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# Development of *E. coli* Expression System to Overproduce a Harmful Protein, Carboxypeptidase *Taq*.

**Sang-Hyeon Lee**†

*Major in Bioscience and Biotechnology, Division of New Materials and Biotechnology Chemistry, Silla University, San 1-1, Kwaebop-dong, Sasang-gu, Pusan, 617-736.*

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**Abstract** The *E. coli* expression system to overproduce a harmful protein, *carboxypeptidase Taq* was developed. Since expression plasmid pCK305N containing the colicin promoter already has the initiation codon on the restriction site, the initiation codon of the CPase *Taq* gene was removed. Expression plasmid pCP4-col includes the entire CPase *Taq* gene, which is directed by the colicin promoter. *E. coli* cells harboring pCP-col produced a high amount of the enzyme when they were cultured in the present of mitomycin C (0.4 $\mu$g/ml). An amount of purified enzyme produced by pCP4-col directed by the colicin promoter was 10.5 mg. This result indicated that the novel *E. coli* expression system controlled by the colicin promoter could produce almost twice amounts of CPase *Taq* than the conventional system controlled by the *tac* promoter.

**Key words**: carboxypeptidase, zinc-dependent, thermostable, colicin promoter

## Introduction

Carboxypeptidase (CPase) *Taq* is a thermostable zinc-dependent metallo-carboxypeptidase and has a broad substrate specificity [1]. The optimum temperature for its reaction is 80℃ [1]. We previously described the cloning, sequencing and expression in *E. coli* cells of the CPase *Taq* gene [2]. The deduced amino acid sequence of CPase *Taq* exhibits no similarity to any protein sequences [2], including those of other metallo-carboxypeptidases like CPases A and B, suggesting that it is a novel type of metallo-carboxypeptidase. On the other hand, a conserved active-site motif (His-Glu-X-X-His) of zinc-dependent endopeptidases and aminopeptidases was found in the deduced amino acid sequence of the enzyme [2,3].

Since CPase *Taq* is a proteolytic enzyme, the production of the enzyme in *E. coli* cells might be harmful to bacterial growth. In general, the conventional expression systems may not be suitable to express proteolytic enzymes. Overproduction of the enzyme in *E. coli* cells is very helpful for its purification with better purity and economical viewpoint. These concerns suggest that a novel expression system for the expression of harmful proteins will be needed.

In this paper, I develop the expression system, which directed by the colicin promoter to express CPase *Taq*. This expression system would be useful to express the harmful proteins, such as a protease *etc.*

†Corresponding author
Phone: 82-51-999-5624, Fax: 82-51-999-5176
E-mail: slee@silla.ac.kr

## MATERIALS AND METHODS

### Enzymes and reagents for gene engineering.

T4 polynucleotide kinase, T4 DNA ligase, a DNA blunting kit, and agarose powder were purchased from Takara Korea (Seoul, Korea). Restriction enzymes were obtained from Takara Korea (Seoul, Korea). Mitomycin C was purchased from Wako Chemical Inc. (Tokyo, Japan).

### Cells, plasmid vectors, and culture conditions.

*E. coli* MV1184 [*ara* $\Delta$*(lac-proAB) rpsL thi* $\phi$80*lacZ*$\Delta$*M15* $\Delta$*(srl-recA)*306::*Tn*10(*Tc*$^r$)/*F'*:*traD*36 *proAB lacIqZ*$\Delta$*M15*], CJ236 [*dut ung thi relA: pCJ*105(*Cm*$^r$)], and bacteriophages M13mp19 were obtained from Takara Korea. *E. coli* W3110 was originally isolated. Host cells were grown in LB medium (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl) at 37℃. Ampicillin (100 mg/ml) was added when needed. Plates were solidified with 1.5% agar.

### Oligonucleotide-directed mutagenesis.

To introduce *Sca*I site into the initiation and second codons in the CPase *Taq* gene, oligonucleotide-directed mutagenesis was performed according to Kunkel [4] using a MUTAGENE *in vitro* mutagenesis kit (Bio-Rad). The entire region of the DNA fragment of the gene was sequenced to prove that only the mutation expected had occurred.

### Expression of the CPase *Taq* gene in *E. coli* and purification of the enzyme

*E. coli* W3110 cells harboring the expression plasmid for the CPase *Taq* gene (pCP4-col), which is controlled by the colicin promoter were grown in 1 L LB medium containing ampicillin (100 $\mu$g/ml) at 37℃ for 30 min and then mitomycin C (0.4 $\mu$g/ml) was added to induce the colicin promoter. After 9 hrs from mitomycin C induction, the cells were collected by centrifugation and then washed with 0.05 M Tris-HCl buffer (pH 7.2). The washed cells were suspended in a 10-fold volume of the same buffer and then disrupted by sonication. The sonicated sample was centrifuged at 20,000×g for 20 min, and then the supernatant obtained was treated at 70℃ for 1 h and centrifuged again at 20,000 ×g for 20 min to remove denatured proteins. The supernatant was brought to 30% saturation with ammonium sulfate and then stirred at 4℃ for 30 min. After the precipitate had been removed by centrifugation at 20,000×g for 20 min, the supernatant obtained was put on each column (1.6 ×8 cm) of butyl-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 7.2) 30% saturated with ammonium sulfate. The column was washed with the same buffer 20% saturated with ammonium sulfate. Proteins were eluted with the same buffer 10% saturated with ammonium sulfate, in a total volume of 50 ml, at the flow rate of 3 ml per min. The eluate was dialyzed against 50 mM Tris-HCl buffer (pH 7.2), and then put on a column (1.6×8 cm) of DEAE-Toyopearl 650S (Tosoh) equilibrated with 50 mM Tris-HCl buffer (pH 7.2). The column was washed with the same buffer containing 50 mM NaCl. Proteins were eluted with the same buffer containing 100mM NaCl, in a total volume of 50 ml, at the flow rate of 3 ml per min. The protein concentrations were measured with the BCA protein assay reagent (Pierce Chemical Co.), with bovine serum albumin as the standard protein.

### Enzyme activity assay

The enzyme activity was assayed by the ninhydrin method of Rosen [5] with 0.5 mM Cbz-Phe-Tyr (Protein Research Foundation, Osaka, Japan) as the substrate, at 70℃ for 30 min in 50 mM N-2-hydroxyethylpiperazine-N-3-propanesulfonic acid (HEPPS)-NaOH buffer (pH 8.5). One unit of enzyme activity was defined as the amount of enzyme that produced a ninhydrin-positive substance corresponding to 1 mmol of tyrosine per minute. The specific activity was expressed as units (U) per milligram of protein.

### SDS-PAGE

SDS-PAGE was performed by the method of Laemmli [6] with an 11% polyacrylamide gel. The enzyme solution was mixed with the sample buffer and then incubated at 37℃ for 30 min before being put on the gel.

## RESULTS AND DISCUSSION

### Construction of the CPase *Taq* expression plasmid, which is directed by the colicin promoter.

Expression plasmid pCK305N containing the colicin promoter already has the initiation codon on an *Nco*I site (Fig. 1). Therefore, to insert the CPase *Taq* gene into pCK305N, it is necessary to remove the initiation codon of the CPase *Taq* gene. Plasmid pCP3-7 carrying the CPase *Taq* gene [3] was digested with *Eco*RI and *Pst*I, the 250-bp fragment containing the 5'-terminal 250-bp coding region of the gene was subcloned into M13mp19, and pEP250M was obtained (Fig. 1). Site-directed mutagenesis was performed using the 25-mer synthetic oligonucleotide shown in the box to remove the initiation codon of the gene and to introduce an *Sca*I site as indicated, and pEP250M-S was obtained (Fig. 1). The 250-bp *Eco*RI-*Pst*I fragment of pCP3-7 was replaced with the 250-bp *Eco*RI-*Pst*I fragment of pEP250M-S, and pCP4-7 was obtained. Finally, pCK305N was digested with *Nco*I and *Eco*RI. After blunting the *Nco*I and *Eco*RI sites of pCK305N, the 2,200-bp *Sca*I-*Ssp*I fragment of pCP4-7

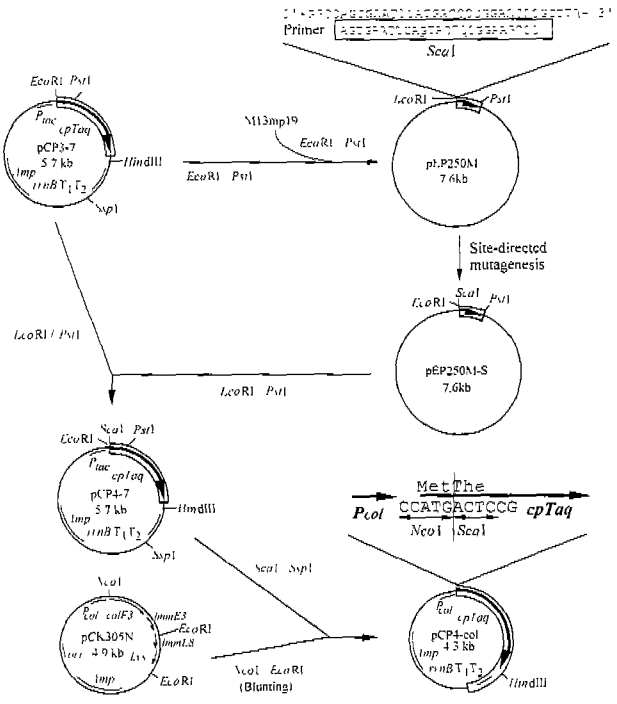


**Fig. 1.** Construction of the expression plasmid pCP4-col for the CPase *Taq* gene.

The position and direction of the entire CPase *Taq* gene (cpTaq) is indicated by thick arrows. *Amp*, ampicillin resistance gene; $P_{tac}$, *tac* promoter; $P_{col}$, colicin promoter; *rrnB*$T_1T_2$, ribosomal RNA transcription termnators 1 and 2; *colE3*, gene encoding colicin E3; *immE3*, gene encoding immunity protein, which protects the host from the colicin E3; *immE8* gene encoding immunity protein, which protects the host from the colicin E8; *Lys*, gene encoding lysozyme; *ori*, replication origin of the plasmid.

was inserted, and CPase *Taq*-expression vector pCP4-col, which is directed by the colicin promoter was obtained.

When pCK305N was digested with *Nco*I and *Eco*RI, the ribosomal RNA transcription terminator was removed (Fig. 1). The 2,200-bp *Sca*I-*Ssp*I fragment of pCP4-7 includes the ribosomal RNA transcription terminator ($rrnBT_1T_2$) (Fig. 1), and this terminator would be used effectively in pCP4-col.

## Expression of the CPase *Taq* gene in *E. coli* and purification of the enzyme.

*E. coli* cells harboring pCP4-col produced a high amount of CPase *Taq* (Fig. 2). CPase *Taq* was effectively purified by heat treatment with a great yield, and successive butyl-Toyopearl and DEAE-Toyopearl chromatographies gave a purified preparation of the enzyme protein (Table I and Fig. 2). The mobility of the purified CPase *Taq* produced in *E. coli* (Fig. 2, lane 4) was identical with that of the CPase *Taq* purified from *T. aquaticus* YT-1 (Fig. 2, lane 5). The specific activities of the two purified enzymes were almost identical (Table 1 and reference 4).

*E. coli* cells harboring pCP4-col produced higher amount of CPase *Taq* than *E. coli* cells haboring pCP1-7 (Table 1 and reference 4). Amounts of purified enzymes produced by pCP4-col, which is directed by the colicin promoter and pCP1-7, which is directed by the *tac* promoter were 10.5 mg and 6 mg, respectively (Table 1 and reference 4). This result indicated that the colicin promoter could produce almost twice amounts of CPase *Taq* than the *tac* promoter. Since mitomycin C is an inhibitor of the DNA synthesis, the colicin promoter is effectively induced by mitomycin C through the SOS response [7-10]. I also have tested the expression system, which directed by strong T7 promoter

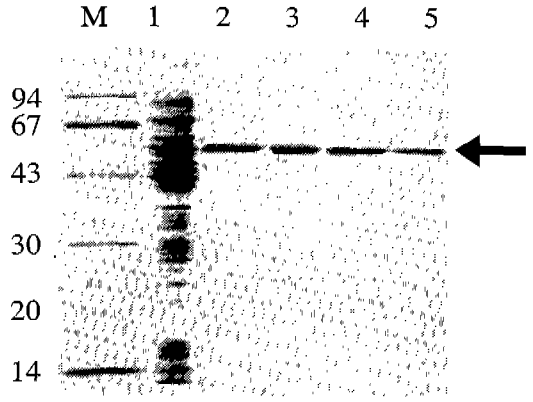


**Fig. 2.** SDS-PAGE of CPase *Taq* produced in *E. coli* cells harboring pCP4-col.
Lane M. size standard marker: lane 1. the supernatant obtained on centrifugation after disruption of the cells by sonication: lane 2. soluble fraction obtained on centrifugation after heat treatment at 70℃ of the lane 1 material: lane 3. sample after butyl-Toyopearl chromatography; lane 4. sample after DEAE-Toyopearl chromatography: lane 5. purified CPase *Taq* from *T. aquaticus* TY-1. The arrow indicates the position of CPase *Taq*.

**Table 1.** Purification of CPase *Taq* from *E. coli* cells harboring pCP4-col, which is carrying the CPase *Taq* gene.

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) |
|---|---|---|---|---|
| Cell-free extract | 550.0 | 47,300 | 86 | 100 |
| Heat treatment | 154.0 | 40,360 | 262 | 85 |
| Butyl-Toyopearl | 20.0 | 29,600 | 1,480 | 63 |
| DEAE-Toyopearl | 10.5 | 27,730 | 2,641 | 59 |

to express CPase *Taq*, however, no remarkable expression of the enzyme was observed (data not shown).

Here these results indicate that this expression system, which is directed by the colicin promoter would be useful to express the harmful proteins, such as a protease *etc.*

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## REFERANCES

1. Lee, S.-H., E. Minagawa, H. Taguchi, H. Matsuzawa, T. Ohta, S. Kaminogawa, and K. Yamauchi. 1992. Purification and characterization of a thermostable carboxypeptidase (carboxypeptidase *Taq*) from *Thermus aquaticus* YT-1. *Biosci. Biotech. Biochem.*, **56**, 1839-1844.
2. Lee, S.-H., H. Taguchi, E. Yoshimura, E. Minagawa, S. Kaminogawa, T. Ohta, and H. Matsuzawa. 1994. Carboxypeptidase *Taq*, a thermostable zinc enzyme, from *Thermus aquaticus* YT-1: Molecular cloning, sequencing, and expression of the encoding gene in *Escherichia coli*. *Biosci. Biotech. Biochem.*, **58**, 1490-1495.
3. Lee, S.-H., H. Taguchi, E. Yoshimura, E. Minagawa, S. Kaminogawa, T. Ohta and H. Matsuzawa. 1996. The active site of carboxypeptidase *Taq* possesses the active-site motif His-Glu-X-X-His of zinc-dependent endopeptidases and aminopeptidases. *Protein Engineer.*, **9**, 467-469.
4. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA*, **82**, 488-492.
5. Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.*, **67**, 10-15.
6. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
7. Waleh, N. S., I. Sohel, J. B. Lazar, D. V. Hudson, P. Sze, P. A. Underhill, P. H. Johnson. 1992. Expression of synthetic genes encoding fused proteins under tight control of modified regulatory regions of the colicin operon. *Gene* **117**, 7-14.
8. Cavard, D., S. P. Howard, R. Lloubes, C. Lazdunski. High-level expression of the colicin A lysis protein. *Mol. Gen. Genet.* 217, 511-519.
9. Parker, R. C. 1986. Mitomycin C-induced bidirectional transcription from the colicin E1 promoter region in plasmid ColE1. *Biochim. Biophys. Acta* **868**, 39-44.
10. Waleh, N. S. and P. H. Johnson. 1985. Structural and functional organization of the colicin E1 operon. *Proc. Natl. Acad. Sci. USA* **82**, 8389-8393.