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Solubilities and Activities of Chloramphenicol Acetyltransferase and β-Lactamase Overproduced by the T7 Expression System in *Escherichia coli*

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Overproduced proteins in many cases result in forming insoluble inclusion bodies, and their formation might be due to high concentration of protein. To investigate how proteins become insoluble, chloramphenicol acetyltransferase (CAT) and β-lactamase were overproduced, and their solubilities and activities were determined. CAT was accumulated from 9 to 45% of total cellular protein in a fully soluble form without inclusion body formation. CAT specific activity was shown to be proportional to the amount of the protein produced. Moderately produced β-lactamase by the phage T7 expression system at 30°C comprised only mature forms in a soluble form. However, overproduced β-lactamase at 37°C became insoluble. Most precursor forms of β-lactamase in the cytoplasm were insoluble, whereas majority of the mature forms in the periplasm space were soluble. Also, chaperone GroE proteins which assist proper protein folding and translocation did not increase β-lactamase solubility significantly under the experimental condition. It seems that the formation of inclusion bodies in the cell is related to the nature of protein itself rather than just to high concentration of protein.

KEY WORDS □ chloramphenicol acetyltransferase, β-lactamase, inclusion body, T7 expression system

It became possible to overproduce proteins with the help of recombinant DNA techniques. However, some overproduced proteins form inclusion bodies in an inactive form (10). There were many studies about how inclusion bodies are formed. One typical hypothesis is that high concentration of protein in the cell results in inclusion body formation.

In this study, two cases were shown. One was chloramphenicol acetyltransferase (CAT) that was still soluble even when highly overproduced. The other was β-lactamase that became insoluble when overproduced. The fact that overproduced CAT is soluble and active is contradictory to the hypothesis that proteins become insoluble when overproduced. Chaperone GroE was known to affect protein folding (12). Effect of GroE proteins on β-lactamase solubility was investigated by overproducing the GroE proteins.

Whether overproduced proteins form inclusion bodies or not seems to be ultimately connected with the nature of protein itself rather than just with the rate of translation and the resultant high concentration of protein in the cell.

MATERIALS AND METHODS

Bacteria and plasmids

E. coli BL21 is devoid of the OmpT and Lon proteases (6). *E. coli* BL21(DE3) carries the phage T7 RNA polymerase gene under the IPTG inducible *lacUV5* promoter in the chromosome (15). Constructions of plasmid pET3CAT (7), pBK (7), pET7 (16), and pGroESL (5) have been previously described. Constructions of BL21(DE3, pET3CAT) and BL21(pBK, pET3CAT) have been described (7). BL21(DE3, pET7) was constructed by transforming BL21(DE3) with pET7. Construction of BL21(DE3, pET7, pGroESL) was achieved by transforming BL21(DE3, pET7) with pGroESL.

Induction of the T7 expression system

A single colony of *E. coli* cells was inoculated into Luria Broth (LB) media containing 50 μg/ml ampicillin, or 50 μg/ml kanamycin, or 20 μg/ml chloramphenicol depending on selection markers on each plasmids. T7 RNA polymerase was induced with 0.5 mM IPTG in BL21(DE3) cells containing appropriate plasmids when the cultre

reached an A_{600} of 0.4~0.5, and cells were harvested 2~4 h after induction.

Fractionation of induced cells

Induced and harvested cells suspended in 50 mM Tris-HCl (pH 7.8) were broken by sonication several seconds using Branson sonifier (VWR Model 350). The lysates were separated into pellet and supernatant fractions by centrifugation at 12,000 rpm, 4°C for 10 min.

Quantification of CAT

CAT specific activity was measured by the standard method previously described by Shaw (13). CAT has a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ at 412 nm. One unit of specific activity is defined as the amount of enzyme that acetylates one μmole of chloramphenicol per min per mg of total soluble protein. Protein concentration was determined by the method of Bradford (11). The same amounts of total soluble protein were run in SDS-PAGE, which was later stained with Coomassie brilliant blue R-250. To determine CAT percentage in total soluble protein, each lane of the stained SDS-PAGE gels was scanned by a laser densitometer.

Quantification of β-lactamase

Cells broken by sonication were separated into insoluble pellet and soluble supernatant fractions by centrifugation. Specific activity of β-lactamase in the supernatant fractions was determined by measuring the rate of degradation of penicillin G as indicated by the decrease in A_{232} of the solution, and using 1,700 $M^{-1}cm^{-1}$ as molar extinction coefficient (14). One unit of β-lactamase degrades one μmole of penicillin G per min per mg of total soluble protein at 37°C.

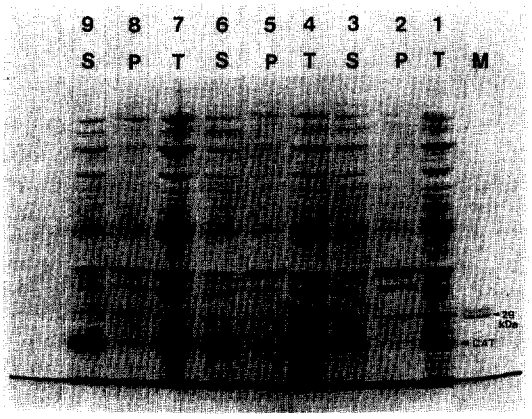

Fig. 1. *Solubility of CAT.*
Cultures were induced with 0.5 mM IPTG at A_{600} of 0.4 at 30 (lane 4) and 37°C (lanes 1, 7). Aeration continued for another 2 h. Cells were broken by sonication. The lysates (T) were separated into pellet (P) and supernatant (S) fractions by centrifugation. Marker protein (29 kDa) is shown in lane M. BL21(DE3, pET3CAT) cells cultured at 37°C were run in lanes 1-3 on SDS-PAGE, and BL21(pBK, pET3CAT) at 30°C in lanes 4-6. Lanes 7-9 show BL21(pBK, pET3CAT) cultured at 37°C.

RESULTS AND DISCUSSION

CAT solubility

There is a report that elogation factor Tu was soluble when expressed under its native promoter, while its fivefold overproduction led to most of inclusion body formation (9). In many cases, overproduced proteins form inclusion bodies, resulting in loss of biological activity. CAT under the phage T7 promoter was overexpressed by inducing T7 RNA polymerase from chromosome (Fig. 1, lane 1) or from plasmid pBK (lanes 4 and 7). In pET3CAT, the promoterless cat gene is preceded by the T7 promoter. Induced T7 RNA polymerase recognized the T7 promoter and expressed CAT.

To determine whether CAT overproduced in the T7 expression system forms inclusion bodies or not, induced cells by IPTG were broken and fractionated into supernatant and pellets. All the CATs, as shown by SDS-PAGE in lanes 3, 6, and 9, were found in supernatants, not in pellets (lanes 2, 5, and 8), indicating that overproduced CAT was completely soluble.

Overproduced CAT activities

To determine whether overproduced CAT has specific activity equivalent to its amount, CAT was produced to various levels by different expression conditions, and then its specific activity and CAT percentage of total soluble protein were determined.

The cat gene under *E. coli* promoter was expressed by *E. coli* RNA polymerase at 37 and 42°C (Fig. 2, lanes 2 and 3). In BL21(DE3, pET3 CAT), CAT was accumulated by T7 RNA polymerase transcription to 42% of total protein after IPTG induction for 4 h at 37°C (lane 4). To obtain various levels of CAT, BL21(DE3, pET3 CAT) was cultured and induced at 25, 30, and 37°C (lanes 5-7)

CAT was accumulated to various levels from 9 to 45% of total protein at different expression conditions. Even fivefold overproduction did not result in inclusion body formation. All the CAT produced was found only from soluble supernatant fraction, as analyzed by SDS-PAGE (data not shown). CAT specific activity was determined in enzyme unit per mg of total soluble protein. CAT specific activity was linearly proportional to CAT percentage of total protein (Fig. 3), which was determined by densitometric analysis on each lane. The results indicate that

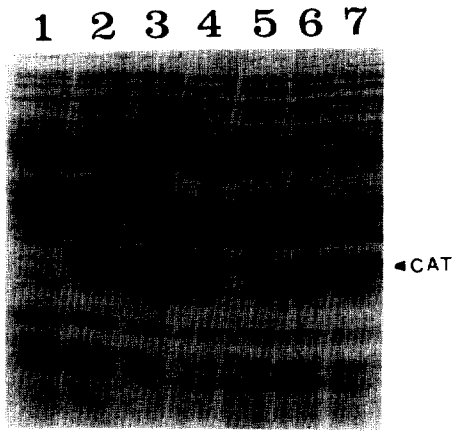

Fig. 2. *Various levels of CAT expression.*
Total soluble protein from supernatant fractions of broken cells by sonication was run on SDS-PAGE. BL21(DE3) as a control (lane 1) and BL21(DE3, pACYC184) cultured overnight at 37°C (lane 2), and BL21(pACYC 184) cultured overnight at 42°C (lane 3) were run with BL21(DE3, pET3CAT) induced by IPTG for 4 h at 37°C (lane 4). BL21(DE3, pET3CAT) cells were cultured and induced by IPTG for 2 h at 25, 30, 37°C (lanes 5-7, respectively).

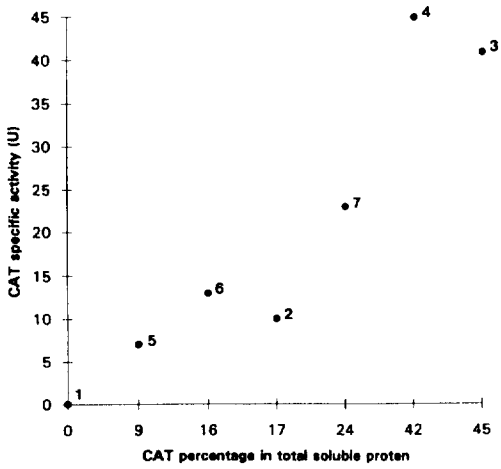

Fig. 3. *Correlation between CAT amount and activity.*
Densitometric analysis was done from lane 1 to 7 for SDS-PAGE in Fig. 2. CAT amount in total soluble protein and corresponding activity are shown as a circle. Numbers beside circles indicate lane numbers in Fig. 2.

overproduced CAT was soluble and active even at 45% of total protein.

Solubility of overproduced β-lactamase

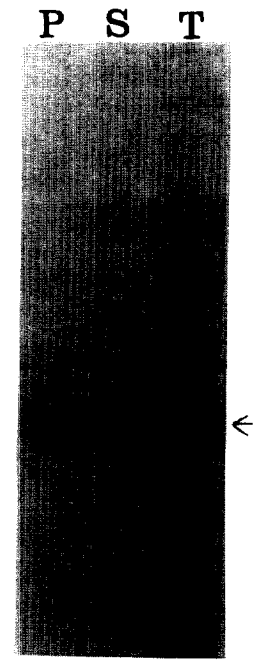

Fig. 4. *Solubility of β-lactamase.*
BL21(DE3, pET7) cells were cultured and induced by IPTG at 30°C. Induced cells (T) were broken by sonication and centrifuged into supernatant (S) and pellet (P) fractions. The arrow on SDS-PAGE indicates mature β-lactamase.

To overexpress the β-lactamase gene, the T7 expression system was used. Also, solubility of β-lactamase produced in two different culture conditions was compared. Plasmid pET7 carries the β-lactamase gene under control of phage T7 gene 10 promoter. The β-lactamase gene is located about 600 bp away from the T7 promoter. The location of T7 promoter is unimportant, since T7 RNA polymerase will actively circumvent a plasmid several times without termination. In BL 21(DE3, pET7) cells, β-lactamase will be produced by transcription of T7 RNA polymerase induced by IPTG.

BL21(DE3, pET7) cells cultured and induced by IPTG at 30°C produced only single mature band for β-lactamase. β-Lactamase activities in induced cells (76 U) showed 3 times those in uninduced cells (25 U). Enzyme activities in uninduced cells were due to incomplete repression of the LacI repressor which binds to lac operator located upstream from the T7 RNA polymerase gene. The mature β-lactamase was completely soluble as shown by SDS-PAGE in Fig. 4. However, the cells induced at 37°C produced two prominent bands (Fig. 5, lane 2). They were

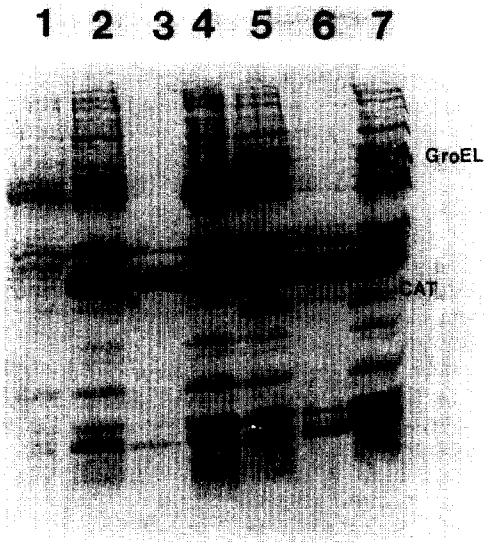

Fig. 5. *GroEL effect on β-lactamase solubility.* BL21(DE3, pET7) cells uninduced (lane 1) or induced (lane 2) by IPTG at 37°C were run on SDS-PAGE with pellet fraction (lane 3) and soluble one (lane 4) of induced cells. BL 21(DE3, pET7, pGroESL) cells induced (lane 5) were run with pellet (lane 6) and soluble fraction (lane 7). p and m indicate precursor and mature forms for β-lactamase.

precursor and mature bands for β-lactamase. Proper fractionation of cells was proved by the fact that CAT, which is inherently soluble, was found only in a soluble fraction, not in an insoluble one (lanes 5-7). It was interesting that most overproduced precursor forms for β-lactamase were found in pellet fractions, and most mature forms in the periplasm (lanes 3 and 4). Inclusion body formation at 37°C seemed to be due to β-lactamase overproduction, while relatively small production at 30°C caused complete solubility of β-lactamase. The fact that overproduced mature forms for β-lactamase in the periplasmic space was largely soluble was surprising, since overproduced β-lactamase under tac promoter control was mostly insoluble in the periplasmic space (3). This might be due to differences of expression systems. Sometimes, it is tedious and time-consuming to obtain biologically active forms from inclusion bodies by urea or guanidinium hydrochloride treatment. It is quite significant biotechnologically to obtain large amount of soluble and mature forms for β-lactamase by the T7 expression system.

GroEL effect on β-lactamase solubility

Interaction between β-lactamase and chaperone GroEL was demonstrated *in vitro* (2) and *in vivo* (8). Chaperone affects protein folding, assembly, and translocation across the cell membrane (12). To determine if overproduced GroE proteins influence β-lactamase solubility, BL21(DE3, pET7) cells were transformed with plasmid pGroESL which contains the *groE* operon, *groEL* and *groES*. BL21(DE3, pET7, pGroESL) cells were cultured to an A_{600} of 0.5 at 37°C and induced by IPTG for 3 h. The overproduced GroE proteins did not change significantly insoluble precursor forms for β-lactamase in the cytoplasm as shown in lane 6 of Fig. 5 at this experimental condition.

Buchner (4) reported that a sixfold molar excess of GroEL over denatured citrate synthase led to a tenfold increase in the yield of reactivated protein by suppressing aggregation, compared with renaturation in the absence of GroEL. A molar ratio of GroEL over β-lactamase was seemingly below one. So, excess GroEL over β-lactamase may be needed to suppress aggregation of β-lactamase precursor forms in the cytoplasm. It was demonstrated that chaperone DnaK overproduction prevented inclusion body formation of human growth hormone significantly, and slow production of target protein for DnaK was more effective in obtaining soluble and active proteins (1). Slow β-lactamase production by the T7 expression system in the presence of GroEL may be effective in obtaining soluble and active enzyme, or using another chaperone such as DanK will be also effective.

ACKNOWLEDGEMENT

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초 록: 대장균에서의 T7 발현체계에 의하여 과잉생산된 클로람페니콜 아세틸전이효소와 베타-락타메이즈의 수용성과 활성

김한복 (호서대학교 자연과학대학 생화학과)

단백질이 어떻게 비수용성이 되는지를 알기위해, 클로람페니콜 아세틸전이효소와 베타-락타메이즈를 과잉생산하여 그들의 수용성과 활성을 측정하였다. 클로람페니콜 아세틸전이효소는 총 단백질의 9에서 45%를 차지하였으며, inclusion body 형성없이 완전히 수용성이었으며, 효소활성은 만들어진 양과 비례하였다. 또한 30°C에서 T7 발현체계에 의해 생성된 베타-락타메이즈는 수용성의 숙성체였으나, 37°C에서는 비수용성이 되었다. 세포질에 있는 대부분의 베타-락타메이즈는 비수용성이었고, 페리플라즘 공간에서는 대부분이 수용성이었다. 단백질의 올바른 폴딩을 도와주는 chaperone의 일종인 GroEL 단백질은 본 실험조건에서는 베타-락타메이즈의 수용성을 별로 높이지는 못했다. 세포 내에서 inclusion body의 형성은 단백질의 높은 농도보다는 각각 단백질 자체의 특성과 관련된 듯하다.