한국생물공학회지 제6권 제1호
Korean J. Biotechnol. Bioeng.
Vol. 6. No. 1. 111-114(1991)

단보(Note)

Effects of a Processing Inhibitor on the Overproduction of Plasmid Encoded β-lactamase in *E. coli*

W. K. Hong and E. K. Kim
Department of Biological Engineering, Inha University Inchon, Korea

대장균 β-lactamase의 대량생산시 Processing Inhibitor의 영향

홍 원 경 · 김 은 기
인하대학교 생물공학과

ABSTRACT

The effects of the precursor processing inhibitor, carbonylcyanide-chlorophenyl hydrazone (CCCP), are investigated on the production of soluble β-lactamase and the formation of the inclusion body when β-lactamase is overproduced by induction with isopropyl thiogalactoside (IPTG). When cells are treated by CCCP, more soluble β-lactamase is produced. In this case, no difference in the amount of inclusion body is observed.

INTRODUCTION

Development of recombinant technology provided great opportunity for the mass production of useful products such as interferon, insulin, TPA etc.(1). For the industrial production of such products, yeast of *E. coli* is preferred host. Strong inducible promoters are usually employed to improve the overall expression efficiency. When the cloned gene is expressed strongly, sometimes, overproduced proteins aggregate and form inactive, so called 'inclusion body', usually in the cytoplasm(2).

Refolding process of such inclusion body, in the industrial process, includes series of complicated, expensive steps. In order to prevent the formation of inclusion body and to increase the yield of soluble protein, signal sequences are attached upstream of the "target gene". In this case, precursor proteins are processed at the membrane by the signal peptidase and secreted into the periplasm or excreted into the medium(3). However, even though processed proteins are secreted into the periplasm, formation of inclusion body is, sometimes, observed in the periplasm when the proteins are overproduced(4).

Very few studies were performed to elucidate the reasons of inclusion body formation in the periplasm and to increase the soluble protein yield. Recently, George et al. showed that addition of the sucrose to the induced *E. coli* cells, during the overproduction of β lactamase, increased soluble β-lactamase yield three fold with less formation of the inclusion body. These group suggested the possible causes as either protein stabilizing effects of the sucrose or decreased β-lactamase precursor processing rate (7). Daniels et al. showed that, CCCP, one of the proton ionophore, inhibited the processing of β lactamase precursor, resulting in the decrease in the processed β-lactamase during the normal, constitutive synthesis of β lactamase (6).

In our present study, we attempted to investigate the effects of β-lactamase processing inhibitor, CCCP, on the inclusion body formation and the yield of soluble β-lactamase during the β lactamase overproduction.

MASTERIALS AND METHODS

Strain:

E. coli RB791($lacI^q$), a derivative of *E. coli* W3110, harvoring plasmid pKN, was obtained from Cornell University. Plasmid pKN is pBR322 modified by placement of the tac promoter upstream of the β-lactamase gene and insertion of a neomycin resistance gene between the Hind Ⅲ and BamHI sites(4).

Culture:

Standard M9 medium complemented with glucose (5 g / *l*), casamino acid(2 g / *l*) and neomycin sulfate (50mg / *l*) was used. Cells grown overnight in this medium were inoculated into the 20ml medium (250ml flask) with initial optical density of 0.05(OD 600) and cultivated in the rotary shaking incubator (37℃, 200 rpm). Synthesis of β-latamase was induced by addition of IPTG to a concentration of 0.1mM to cultures of an O.D. of 0.5. After 24 hrs of growth, 5 mls of cells were centrifuged at 10,000 g for 5 mins. The supernatant was saved as the " supernatant fraction", and the pellet was washed in 0.33 M Tris-HCl buffer, pH 7.5. After resuspending the cell pellet in the same buffer, cells were broken by mini-bead beater (Seolin, Korea). The lysates were centrifuged at 13,000 g for 10 mins to precipate the insoluble material including the inclusion body. Supernatant in this step was saved as the "periplasmic fraction". "Total soluble β-lactamase" includes the "supernatant fraction" and the " periplasmic fraction". Appropriate concentration of CCCP is added with IPTG addition as a precursor processing inhibitor.

Assays:

Soluble β-lactamase was assayed by monitoring the decrease of O. D. (240) of a 50mM K_2HPO_4(pH 7.0) solution containing 0.5mg / ml penicillin G and the sample of unknown β-lactamase concentration.(5). Solubilization and the refolding of the inclusion body was performed as follows. After resuspending the insoluble material in 1.0ml of buffer (50mM Tris-HCl pH 7.9, 250mM KCl, 1mM DTT and 0.1 mM EDTA) containing 6N guanidine HCl, the mixture was incubated on ice for 45 mins. After diluting the mixture 10 fold by dropwise addition of the cold Tris buffer, the solution was dialyzed against 1 liter of the same buffer. The dialysate was centrifuged at 13,300 g for 25 mins to separate solubilized β-lactamase from cell debris. (9). Activity of refolded β-latamase was employed as the relative amount of the inclusion body due to the semi-quantitative nature of the densitometry for the measurement of inclusion body in the denaturing protein gel.

RESULTS AND DISCUSSIONS

To find the effect of CCCP treatment during the overproduction of β-lactamase, CCCP (10μM) was added at the point of IPTG addition. As shown in the Figure 1, production rate of total soluble β-lactamase decreased with the addition of CCCP.

Same phenomenum, decreased β-lactamase production with CCCP treatment, was reported by Daniels et al. during the normal, constitutive synthesis. (6). These group measured the concentrations of the precursor form and processed form of β-lactamase using Western-blot technique and found that CCCP inhibited the precursor processing. A model was suggested that CCCP would disturb the membrane potentials which inhibit the correct orientation of precursor into the membrane where signal peptidases are located (6).

Therefore it seemed probable, in our study, that decreased production rate of processed, soluble β-lactmase was

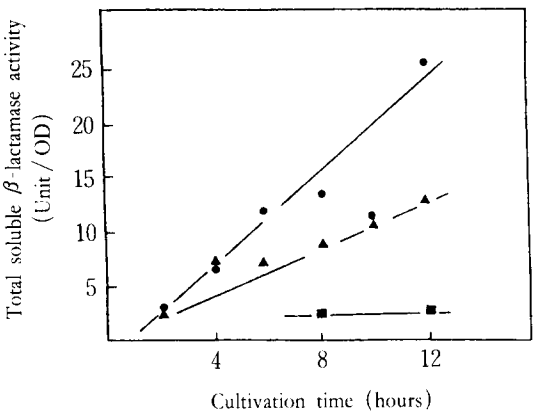

Fig. 1. Time course synthesis of β-lactamase with CCCP treatment.
(●) IPTG(0.1 mM) only
(▲) IPTG(0.1 mM)+CCCP(10uM)
(■) Control (No IPTG, No CCCP)

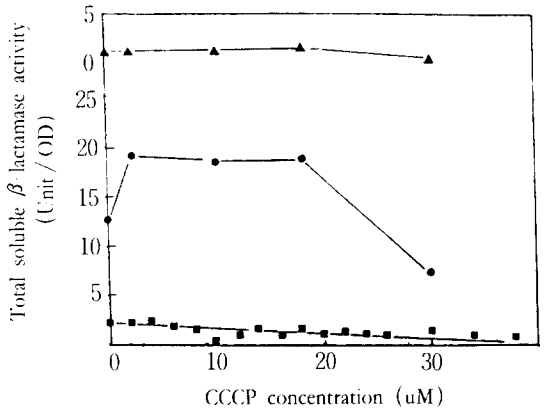

Fig. 2. Effects of CCCP on the overproduction of β-lactamase.
(●) Total soluble activity with IPTG (0.1mM)
(▲) Relative concentration of inclusion body measured as refolded activity (0.1 mM IPTG)
(■) Total soluble activity without IPTG+ CCCP

due to the inhibition of precursor processing rate by CCCP treatment.

Since β-lactamase is produced constituitively without IPTG induction as shown in the Figure 1, it seemed apparent that production of β-lactamase would be decreased as the CCCP concentration increased without IPTG induction (Figure 2). However, when β-lactamase was overproduced (−10 times of constituitive synthesis) upon IPTG induction, yield of total soluble β-lactamase increased as the CCCP concentration increased upto 20μM. At this concentration of CCCP, about 70% of β-lactamse precursor processing was reported to be inhibited (6).

As the CCCP concentration increased above 20uM, inhibition of the processing seemed to be so severe that the absolute amount of processed β-lactamase decreased. Therefore, increased production of soluble β-lactamase with the CCCP treatment could be observed in the range of 20μM CCCP.

This result showed that more soluble β-lactamase was formed in the periplasm or medium, even though processing rate of the β-lactamase decreased due to the CCCP treatment.

When the amounts of inclusion bodies were determined relatively by measuring the refolded β-lactamase activity, no differences in the amount of inclusion bodies were observed as the CCCP concentration increased (Fig. 2).

Therefore it seemed probable that the processing rate has no direct relations with the inclusion body formation in the periplasm. Accordingly increased production of soluble β-lactamase was not attributed to the decreased formation of inclusion body, since no differences in the amount of inclusion body were observed.

George et al. showed that addition of non-metabolizable sucrose into the medium (0.3M) increased yield of total soluble β-lactamase three times with less formation of inclusion body (7). Since sucrose is well known protein stabilizer at high concentration (0.3M), this sucrose night stabilize the overproduced proteins in the periplasm.

Also in their experiment, accumulation of precursor was observed at high sucrose concentration which possibly indicated the inhibition of precursor processing. Therefore this group suggested that the reasons of two phemomena, decreased inclusion body formation and incresed soluble β-lactamase production, might be either the decreased processing rate of the precursor or the increased stability of proteins by the sucrose present in the periplasm.

However, as shown in our present study, decreased rate of precursor processing had little effects on the inclusion body formation. Also we found in our laboratory that addition of NaCl as a protein stabilizer increased the soluble β-lactamase yield significantly. We are currently investigating whether other protein stabilizers generally increase the soluble β-lactamase yield and affect the inclusion body formation.

The reason why more soluble proteins are produced with decreased processing rate, as shown in this study, is not clear at this moment. However it is interesting that more soluble β-lactamase were produced at lower temperature (20℃) than higher temperature (37℃) (9). Same phenomena were also observed in the production of interferon in the cytoplasm (1). Therefore both the decreased processing rate, as shown in this study, and decreased synthesis rate at lower temperature, might provide, in common, favorable environments for the production of soluble proteins.

ACKNOWLEDGEMENT

This work was supported by the financial support of Korea Science and Engineering Foundation, for which the authors are very grateful.

요 약

대장균의 plasmid상의 β-lactamase를 IPTG induction으로 대량 생산할 때 precursor processing inhibitor (CCCP)를 가하여 β-lactamase의 soluble fraction과 insoluble fraction (inclusion body)의 생산성을 비교하였다. CCCP로 처리한 경우가 더 많은 soluble β-lactamase를 생성하였으나, inclusion body의 양에는 큰 차이가 없었다. 이것은 β-lactamase precursor processing 속도를 낮출 경우 soluble β-lactamase가 더많이 생성된다는 것을 보여주었다.

REFERENCES

1. S. K. Sharma (1986) *J. Biotechnol.* 4: 119−124
2. T. Mizukami, Y. Komatsu, N. Hori and T. Oka (1986) *Biotechnol. Lett.* 8: 605−610
3. G. Georgiou, M. L. Shuler and D. B. Wilson (1986) *Biotech. Bioeng.* 32: 741−748
4. G. Georgiou, M. L. Shuler (1986) *Appl. Environ. Microbiol.* 52: 1157−1161
5. J. J. Chalmers, M. L. Shuler and D. B. Wilson (1985) *Biotech. Progress* 1: 75−79
6. C. J. Daniels, D. G. Bole and D. L. Oxender (1981) *Proc. Natl. Acad. Sci USA* 78(9): 5396−5400
7. G. A. Bowden and G. Georgiou (1988) *Biotechnology Progress* 4(2) 97−101
8. C. H. Shein (1990) *Biotechnology* 8: 308−317
9. J. J. Chalmers, E. K. Kim(1990) *Appl. Env. Microbiol* 56(1) 104−111

(Received; February 19, 1991, Revised; March 8, 1991, Accepted; April 30, 1991)