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Introduction of Mutation at Threonine-56 of *Escherichia coli* Ornithine Transcarbamylase

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Escherichia coli ornithine transcarbamylase catalyzes the condensation reaction of ornithine and carbamyl phosphate to produce citrulline. To study the structure and function relationship of the enzyme, Threonine-56 was selected to mutate. This residue has been suggested to be located at the binding pocket of carbamyl phosphate. Threonine-56 is one of amino acids in the consensus stretch of phosphate binding proteins. It has been substituted to serine, valine and glycine to observe the size and charge effect. Site-directed mutagenesis by the polymerase chain reaction (PCR) was applied. Each mutant *argI* gene was obtained, and was ligated to the prokaryotic expression vector pKK223-3. TB 2 *E. coli* strain which does not have ornithine transcarbamylase activity has been used to express mutant *argI* genes. The result of DNA gel electrophoresis after *Hind* III/*Eco* R1 digestion of cloned mutant genes indicated that mutant clones were successfully constructed. For further work, DNA sequencing and purification of mutant enzymes will be performed.

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INTRODUCTION

Escherichia coli ornithine transcarbamylase catalyzes the condensation reaction of ornithine and carbamyl phosphate to produce citrulline. Ornithine transcarbamylases of *E. coli*, mouse and human are composed of three identical subunits and their molecular weights are around 110,000 daltons (Marshall & Cohen, 1980). The substrate binding mechanism is Bi Bi ordered (Legrain & Stalon, 1976). The first substrate carbamyl phosphate binds enzyme and then ornithine binds. When carbamyl phosphate binds to the enzyme, the enzyme isomerizes to open the binding pocket for incoming ornithine. For *E. coli*, one of amino acid residues which are responsible for substrate-mediated isomerization has been reported. Arginine-57 induces the induced-fit

type isomerization upon the binding of carbamyl phosphate (Kuo *et al.*, 1988). Mutation of this residue to glycine did not mediated productive conformational change resulting in the decrease of enzymatic specific activity 21,000 times.

For ornithine transcarbamylase, the mechanisms for catalytic reaction at the molecular level, and structural details have not been reported yet. In this research, Threonine-56 at the active site, was mutated to serine, valine and glycine to study the structural and functional role of this residue.

MATERIALS AND METHODS

Taq DNA polymerase which had proofreading capability, restriction enzymes such as *Hind* III and *Eco R*1, were purchased from New England Biolab (USA). Mutagenic oligodeoxynucleotide primers were synthesized at the Bionia (Korea). Prokaryotic expression vector, pKK223-3 was obtained from Pharmacia (Sweden). Matrex Blue Gel A for affinity chromatography was purchased from Amicon (USA). Plasmid purification kit and PCR purification kit were obtained from Promega (USA). Other chemical reagents for cloning and enzyme purification such as KCl, Tris, ammonium sulfate, sodium acetate were purchased from SIGMA (USA). Thermal cycler and Gel electrophoresis kit were products of Bio-Rad(USA).

Mutant *argI* genes were obtained by the application of site-directed mutagenesis. The codon to be mutated was located at the middle of corresponding mutagenic primer as shown in Table 1. The DNA sequence of N-terminal primer was 5'-CA GGT GAA TTC ATG TCC GGG TTT TAT CAT AAG CAT-3'. The DNA sequence of N-terminal primer was 5'-CA TGT AAG CTT TTA TTT ACT GAG CGT CGC GAC CAT-3'. Restriction enzyme sites of *Eco R*1 and *Hind* III to ligate amplyfied DNA on the Prokaryotic expression vector, pKK223-3, were introducd as underlined in each primr. PCR was performed with Genecycler (BioRad, USA). In the reaction mixture, there were 2μg of *E. coli* genomic DNA, 10$\mu\ell$ of 2mM dNTP, 100pmole of each N-terminal and C-terminal primers, 10 $\mu\ell$ of 10 x reaction buffer, 2.5unit of Taq DNA polymerase in total 100$\mu\ell$ of volume. Reaction was cycled 25 times. Each cycle was consisted of 30 seconds of denaturation at 94℃, 1 min of annealing reaction at 55℃, and 2 min of extension at 72℃. Further details for DNA amplification was described in Molecular Cloning (1989). The synthesis of intermediate DNA was confirmed on the agarose DNA gel electrophoresis. Full length of mutant *argI* genes were amplified with 5'-, and 3-'intermediate DNAs and N-, and C-terminal primers. Since *Eco R*1 or *Hind* III enzyme sites were introduced at the end of each N-, and C-terminal primers, mutant *argI* genes and prokaryotic expression vector

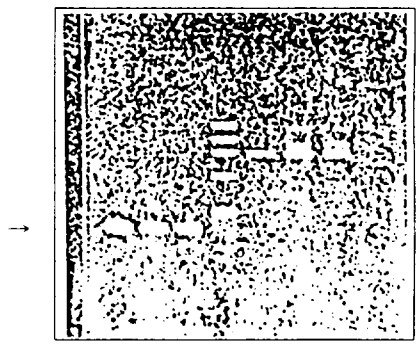
pKK223-3 were digested with *Eco R*1 and/or *Hind* III, and ligated. Recombinated plasmid was transformed for the TB2 *E. coli* strain which lacked activity of ornithine transcarbamylase.

The recombinant DNA was selected in the LB agar plate in the presence of 50μg of ampicillin. Positive clones were picked and cultured in the LB/ampicillin medium over night, and plasmid were purified by the alkali lysis method. Purified recombinant DNA was double digested with *Eco R*1 and *Hind* III, and was electrophoresised.

RESULTS AND DISCUSSION

To synthesize the mutant Thr-56 ornithine transcarbamylase, site-directed mutagenesis by PCR was applied. Mutant *argI* genes were amplyfied in stepwise manner. 5′-side DNA fragment was synthesized by the use of corresponding mutagenic primers and N-terminal primer. 3′-side DNA fragment was synthesized by the use of corresponding mutagenic primers and C-terminal primer. Full size *argI* genes were amplified with 5′- and 3′-side intermediates, and N-, and C-terminal primers. The results were following.

Figure 1. 5′- and 3′-DNA Intermediates Made By PCR.



1 2 3 M 4 5 6

1. 5′-intermediate of Thr-56 to Ser.
2. 5′-intermediate of Thr-56 to Val.
3. 5′-intermediate of Thr-56 to Gly.

M. 1.35, 1.08, 0.87, 0.60, 0.31, 0.28, 0.23, 0.19kbs

4. 3′-intermediate of Thr-56 to Ser.
5. 3′-intermediate of Thr-56 to Val.
6. 3′-intermediate of Thr-56 to Gly.

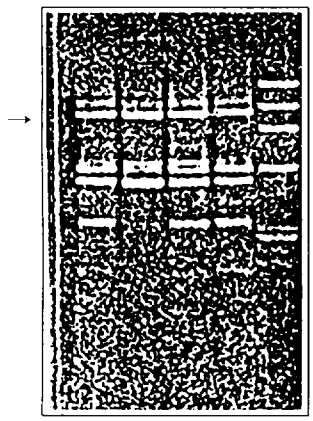
Table 1. Amino acid & Mutagenic primers

	Amino acid	Condon	Oligodeoxynucleotides(5′-3′)
Wild type	Thr 56	ACT	
Mutant	Ser 56+	AGC	TTC GAA AAA GAC TCG AGC CGT ACC CGA TGC TCT
	Ser 56-		TCG GGT ACG GCT CGA GTC TTT TTC GAA GAT GAG
	Gly 56+	GGG	TTC GAA AAA GAC TCG GGG CGT ACC CGA TGC TCT
	Gly 56-		TCG GGT ACG CCC CGA GTC TTT TTC GAA GAT GAG
	Val 56+	GTC	TTC GAA AAA GAC TCG GTC CGT ACC CGA TGC TCT
	Val 56-		TCG GGT ACG GAC CGA GTC TTT TTC GAA GAT GAG

Amino acid and DNA sequences were based on the *argI* gene sequences from Bencini *et al.*, (1983) and Kuo *et al.*(1988).

Figure 1 showed the result of DNA gel electrophoresis after the synthesis of 5′- and 3′-side DNA fragments. The DNA bands in the gel indicates 0.2kb of 5′-side intermediate and 0.8kb of 3′-side intermediate DNAs were synthesized (bands were indicated by the arrow).

Figure 2. Full Length Mutant *argI* Genes Amplified By PCR.



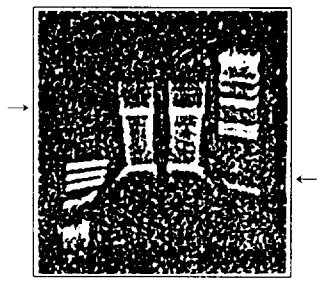
1 2 3 4

1. Mutant *argI* of Thr-56 to Ser.
2. Mutant *argI* of Thr-56 to Val
3. Mutant *argI* of Thr-56 to Gly
4. Marker; same as marker in Fig. 1

Figure 2 showed the result of DNA gel electrophoresis of full length mutant genes. As shown in the gel, 1.0kb of Ser-56, Val-56 and Gly-56 mutant genes(bands were indicated by the arrow) were successfully amplified from corresponding 5′- and 3′-intermediates.

Figure 3 showed the result of DNA gel electrophoresis after the *Eco R* 1/*Hind* III double digestion of cloned mutant *argI* genes. pKK223-3, expression vector has been shown at the 4.5kb position, and mutant *argI* DNA were shown at the 1.0kb bands (bands were indicated by the arrow). Only Ser-56 and Val-56 were cloned yet.

Figure 3. *Hind* Ⅲ/*Eco R*1 Digestion of pKK(mutant *argI*).



1 2

1. Thr-56 to Ser.
2. Thr-56 to Val.

In conclusion, clones which had point mutation at Thr-56 to serine and valine were obtained. For further work, however, another mutant Gly-56 clone has to be obtained. DNA sequencing for confirming correct mutation protein purification, and enzyme kinetics will be performed to understand the structure and function relationships of ornithine transcarbamylase.

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초록

Escherichia coli 오니틴 트랜스카바미라제의 Threonine-56에 돌연변이 도입

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Escherichia coli 오니틴 트랜스카바미라제의 구조와 기능간의 상관관계를 연구하기 위하여 Threonie-56을 point mutation 시켰다. 이 잔기는 인산 결합 단백질에서 공통적으로 발견되는 것으로서, 카바밀인산의 결합자리에 위치한다고 알려져 왔다. Threonie- 56을 전하와 크기의 효과를 알아보기 위하여 serine, valine과 glycine으로 치환시켰다. 돌연변이 시스템으로는 PCR을 이용한 site-directed mutagenesis를 사용하였다. 증폭된 돌연변이 유전자들은 각각 발현벡타인 pKK223-3에 접합시켜서 오니틴 트랜스카바밀라제의 활성이 없는 *Escherichia coli* TB 2세포에 형질전환 시켰다. DNA 아가로스 전기영동의 결과는 각각의 돌연변이 유전자들이 클로닝되었음을 보여주었다. 앞으로는 DNA염기서열 결정, 효소의 추출과 성질분석으로 Threonine-56의 구조와 기능상에서의 역할을 밝히고자 한다.