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# Cloning and Expression of *Escherichia coli* Ornithine Transcarbamylase Gene, *arg*I

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Abstract : *Escherichia Coli* ornithine transcarbamylase is the enzyme which catalyzes the L-citrulline biosynthesis from L-ornithine and carbamyl phosphate. To facilitate the purification of enzyme which will be used for many biochemical studies such as structure and function·relationships and catalytic mechanisms, the cloning and expression of *E. coli arg*I gene for ornithine transcarbamylase was conducted. *arg*I was amplified from genomic DNA of *E. coli* strain of DH5α by polymerization chain reaction (PCR) method. The amplified *arg*I gene was ligated to the prokaryotic expression vector pKK223-3 and used for transformation of *E. coli* TB2 which was deficient of ornithine transcarbamylase. The over-produced enzyme by the transformant was purified by ammonium sulfate fractionation, heat denaturation and affinity chromatography. The result of SDS denaturation gel electrophoresis for the purified enzyme showed a single band of about 38 kDa of ornithine transcarbamylase. Kinetic data for the expressed enzyme gave almost the same values as those of the wild type enzyme. The $k_{cat}$ of the enzyme was $1.0\times10^5\,min^{-1}$, and $K_m$s for ornithine and carbamyl phosphate were 0.35 mM and 0.06 mM, respectively(Received April 6, 1995; accepted April 28, 1995).

## Introduction

Ornithine transcarbamylase (EC 2.1.3.3) catalyzes the biosynthesis of L-citrulline by the condensation of L-ornithine and carbamyl phosphate. The bacterial enzyme is located in the pathway leading to arginine biosynthesis. In mammals, ornithine transcarbamylase is one of the enzymes in the urea cycle. Ornithine transcarbamylases obtained from most of the sources so far studied are trimers composed of identical subunits. Its molecular weight is about 110 kilo dalton(kDa).[1]

Ornithine transcarbamylase normally observes Michaelis-Menten kinetics for both substrates, L-ornithine and carbamyl phosphate.[2] Substrate binding follows Bi-Bi sequential mechanism.[3,4] Carbamyl phosphate binds to the enzyme first, and L-orntihne binds to the binary complex of enzyme and carbamyl phosphate. Actually, carbamyl phosphate opens the binding pocket of L-ornithine.[1] The enzyme is not known to be an allosteric enzyme. Zinc(II) ion, however, mediates the regulation of enzymic activity in two ways.[5] The action of zinc ion depends on the incubation pattern of the enzyme with ligands. The metal ion behaves as a classical reversible inhibitor to the binary complex of the enzyme and carbamyl phosphate. Zinc acts as an allosteric cofactor in ornithine saturation giving a positive cooperativity. On the other hand, zinc ion inhibits the enzyme irreversibly when it binds to the enzyme first. This irreversible inhibition is incubation-time dependent.

The three-dimensional structure of the ornithine transcarbamylase has not been determined yet. Several amino acid residues such as arginine, lysine, and cysteine have been proposed as critical ligands for enzyme functions.[6,7] Arginine-57 plays an important role in carbamyl phosphate-induced enzyme isomerization.[1] Cysteine-273 is a ligand of L-ornithine and zinc ion.[8] The overall pictures for the catalysis and ligand binding at the active site have not been well elucidated. Therefore, the protein over-production system is needed to obtain enough enzymes to study the structure and function relationships, and the mechanistic details of ornithine transcarbamylase. In this study, *E. coli arg*I gene for ornithine transcarbamylase was cloned and over-expressed.

## Materials and Methods

### Materials

Oligodeoxynucleotides and *Taq* DNA polymerase for polymerase chain reaction (PCR) were obtained from Korea Biotech. Restriction enzymes and deoxynucleotides were purchased from BM Korea, and prokaryotic expression vector pKK223-3 was obtained from Pharmacia. Matrex Blue Gel A for affinity chromatography and Microcon for the filtration of DNA fragments were obtai-

Key words : Ornithine transcarbamylase, polymerase chain reaction, gene expression, protein purification, enzyme kinetics
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ned from Amicon(USA). Bio-Rad's Coomassi Blue dye was used to determine the protein concentration. Other chemicals for DNA work were purchased from SIGMA.

### Preparation of *E. coli* genomic DNA

*E. coli* genomic DNA was purified from DH5α strain [*hsd*R *rec*A1 Δ(*arg*F-*lac*ZYA)U169 φ80d*lac*ZΔM15]. A single colony of DH5α was obtained from LB plate, and used as an inoculum for over-night culture in LB broth. Bacterial cells were precipitated in the microcentrifuge. The precipitate was resuspended in buffer containing 567 μl of TE, 30 μl of 10 % SDS, and 3 μl of 20 mg/ml of proteinase K, and then incubated for 1 hr at 37℃. 100 μl of 5 M NaCl was added and mixed. Subsequently, 80 μl of CTAB/NaCl solution was added and the lytic mixture was further incubated for 10 min at 65℃. The cell lysate was extracted with chloroform/isoamyl alcohol first, and then phenol/chloroform/isoamyl alcohol. Genomic DNA was precipitated by adding 0.6 volume of isopropyl alcohol, and the DNA precipitate was washed with 70% ethanol. The genomic DNA was resuspended in 100 μl of TE.

### Amplification and cloning of *arg*I gene

Two oligodeoxynucleotides to amplify the *arg*I gene were prepared. The sequences of the N-terminal primer was 5'-CA GGT *GAA TTC* ATG TCC GGG TTT TAT CAT AAG CAT-3'. The sequences of the C-terminal primer was 5'-CA TGT *AAG CTT* TTA TTT ACT GAG CGT CGC GAC CAT-3'. For the directional cloning to the prokaryotic expression vector, pKK223-3, restriction enzyme sites of *Eco* R1 and *Hind* III were added to the end of N-terminal and C-terminal sequences, respectively. The sequences of enzyme sites were underlined. PCR was performed with Thermocycler. *arg*I gene was amplified in the 100 μl of solution which contained 2 μg of genomic DNA, 10 μl of 2 mM dNTP, 100 pmole each of N-terminal and C-terminal primer, 10 μl of 10X reaction buffer supplied by manufacturer, and 2.5 units of Taq DNA polymerase. Denaturation temperature was 94℃, annealing temperature was 55℃, and extension temperature was 72℃. Details of the amplification followed the protocols in Molecular Cloning.[9] Amplified *arg*I gene was confirmed by 1 % agarose gel electrophoresis.

DNAs of *arg*I gene amplified and expression vector pKK223-3 were digested with *Eco* R1 and *Hind* III. Double-digested DNA fragments were filtered with Amicon's Microcon-30 and ligated with T4 DNA polymerase. The recombinant plasmid with *arg*I gene, then, was used for transformation of TB 2[*E. coli* K-12 : Δ(*arg*I-*pyr*BI)*arg*F$^-$] cell by the shot-gun method, and the resulting transformation mixture was spread on the M9 minimal plate containing 1 mM of uracil and 50 μg/ml of ampicillin. The colonies were analyzed to obtain the recombinant plasmids after growth in the LB broth containing 50 μg/ml of ampicillin.

### Purification of ornithine transcarbamylase

A colony of TB 2 cell containing the recombinant plasmid(pKK/*arg*I) was selected and picked from the M9/uracil/ampicillin/agar plate, and cultured in 1 liter of LB broth with 50 μg/ml of ampicillin for overnight at 37℃. The cells were precipitated in the centrifuge at 5000 rpm for 20 min. The precipitate was suspended in two volumes of breakage buffer(100 mM $KH_2PO_4$/40 mM L-ornithine, pH 7.5). Cells were lysed with ultrasonicator for 15 min, and centrifuged at 12000 rpm for 20 min. The supernatant was heated in a 65℃ water bath for 10 min and cooled down in an ice bath. The supernatant collected after heat denaturation was brought to 40% ammonium sulfate saturation, then centrifuged. The resulting supernatant was brought to 90% ammonium sulfate saturation, then recentrifuged. The precipitate was resuspended in 60% ammonium sulfate/50 mM potassium phosphate solution, pH 7.5. The suspension was stirred for 30 min and then centrifuged. The precipitate was dissolved and dialyzed in 20 mM Tris acetate buffer, pH 7.5. The dialyzed enzyme solution was applied to Matrex Blue Gel A column eluting with 0~0.5 M KCl gradient at room temperature. The enzyme fraction were pooled and dialyzed against 10 mM Tris acetate, pH 7.5, and concentrated with an Amicon's protein concentrator. The homogeneity of the enzyme was identified by observation of a single band on Sodium dodecylsulfate polyacrylamide gel stained with Coomassie Blue according to the method of Laemmli.[11]

### Enzyme assays

Steady-state initial velocity assays were performed as described previously.[2,10] Enzymic reactions for both ornithine and carbamyl phosphate saturations were usually initiated by the addition of carbamyl phosphate to the reaction mixture. Initial velocity was taken to be the rate of L-citrulline production as determined by the method of Pastra-Landis. The enzymic reactions were quenched by the addition of color reagent which contained 1 volume of 10% 2, 3-butadionmonoxime in 5% acetic acid and 2 volume of pre-made antipyrine solution in 50 % sulfuric acid to each assay tube.

Initial velocity data were fit to the Michaelis-Menten equation by nonlinear regression methods

$$vo = k_{cat}[E]S/(Km + S)$$

In this equation, $vo$ is the initial velocity, $k_{cat}$ is the specific activity, S is a substrate concentration, [E] is

enzyme concentration, and $K_m$ is the apparent substrate concentration to give a rate of half $V_{max}$.

## Results and Discussion

To clone the *arg*I gene of ornithine transcarbamylase, PCR was applied to amplify the *arg*I gene from the wild-type *E. coli* genomic DNA. Oligodeoxynucleotide primers for the amplification had *Eco* R1 or *Hind* III restriction enzyme site to facilitate cloning in the expression vector, pKK223-3. Each primer had 5 extra bases at the 5′ side of the restriction enzyme site to be a good substrate for the enzyme. When PCR was done, agarose gel electrophoresis was applied to check whether 1.0 kilo bases(kb) of *arg*I gene was amplified as shown in Fig. 1. The template DNA for lane 1 and 2 was the genomic DNA of *E. coli* strain DH5α. Total DNA of TB 2 cell lysate after the plasmid containing *arg*I gene cured was also used as the template DNA for the lane 3 as a negative control. The results of the gel electrophoresis clearly showed the single band of 1.0 kb expected to be amplified *arg*I genes as shown in lane 1 and 2. Lane 3 showed no band indicating that TB 2 cell was cured of the foreign plasmid, and that there was no *arg*I gene for ornithine transcarbamylase in the cell.

To express the wild-type *arg*I gene, *E. coli* TB 2 was used as a host cell. It was not able to grow in the medium without uracil and arginine due to the deletion of both *pyr*BI and *arg*I genes. *pyr*BI were genes of *E. coli* aspartate transcarbamylase. Since we did not have a TB 2 strain except TB 2 containing mutant *arg*I/pUC 18·plasmid, plasmid curing was performed by successive rich-medium cultures. By comparing colonies appeared on the M9 plate containing uracil and arginine with those on the same plate without arginine at the last stage of screening, TB 2 cells cured of the plasmid were obtained. In addition, the result of PCR in Fig. 1(Lane 3) conclusively indicated that mutant *arg*I/pUC 18 plasmid was absent from the cured TB 2 cell.

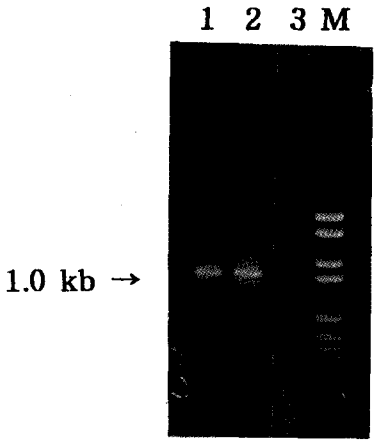


Fig. 1. The results of the agarose gel electrophoresis for PCR product of *arg*I gene. Lanes 1 and 2; genomic DNA of DH5α as a template for *arg*I gene amplification. Lane 3; total DNA of TB 2 cell lysate as a template for PCR. The TB 2 cells used in this experiment were supposed not to have a mutant *arg*I/pUC 18 plasmid since the plasmid was cured. Sizes of the molecular marker were 2.18 kb, 1.77 kb, 1.23 kb, 1.03 kb, 0.65 kb, 0.52 kb, 0.45 kb, 0.39 kb, and so on from the top DNA band.

Competent TB 2 cells were transformed with the recombinant plasmid of *arg*I and pKK223-3(pKK/*arg*I). The cloned plasmids were purified from the transformants and digested with *Eco* R1 and *Hind* III. The result is shown in Fig. 2. Lane 1 showed two bands of cloned *arg*I gene(1.0 kb) and the pKK223-3 vector(4.5 kb) after *Eco* R1/*Hind* III double-digestion of pKK/*arg*I. The DNA band shown in lane 2 was PCR product of *arg*I gene.

To check whether the cloned *arg*I gene was functionally expressed, the host TB 2 cell and the TB 2 containing pKK/*arg*I plasmid were grown at various conditions. The results are shown in Table 1. A TB 2 cell lacking functional gene for ornithine transcarbamylase did not grow in the M9 minimal medium without arginine, while a TB 2 transformant with pKK/*arg*I plasmid grew well under the same condition. Since arginine is the penulti-

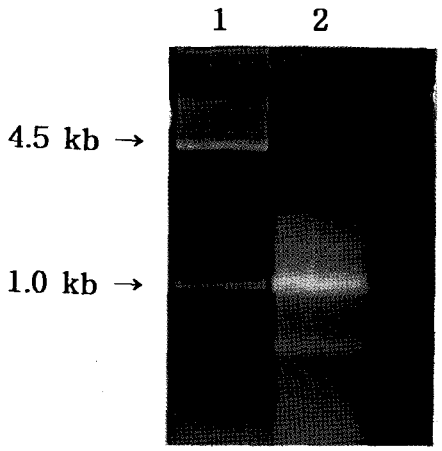


Fig. 2. The results of the agarose gel electrophoresis for pKK/*arg*I plasmid. Lane 1; pKK/*arg*I digested with *Eco* R1 and *Hind* III. Lane 2; The PCR product of *arg*I gene as shown in lane 1 of Fig. 1.

Table 1. The viabilities of TB 2 strains under various growth conditions

| Strains | Growth conditions | | | | |
|---|---|---|---|---|---|
| | LB | LB/amp | M9/uracil/arg | M9/uracil | M9/uracil/amp |
| TB 2 | O | X | O | X | X |
| TB 2 (pKK/*arg*I) | O | O | O | O | O |

amp; ampicillin, arg; arginine. O indicates that the bacterial cells could grow on the media, and X means that the cells were not able to grow on the media.

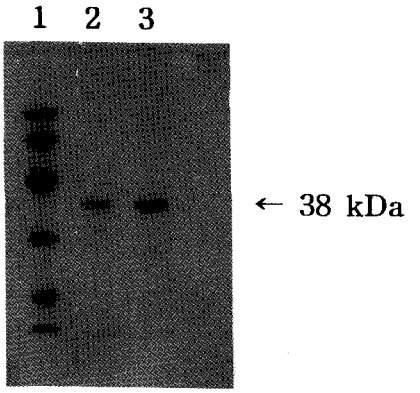


**Fig. 3. The results of the SDS polyacrylamide gel electrophoresis for the expressed *arg*I gene product.** Lanes 2 and 3; Two protein fractions of which had activity of ornithine transcarbamylase were applied on the 7.5% SDS polyacrylamide gel. They were collected by the use of the Matrex Blue Gel A column. Lane 1 was the protein molecular size marker. The sizes of the molecular marker were 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, and 14.4 kDa from the top protein band. The calculated molecular size of the fractionated protein was 38 kDa.

mate product of biosynthetic pathway involved ornithine transcarbamylase, the results support that the cloned *arg*I gene is functional for the synthesis of ornithine transcarbamylase.

Ornithine transcarbamylase produced in the TB 2 transformant was purified by ammonium sulfate fractionation, heat denaturation, and Amicon's Matrex Blue Gel A affinity chromatography. The purity and the molecular size of the enzyme were determined on the SDS denaturing polyacrylamide gel electrophoresis. The result is shown in Fig. 3. The estimated size of *arg*I gene product as judged by the protein migration on the gel was about 38 kDa, while the size of the one subunit of ornithine transcarbamylase deduced from the wild-type DNA coding sequences was about 37 kDa. Since there was only 4% difference in the molecular size between them, the protein of 38 kDa was assumed to be an expressed *arg*I gene product.

The kinetic parameters such as $k_{cat}$ and $K_m$ of ornithine transcarbamylase expressed from the cloned *arg*I were also obtained and compared with those of the wild-type ornithine transcarbamylase. The results are shown in Table 2. The data indicated that the kinetic parameters were very much same between the wild-type and the cloned enzymes, even though there were slight differences.

Combined results obtained from the PCR amplification of a bacterial gene, M9 minimal medium screening, SDS PAGE, and kinetic parameters of ornithine transcarbamylase directly suggested that the *arg*I gene of *E. coli* ornithine transcarbamylase was successfully cloned and expressed. For further work, DNA sequencing of *arg*I gene will be performed. The establishment of the overproduction system for ornithine transcarbamylase will facilitate the study of the architecture of catalysis by ornithine transcarbamylase.

Table 2. The comparison of kinetic parameters of *E. coli* ornithine transcarbamylase

| Sources | $k_{cat}$ ($min^{-1}$) | $Km^{orn}$ (mM)[a] | $Km^{cp}$ (mM)[b] |
|---|---|---|---|
| *E. coli* K-12 | $1.4\times10^5$ | 0.32 | 0.05 |
| TB 2 (pKK/*arg*I) | $1.0\times10^5$ | 0.35 | 0.06 |

$Km^{orn}$, Michaelis constant for L-ornithine; $Km^{cp}$, Michaelis constant for carbamyl phosphate; $k_{cat}$, turnover number of the enzyme. [a]The enzymic reaction was performed at 2 mM carbamyl phosphate in Tris acetate buffer, pH 8.5 for 5 min at 25℃. The range of L-ornithine concentration was 0~6 mM. [b]The enzymic reaction was performed at 4 mM of L-ornithine in 50 mM Tris acetate buffer for 5 min at 25℃. The range of carbamyl phosphate concentration was 0~2 mM.

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***Escherichia coli* 오르니틴 트란스카바밀라제의 유전자 *arg*I의 클로닝 및 발현**

류기중, 유장걸, 고영환, 김찬식, 송성준, 오영선, 이선주*(제주대학교 방사능이용연구소)

**초록** : *Escherichia coli*의 오르니틴 트란스카바밀라제는 오르니틴과 카바밀인산으로부터 시트룰린의 합성을 촉진시키는 효소이다. 이 효소의 기능과 구조와의 상관관계, 반응메카니즘등 생화학적 연구를 하기 위하여 대량의 효소를 추출할 필요가 있다. 본 연구는 오르니틴 트란스카바밀라제의 대량생산 시스템을 확립하기 위하여 *E. coli arg*I 유전자를 *E. coli* DH5α 세포의 염색체 DNA를 추출한 후에 PCR 방법으로 증폭시켜 얻었다. 증폭된 *arg*I 유전자를 단핵생물 단백질 발현벡터인 pKK223-3에 접합시킨 후, 오르니틴 트란스카바밀라제가 존재하지 않은 *E. coli* TB2 세포에 클로닝 시켰다. 이 세포로부터 생산된 오르니틴 트란스카바밀라제는 암모늄염에 의한 분할, 열변성, 크로마토그래피등을 사용하여 순수하게 분리하였다. SDS 단백질 전기영동 결과 약 38 kDa 크기의 효소가 순수하게 얻어졌다. 반응속도론적 실험결과 $k_{cat}$은 $1\times10^{5}m^{-1}$, $K_M$은 오르니틴에 대하여는 0.35 mM, 카바밀인산에 관하여는 0.06 mM이 각각 얻어졌다. 이 결과는 야생형 오르니틴 트란스카바밀라제의 반응속도 인자들과 비슷한 값이다. 본 연구는 이들 결과로부터 오르니틴 트란스카바밀라제의 기능을 하는 *E. coli arg*I 유전자가 클로닝 되었음을 확인하였다.

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*연락저자