previous study [4] and constructed in GenScript USA Inc. This sequence has 2355 base pairs length (including 6xHis tag) and was obtained from modified of *AcyII* gene from *Pseudomonas* SE83. The codon optimization was performed to obtain optimal gene sequences expression in *E. coli* BL21 (DE3) cells. The gene was cloned into pET21a vector using the *Nde*I restriction enzyme for the N terminal and *Xho*I for the C terminal. The successfully constructed plasmid pETa-S12 was transformed into competent cells *E. coli* BL21(DE3) and spread evenly on an LB agar plates (Contents/L: NaCl 10 g, yeast extraction 5 g, tryptone 10 g, agar 15 g) containing 100 µg/ml ampicillin, and then cultured overnight at 37°C. The transformant plasmid was then isolated and characterized by DNA electrophoresis, Nanodrop, and nucleotide sequencing. The blank empty plasmid was used as negative control.

2.2 Expression of S12 in *E. coli* under different inducing conditions

Single transformant colony was selected and inoculated into 2 ml of LB medium (Contents/L: NaCl 10 g, tryptone 10 g, yeast extraction 5 g) containing 100 µg/ml ampicillin and cultured overnight at 37°C with shaking at 200 rpm. Subsequently, 0.5 ml of the overnight culture was pipetted and inoculated into new 50 ml LB medium with ampicillin (100 µg/ml) in a 250 ml Erlenmeyer and incubated in the 37°C shaker at 200 rpm until it reached an OD_{600} of 0.6-0.8. The expression of CPC acylase was then optimized by variation of several fermentation factors condition, i.e. IPTG concentration as inducer, incubation temperatures and induction time to yield the maximum soluble of CPC acylase. For the first step, the optimal concentration of IPTG was determined. The cells with OD_{600} of 0.6-0.8 were induced with 0.1, 0.3, 0.5, 0.8, and 1.0 mM IPTG, and incubated at 25^{o}C for 24 h shaking at 100 rpm. Furthermore the optimum induction temperature was determined at 20°C, 25°C, 30°C, and 37°C using optimum concentration of IPTG and shaking at 100 rpm for 24 h. Lastly, by using optimum condition of IPTG concentration and incubation temperature, the optimal induction time was determined at 2, 4, 6, and 24h.

2.3 Harvesting cells

The cells from the 50 mL production media were harvested by centrifugation at 12,000 rpm for 2 minutes. The pellets were washed with 0.01 M phosphate buffer of pH 8.0 twice, re-suspended in 5 mL of the same buffer and addition of PMSF and DNAse with final concentration 1 mM and 10 µg/mL respectively. Cells were disrupted by sonication with 25% amplitude for 5 minutes (a pulse of 2 sec followed by rest of 20 sec) in ice bath. Cells debris was removed by centrifugation at 10,000 rpm for 10 minutes at 4ºC, which led to clear supernatant, designated as cell-free extract. This cell-free extract and cells debris were subjected to further study.

2.4 Enzyme purification

Purification of enzymes was performed using one step purification method using Ni-NTA (Thermo Scientific) resins. The column was subjected to a phosphate buffer solution of 20 mM pH 7.4 containing 300 mM sodium chloride and 10 mM imidazole. After the addition of 1 M NaCl (final concentration), the crude extract is fed into a column containing Ni NTA resins. Columns were washed with PBS pH 7.4 buffer containing 25 mM imidazole to obtain an absorbance value at 280 nm equal to the buffer absorbance. The resin-bound protein in the column was eluted with PBS 20 mM pH 7.4 containing 250 mM imidazole. The fractions out of the column were collected and analyzed by SDS PAGE and the enzyme activity. Active fractions are combined and used for further research.

2.5 SDS-PAGE assay

SDS-PAGE assay was used to analyze soluble and insoluble protein. SDS PAGE assay of soluble protein was conducted by pipetting a 10 µl of cell-free extract and added to 2.5 µl of 6x SDS loading sample buffer. While insoluble protein was assayed by re-suspending the cells debris using 2 ml of PBS buffer then 10 µl of the suspension was added to 2.5 µL 1x SDS loading sample buffer. The aliquots were boiled, centrifuged and separated on 10% SDS-PAGE gel. Coomassie Brilliant Blue was used to stain the SDS-PAGE gels to determine the protein profile of CPC acylase.

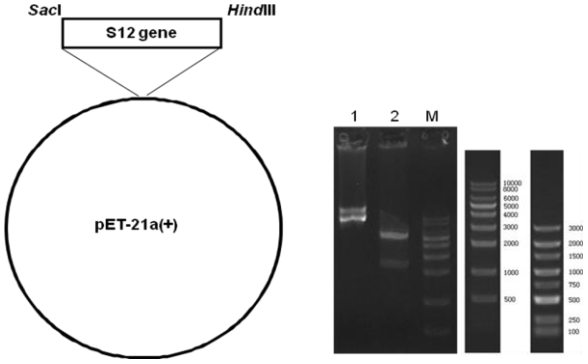
2.6 Image J analysis

The resulting gel was scanned using Cannon MP 278 for subsequent visual analysis. The gray values of soluble and insoluble of target protein (CPC acylase) bands were also measured by Image J, version 1.51j8. Since the CPC acylase consists of two subunits proteins, the α-subunit and β-subunit, hence the comparison of soluble to insoluble protein was calculated only based on β-subunit as representative of target protein expression.

III. RESULTS

3.1 Construction of S12 expression plasmid

The S12 coding sequence was first amplified by PCR from a plasmid containing the full-length S12 gene (Fig. 1A). The S12 gene was inserted in a pET21a plasmid using restriction enzymes *Sac*I and *Hind*III. The insertion gene was confirmed by a double-cut method using the *Sac*I and *Hind*III restriction enzymes (Fig. 2A). Separation of the bands using 1% agarose gel by electrophoresis resulted two bands i.e. the plasmid pET21a and the S12 gene bands with theoretical size of 5500 bp and 2400 bp, subsequently (Fig. 1B). These results proved that the S12 gene has been successfully inserted in a pET21a plasmid.



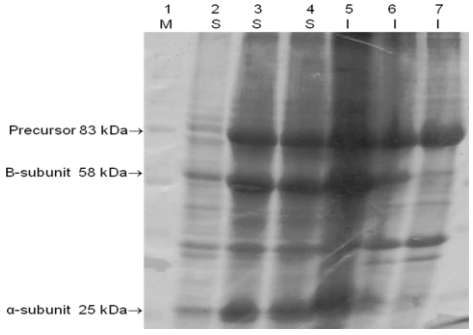
(A) (B)

Figure 1. (A) Construction of recombinant plasmid pET21a containing S12 gene, (B) Double cutting using *Sac*I and *Hind*III enzymes : Lane M : 1 kb ladder, Lane 1 : pET21a-S12 recombinant plasmid, Lane 2 : pET21a-S12 recombinant plasmid was digested by *Sac*I and *Hind*III

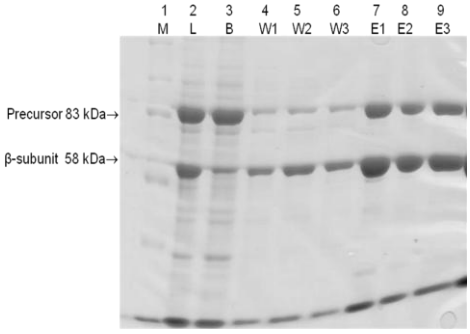
3.2 The expression of CPC acylase

Confirmation of the CPC acylase expression by *E. coli* recombinant was performed as a first step. In the recombinant culture of *E. coli*, previously cultivated using LB medium containing 100 µg/mL of ampicillin at 37^{o}C until it reached OD_{600} 0.8, was induced with 0.1 mM IPTG and then incubated again at 25^{o}C for 24 h. At the end of fermentation, the cells were separated by centrifugation, and then lysed by sonication. After separated the debris by centrifugation, the protein in the supernatant was analyzed using 10 % SDS PAGE and stained with coomassie blue. The protein bands with size 25 and 58 kDa were observed, which are the α-subunit and β-subunit of the cephalosporin acylase.

Furthermore the soluble CPC acylase was confirmed by activity test using cephalosporin C as the substrate. 7-amino cephalosporanic acid (7-ACA) was obtained as the product of bioconversion (data not shown). The SDS PAGE assay also showed that the expression of target protein was majority in soluble form and only small portion was insoluble as inclusion bodies (Fig. 2A). In addition, inactive enzyme precursor with size 83 kD, proteins contain 9 amino acid residues as spacer that are link α-subunit and β-subunit, was also observed. The precursor through self-activation by two steps intramolecular cleavage turns into active proteins [5]. The soluble target protein was then purified by Ni-NTA purification kit as shown in Figure 2B. This results suggest that optimization of overexpression condition was needed to produce maximum soluble target protein.



(A)

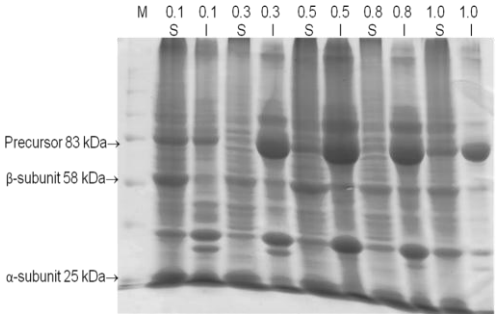


(B)

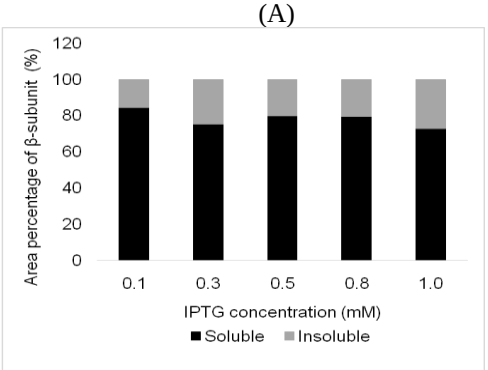
Fig 2. SDS-PAGE assay of soluble CPC acylase. Lane M, protein standard markers. (A) Profile of crude extract CPC acylase, S = Soluble protein in supernatant; I = Insoluble protein in cells pellet; (B) SDS-PAGE assay of purified CPC acylase by one-step purification using nickel-NTA coloumn. The molecular weight of Precursor of S12 is approximately 83 kDa, α-subunit is 25 kDa and β-subunit is 58 kDa; L = lysate, B = binding solution, W1, W2, W3 = washing solution 1,2 and 3, E1, E2, E3 = elution fraction 1, 2 and 3.

3.3 The effect of IPTG concentration

The effect of IPTG concentrations on CPC acylase expression by recombinant *E. coli* were evaluated. The cells with OD_{600} 0.8, prepared as previously mentioned, were induced with varying IPTG concentration from 0.1 – 1 mM. The cells were then incubated again at 25°C for 24 h. The result showed that the expression level of soluble target protein did not appear to vary greatly by IPTG concentration. However the IPTG concentration at 0.1 mM indicated the highest soluble protein as well as the ratio of soluble to insoluble protein target as shown in Fig 3. Induction of 0.1 mM IPTG concentration was used for further study.



(A)



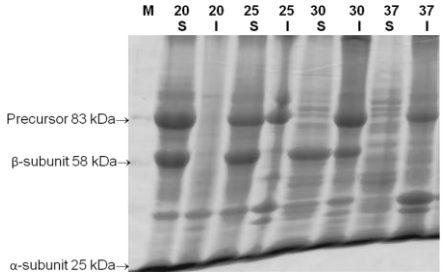
(B)

Fig. 3. (A) SDS PAGE assay of soluble and insoluble protein expression by recombinant *E. coli* induced with different IPTG concentrations ranging from 0.1 mM to 1.0 mM. (S = β-subunit of soluble protein; I = β-subunit of insoluble protein). (B) The percentage of the soluble and insoluble of target protein to the total β-subunit expression (soluble + insoluble target protein).

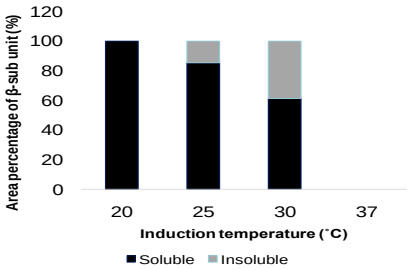
3.4 The effect of temperature

In this study, we used a series of induction temperatures that varied from 20°C to 37°C. The cells with OD_{600} 0.8, were induced with IPTG concentration 0.1 mM. The cells were then incubated again for 24 h with varying temperatur.The results showed that target proteins expression's profiles were influenced by incubation temperatures. The higher incubation temperature results the lower expression of soluble target proteins as shown

in Fig. 4A, as well as the ratio of soluble to insoluble target proteins (S% to P%) (Fig. 4B). The highest amount of soluble target protein (soluble β-subunit) was obtained when the culture was incubated at 20°C, in contrast at high temperature incubation 37°C, no soluble target protein was produced.



(A)

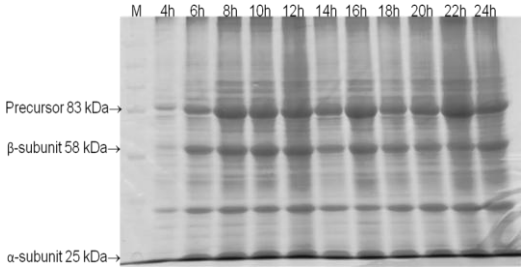


(B)

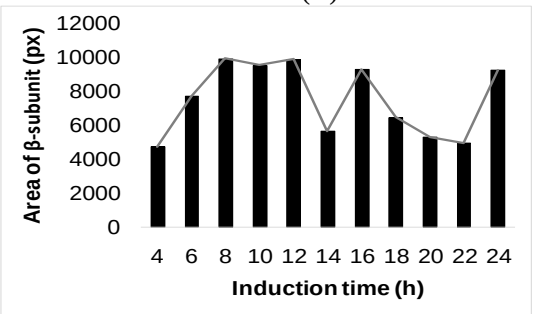
Fig. 4 (A). SDS PAGE assay of protein expression by recombinant *E. coli*, induced at different temperatures ranging from 20°C to 37°C (S = soluble ; I=insoluble). (B) The percentage of soluble and insoluble of β-subunit to total of the β-subunit expression.

3.5 The effect of induction periods

After determining the optimal concentration of IPTG and temperature induction, then we continued to determine the optimal induction periods for protein expression. The cells with OD_{600} 0.8, were induced with IPTG concentration 0.1 mM and incubated again at 25°C for different induction periods. The results showed that the expression of target soluble protein increased as the induction periodes increased and peaked at 12h (Fig 5).



(A)



(B)

Fig. 5 SDS PAGE assay of soluble protein by recombinant *E. coli*, with different induction periods ranging from 4 h to 24 h.

IV. DISCUSSION

The pET21a-S12 was designed carrying 6xHis tag thus we can easily purify CPC asilase. The insertion of this gene in recombinant *E. coli* could result in changing of temporal spatio control. The new recombinant

polypeptides might be synthesized and expressed in the *E. coli* microenvironment, which may be different from the original source in terms of pH, redox potential, osmolarity, cofactor, and folding. Also, in a high degree of expression, hydrophobic stretching in polypeptides occurs more strongly and allows for interaction with similar regions. All of these factors lead to insoluble protein form, which is known as inclusion body [6, 7, 8]. During expression this phenomenon is influenced by factors such as inducer concentration, induction temperature, and duration of induction. In this research, as the first step the effect of IPTG concentration on CPC acylase expression by recombinant *E. coli* was determined. The experiment indicated that the expression level of soluble target protein was not greatly varied starting from IPTG concentration 0.3 to 1.0 mM. However induction with IPTG at 0.1 mM result the highest expression of soluble target protein. High IPTG concentration may toxic effect on cell growth rate, thereby reducing target proteins yields. The temperature before induction almost had no effect on the expression of target protein [9], while temperature incubation after induction has a great effect on expression of target protein [10]. Therefore, we choose to determine the optimum temperature for producing soluble target protein. Our research showed at 20^{o}C of induction temperature all target proteins were expressed in soluble form. Low temperature induction slower rates of protein expression and give newly transcribed recombinant proteins time to fold properly resulting in increased solubility [11]. Increasing temperature lead the formation of inclusion body. The last study was to optimize of induction time. The result indicated that up to 6 h incubation after induction there is no α-subunit and β-subunit expressed and all target proteins were still in the precursors form. The precursor takes time to mature and release the spacer turn into target protein. At 24 hours induction, β- and α-subunit have been formed, but the precursor concentrations are still high. The next challenge of research is to find suitable fermentation condition to obtain as many precursors as possible be converted into soluble target proteins.

V. CONCLUSION

This research showed that expression of cephalosporin acylase by recombinan *E. coli* carrying S12 plasmid was only slightly affected by IPTG concentration, but greatly influence by temperature induction and induction time. The optimal condition of soluble target protein expression was induction with 0.1 mM IPTG, incubation temperature after induction 20^{o}C and incubated for 24h.. Our research has identified optimal conditions needed to obtain a high yield of the soluble S12 fusion protein for cephalosporin acylase expression.

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