

# Solubilization of Cephalosporin Acylase from *Escherichia coli* BL21 (DE3) Inclusion Bodies

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**Abstract.** The 7-aminocephalosporanic acid (7-ACA) is a precursor for semisynthetic cephalosporin antibiotics. The enzymatic conversion of cephalosporin C (CPC) to 7-ACA is cephalosporin acylase (CA). Fragment DNA of CA gene was inserted in pET21a(+) and expressed in *Escherichia coli* BL21(DE3) (*E. coli*). Heterologous expression of foreign genes can produce about 30 % of soluble protein and 70% of insoluble protein as inclusion bodies (IBs) in *E. coli*. IBs are cytoplasmic aggregates of inactive protein and mostly consist of recombinant protein. The formation of IBs in *E. coli* is a challenge for recovery of recombinant protein in industrial scale. Solubilization and refolding are crucial process to obtain active recombinant protein from IBs. The purpose of this research was optimization of solubilization process to increase soluble CA from IBs. The cells of *E. coli* were disrupted by sonication. The pellet of IBs was washed by triton X-100 and solubilized by urea or guanidine HCl (GdnHCl). The transformant of *E. coli* containing pET21a(+)-acyII expressed CA as soluble and insoluble protein (IBs). Several methods were used for solubilization IBs pellet. The two-step denaturing (2DR) was the best method for solubilization IBs pellet.

**Keywords:** cephalosporin acylase, solubilization, inclusion bodies

## 1. Introduction

Cephalosporin C is the second  $\beta$ -lactam antibiotic to be discovered after penicillin that was produced by *Cephalosporin acremonium*. CPC has moderate antibacterial activity and is not potent for clinical use. CPC can be transformed to 7-ACA as precursor for semisynthetic cephalosporin derivative by chemical and enzymatic methods. Now, the enzymatic method (two-step pathway) is widely used because of safety, environmental friendly, specificity and simple reaction, and low cost of production [1-4]. There are two kind of the enzymatic method, two-step pathway using D-amino acid oxidase and glutaryl-7-aminocephalosporanic acid (GL-7-ACA) and one-step pathway using cephalosporin acylase (CA). One-step pathway is more simple process and cost reduction but CA is very low efficiency [3-6]. In recent years, research of CA is developed to increase activity, stability and production.

Based on their gene structure, sequence, molecular mass, and enzyme properties CAs have been classified into five classes. All members are very similar on the basis of biochemical properties, substrate specificity and sequence conservation (they have > 90% of nucleic acid or amino acid



sequence identity). P130 from *Pseudomonas* sp. 130 as a member of class I and N176 from *Pseudomonas* sp. N176 as the member of class III have the highest activity to CPC [7-9].

There are several expression systems to produce recombinant protein, such as bacteria, yeast, insect cells, mammalian cells, and cell-free systems [10]. *E. coli* is frequently used to produce recombinant proteins because of grown on inexpensive and simple media, rapid grown and high density cell in bioreactor, and easy to scale up production [11]. Heterologous expression of foreign genes can produce about 30 % of soluble protein and 70% of insoluble protein as inclusion bodies (IBs) in *E. coli* [11]. If the expression levels of protein higher than 2% the total cellular proteins, it will lead to formation of IBs. IBs are cytoplasmic aggregates of inactive protein and mostly consist of recombinant protein and its size is from 0.2 to 1.5  $\mu\text{m}$ . The aggregates of IBs are deposited at poles [12].

To obtain soluble active protein from IBs, IBs need to purification with detergent solutions, solubilization in denaturant solutions, and refolding [10-12]. Solubilization can convert aggregates of protein to monomolecular dispersion and minimum non-native intra- or inter-chain interaction as denatured protein [13, 14]. IBs are denatured and dissolved by urea, guanidine hydrochloride (Gdn-HCl), and thiocyanate salt as denaturant or chaotropic agents; sodium *N*-lauroylsarcosine (sarkosyl), lauroyl-L-glutamate, sodium dodecyl sulfate (SDS), *N*-cetyltrimethylammonium chloride as detergent; or cysteine [12-16]. Dithiothreitol or  $\beta$ -mercaptoethanol is added in the solubilization agent to reduce undesirable inter- and/or intra-molecular disulfide bonds or incorrect disulfide bonds [11, 14]. After solubilization, the denatured protein can be refolded into a native conformation by dilution, dialysis, chromatography, solid phase matrix, and microfluidic chips [10, 17]. The aim of this study was process solubilization of cephalosporin acylase IBs.

## 2. Materials and methods

### 2.1. Expression of cephalosporin acylase

The selected *E. coli* BL21 (DE3) containing pET21a(+)-acyII was cultured in LB medium containing 100  $\mu\text{g}$  per mL ampicillin on a rotary shaker at 200 rpm for overnight at 37°C. 10% of *E. coli* BL21 (DE3) culture was inoculated in LB medium containing 100  $\mu\text{g}$  per mL ampicillin in logarithmic phase (OD 550 0.5), induced by 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 5 hours of incubation and harvested 4000 rpm for 10 minutes at 4°C.

### 2.2. Washing of IBs

The IBs from 1 L of *E. coli* BL21 (DE3) culture were suspended by 100 mL of 100 mM phosphate buffer pH 8.0 and lysed by sonication on ice. The lysate was centrifuged at 10000 g for 15 minutes at 4°C. The IBs pellet were suspended by using 9 volumes of cell lysis buffer (5 mL of 50 mM Tris-HCl pH 8.0, 5 mL of 10 mM EDTA, 5 mL of 100 mM NaCl, 250  $\mu\text{L}$  of 0.5% Triton X-100, 44 mL of water) and incubated for 5 minutes at room temperature. The suspension was centrifuged at 10000 g for 15 minutes at 4°C. The washed IBs pellets were weighed.

### 2.3. Solubilization of IBs

**2.3.1. Sambrook method [18]** The Washed IBs pellet from 1 L of *E. coli* BL21 (DE3) culture were suspended by 3 mL of inclusion-body solubilization buffer I (50 mM tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl, 8 M urea) per gram wet IBs, freshly added by 4  $\mu\text{L}$  0.1 mM PMSF per gram wet IBs, and incubated for 1 hour in room temperature. The suspension was added by 9 volumes of inclusion-body solubilization buffer II (50 mM  $\text{KH}_2\text{PO}_4$  pH 10.7, 1 mM EDTA pH 8.0, 50 mM NaCl), incubated for 30 minutes at room temperature, adjusted pH 8.0 by 12 M HCl, and incubated for 30 minutes at room temperature. The suspension was centrifuged at 10000 g for 15 minutes at room temperature. The supernatant was soluble of denatured cephalosporin acylase.

**2.3.2. Added Dithiothreitol (DTT).** The Washed IBs pellet from 1 L of *E. coli* BL21 (DE3) culture were suspended by 3 mL of 10 mM Tris-Cl pH 8.5 per gram wet IBs, added by 100  $\mu$ l of 1 M DTT, added by 1 mL of 8M urea, incubated for 3 hours at room temperature in shaker at 100 rpm. The suspension was centrifuged at 10000 g for 15 minutes at room temperature. The supernatant was soluble of denatured cephalosporin acylase.

**2.3.3. Denaturing and refolding (1DR).** The Washed IBs pellet from 1 L of *E. coli* BL21 (DE3) culture were dissolved by 5 mL of extraction buffer II (50 mM Tris-HCl, 50 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 8 M urea, pH 8.0), stirred for 20 minutes, and centrifuged at 30000 g for 30 minutes at 4°C. The supernatant was soluble of denatured cephalosporin acylase [10].

**2.3.4. Two-step denaturing and refolding (2DR).** The Washed IBs pellet from 1 L of *E. coli* BL21 (DE3) culture were dissolved by 5 mL of extraction buffer I (50 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 7 M GdnHCl, pH 8.0). The solution was centrifuged at 30000 g for 30 minutes at 4°C. The supernatant was diluted into 200 mL of dilution buffer (50 mM Tris, 1 mM EDTA, 50 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, pH 8.0) and centrifuged at 30000 g for 30 minutes at 4°C. The IBs pellet were dissolved by 5 mL of extraction buffer II (50 mM Tris-HCl, 50 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 8 M urea, pH 8.0), stirred for 20 minutes, and centrifuged at 30000 g for 30 minutes at 4°C. The supernatant was soluble of denatured cephalosporin acylase [10].

## 2.4. SDS-PAGE

Ten mL of acrylamide solution was added 60  $\mu$ L of 10% ammonium persulfate, mixed, filled the gel casting plates, inserted a comb, and incubated for 20 minutes to polymerize completely. The comb was removed from the gels and the wells were rinsed by running buffer in the electrophoresis apparatus. The samples of soluble cephalosporin acylase were analysed by SDS-PAGE. 15  $\mu$ L of sample was added by 5  $\mu$ L of loading buffer 5X containing 5% of  $\beta$ -mercaptoethanol, boiled for 5 minutes. Samples and standard were loaded onto gel and ran at 150 Volt for 3 hours.

## 2.5. Total protein assay.

The samples of denatured cephalosporin acylase were measured total protein by spectrophotometer. 20  $\mu$ L of sample, added by 1 mL of Bradford reagent, incubated for 5 minutes at room temperature. The absorbance of sample was measured by spectrophotometer at 595 nm. Bovine serum albumin was used as standard protein.

$$\text{Solubilization yield (\%)} = \frac{\text{Denatured CA in supernatant (mg)}}{\text{IBs pellet weight before solubilization (mg)}} \times 100$$

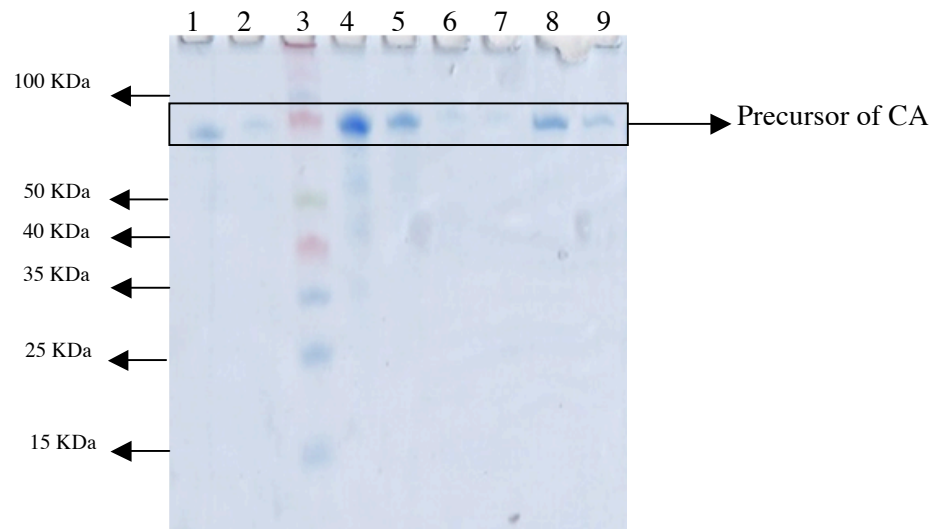
## 3. Results

*Escherichia coli* BL21 (DE3) containing pET21a(+)-acyII expressed CA as insoluble (IBs) and soluble protein. CA (acyII) composes two subunits with molecular weight of 26 KDa as  $\alpha$ -subunit and 57 KDa as  $\beta$ -subunit. Precursor of both subunits has a molecular weight of 83 KDa [19]. After washing, IBs pellet was weighed.

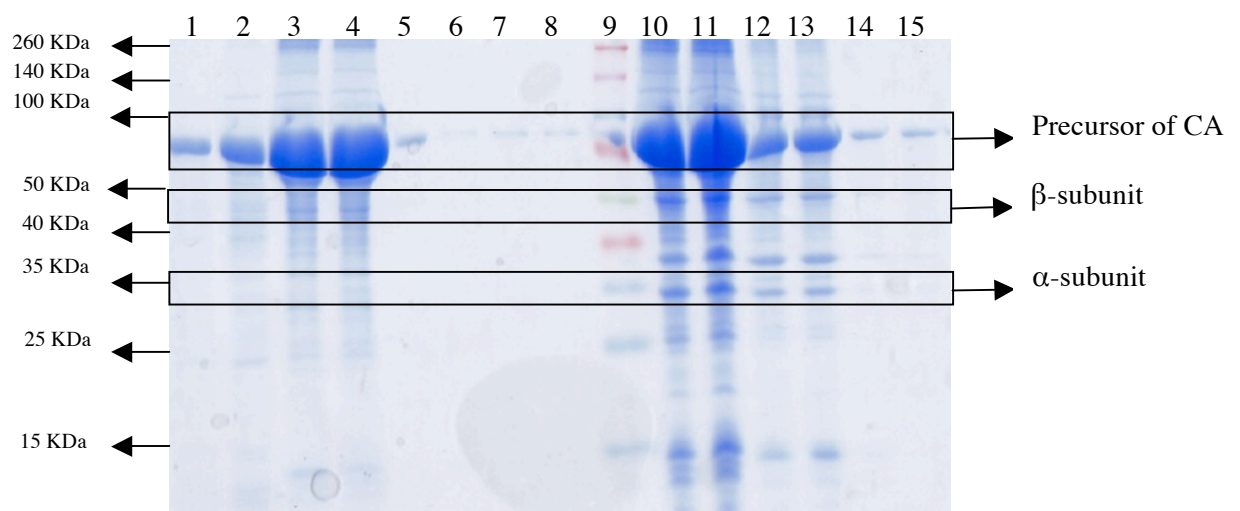
The soluble of IBs was characterized by SDS-PAGE and Bradford assay. The SDS-PAGE showed a precursor of CA was 83 KDa (figure 1 and figure 2). The washing IBs can increase the concentration of soluble IBs (figure 1 lanes 4-5 and lanes 6-7). The band from IBs pellet was evidence for aggregation of CA as IBs and cannot be soluble (figure 2 lanes 10-15). The Sambrook method, added DTT, 1DR method had not  $\alpha$ -subunit and  $\beta$ -subunit of CA bands, because the small concentrations of these subunits cannot be detected by Coomassie Blue staining (<0.3-1  $\mu$ g protein). The  $\alpha$ -subunit and  $\beta$ -subunit of CA bands from the 2DR method can be detected by Coomassie Blue staining (figure 2)

The total solubilization yield was measured by Bradford assay. Solubilization yield of 2DR was the highest solubilization yield (%) of soluble IBs was 22.5% (table 1). A lower concentration of urea

significantly decreased the solubilization yield. 1DR method (8.6%) was higher than Sambrook (7.65) and added DTT method (5.0%) in the solubilization yield. Agitation increased almost 2 fold the solubilization yield in Sambrook method.



**Figure 1.** SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis of soluble protein from IBs. Lanes 1-2 soluble CA from IBs by Sambrook method, 3 molecular weight marker, 4-5 soluble CA from IBs by Sambrook method with agitation, 6-7 soluble CA from IBs by Sambrook method with agitation without washing, 8-9 soluble CA from IBs by added DTT method.



**Figure 2.** SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis of soluble protein from IBs. Lanes 1-2 soluble CA from IBs by 1DR method, 3-4 soluble CA from IBs by 2DR, 5-6 soluble CA from IBs by added DTT method, 7-8 supernatant by 2DR method before diluted, 9 molecular weight marker, 10-11 pellet IBs by 1DR method, 12-13 pellet IBs by 2DR method, 14-15 pellet IBs by added DTT method.

**Table 1.** Concentration of total protein

Method	Total protein Denatured CA in supernatant (mg/mL)	IBs pellet before solubilization (mg/mL)	Solubilization yield (%)
Sambrook	0.406	10.483	3.9
Sambrook*	0.798	10.560	7.6
Added DTT	0.546	10.817	5.0
1DR	0.959	11.197	8.6
2DR	2.677	11.915	22.5

\*Incubation with agitation

#### 4. Discussion

To obtain soluble active CA from IBs, the IBs need to be solubilized and then refolding. The yield of protein refolding can be affected by this impurities. The first step in solubilization of IBs is washing IBs with cell lysis buffer containing triton X-100. IBs pellet were washed to increase yeild of soluble IBs (figure 1. Lanes 4-7) because triton X-100 purified IBs and removed contaminant such as RNA polymares, outer membrane proteins, enzymes, 16 S and 23S rRNA or other nonspecifically adsorbend cell material [12, 13].

Urea or Gdn-HCl is the most commonly used to solubilize the IBs. Urea (Sambrook, added DTT and 1DR method) and 2DR method (combination between Gdn-HCl and urea) were used to soluble IBs. The soluble IBs can be increased by incubation with agitation from Sambrook method (figure 1 lanes 1-2 and lanes 4-5). The concentration of soluble IBs Sambrook method with agitation (0.798 mg/mL) was almost 2 fold than Sambrook method without agitation (0.406 mg/mL) because agitation increase contact between IBs and urea as the denaturant.

In 2DR method, the first denaturing step used Gdn-HCl to dissolve IBs and to precipitate protein solution (soluble IBs) was diluted by dilution buffer. The second denaturing with urea to dissolve IBs. The function of Gdn-HCl is to completely unfold the misfolded secondary structures completely into random coiled structures protein in IBs. After rapid dilution, the soluble IBs might be formed the precipitates. The precipitates are dissolved by urea, the homogeneous partially folded intermediates would allow correct structure in subsequent protein folding process [11]. The result can be showed the band of soluble IBs from the Gdn-HCl was thicker than urea as denaturant agent and the percentage of solubilization yield was the highest (22.5%).

#### 5. Conclusion

*Eschericia coli* BL21 (DE3) containing pET21a(+)-acyII expressed CA as soluble and insoluble protein (IBs). The 2DR method was the highest solubilization yield (22.5%) of CA IBs and Sambrook without agitation was the lowers solubilization yield of CA IBs (3.9%).

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