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Nucleotide Activation of Catabolic Threonine Dehydratase from *Serratia marcescens*

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뉴클레오타이드에 의한 *Serratia marcescens* Catabolic Threonine Dehydratase의 활성화

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Abstract

Serratia marcescens catabolic threonine dehydratase는 streptomycin sulfate treatment, Sephadex G-200 gel filtration, AMP-Sepharose 4B affinity chromatography 등의 방법으로 정제하였는데, 최종 단계에서 회수율은 15.5%이었으며 50배 정제되었다. Native 분자량은 native pore gradient polyacrylamide gel electrophoresis(PAGE) 방법으로는 120,000이었다. SDS-PAGE에 의한 subunit의 분자량은 30,000이었고, 즉 *S. marcescens* 효소는 4개의 동일한 subunit으로 구성된 homo-tetrameric protein임이 판명되었다. *S. marcescens* 효소의 L-threonine에 대한 Km값은 AMP가 있는 조건에서 7.3 mM, AMP가 없는 조건에서 92 mM이었다. *S. marcescens* 효소는 효소 1 mole 당 각각 2 mole의 pyridoxal 5'-phosphate(PLP), 16개의 free -SH group을 가지고 있었다. *S. marcescens* 효소는 AMP의 존재 하에서 α-ketobutyrate, pyruvate, glyoxylate, phosphoenol pyruvate(PEP)에 의해 효소 활성이 억제되었으며, cAMP와 ADP에 의해서는 효소 활성이 증가되었다. 효소학적 성질 면에서 *S. marcescens* 효소는 *E. coli* 효소보다는 *S. typhimurium* 효소와 유사하였다. 한편, *E. coli* 효소는 cAMP에 의하여 효소 활성이 증가되고, *S. typhimurium* 효소는 ADP에 의해 효소 활성이 증가되는 것과 다르게, *S. marcescens* 효소는 cAMP와 ADP 모두 효소 활성이 증가되었다. 따라서 이상의 연구 결과들은 세 enteric bacteria의 catabolic threonine dehydratase가 서로 작은 차이점이 있다는 것을 반영하며, 이러한 사실을 규명하기 위해서는 향후 보다 심층적인 연구를 수행하여야 할 것으로 사료된다.

Key words: *Serratia marcescens*, catabolic threonine dehydratase, cAMP, ADP.

INTRODUCTION

Threonine dehydratase[L-threonine hydro-lyase(deaminating), EC 4.2.1.16.], also known as threonine deaminase, catalyzes the dehydration of L-threonine and yields α-ketobutyrate and ammonia. In contrast to the biosynthetic threonine dehydratase encoded by *ilv*A, catabolic or biodegradative threonine dehydratase encoded by *tdc*B is not sensitive to inhibition by isoleucine and is activated by AMP(Gallagher et al. 2004; Shulman et al. 2008). The *tdc*B gene of *Escherichia coli* has been previously cloned and sequenced previously(Goss et al. 1988; Guillouet et al. 1999). The structure and enzymatic properties of catabolic threonine dehydratase have been studied extensively in *E. coli* and *Salmonella typhimurium*. Several investigators have reported that the native molecular weights of catabolic threonine dehydratases from *E. coli* and *S. typhimurium* are 147,000 and 140,000, respectively, and that the *E. coli* and *S. typhimurium* enzymes are composed of four identical polypeptides with subunit molecular weights of 38,000 and 36,000(Bhadra & Datta 1978). These enzymes are allosterically stimulated by AMP, whereas they

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were inhibited by α-keto acids and certain intermediary metabolites, such as α-ketobutyrate, pyruvate, phosphoenol pyruvate(PEP), and glyoxylate(Simanshu et al. 2006). The *tdc*B expression was subject to cabolite repression to intermediary metabolites(Hobert & Datta 1983). It has also been reported that *E. coli* and *S. typhimurium* enzymes contained 4 moles and 2 moles of pyridoxal phosphate(PLP) per 1 mole of enzyme, respectively, whereas the enzyme from *S. typhimurium*, but not the enzyme from *E. coli*, is stimulated by ADP(Bhadra & Datta 1978). Rat liver threonine dehydratase is inactivated by dialysis, but reactivated by PLP and pyridoxamine phosphate(PMP)(Leoncini et al. 1998).

Although many studies on the catabolic and biosynthetic threonine dehydratses from *E. coli* and *S. typhimurium* have been carried out(Scarselli et al. 2003; Schmitz & Downs 2004), no information is available on the purification, structure, and the enzymatic properties of biosynthetic or catabolic threonine dehydratase from *Serratia* strains(Choi et al. 1996; Choi & Kim 1999; Choi 2000). Accordingly, this report describes the purification and certain properties of the catabolic threonine dehydratase from *Serratia marcescens* ATCC 25419. The properties of catabolic threonine dehydratases from three enteric bacteria are also compared.

MATERIALS AND METHODS

1. Materials

The 5'-AMP Sepharose 4B, Sephadex G-200, Sephadex G-25, streptomycin sulfate, potassium phosphate, AMP, NAD^+, $NADP^+$, dithiothreitol(DTT), phenylmethyl sulfonylfluoride(PMSF), L-threonine, 5,5'-dithiobis (2-nitrobenzoate)(DTNB), phenylhydrazine, pyridoxal phosphate, α-ketobutyrate, α-aminobutyrate, α-hydroxybutyrate, α-ketoglutarate, pyruvate, glyoxylate, phosphoenol pyruvate(PEP), oxalate, ADP, cAMP, ammonium persulfate, acrylamide, N,N'-methylene-bis-acylamide, tetramethylenediamine(TEMED), sodium dodecyl sulfate(SDS), Coomassie brilliant blue R-250 and bovine serum albumin(BSA) were all purchased from Sigma Chemical Co. The brain heart infusion(BHI) was purchased from Difco Laboratories. The molecular weight markers were purchased from Bio-Rad. All other chemicals were reagent grade and commercially available.

2. Bacterial Strain and Culture Conditions

The bacterial strain used in this study, *Serratia marcescens* ATCC 25419, was obtained from Professor Braymer of Louisiana State University. Stock cultures were maintained on BHI agar slants. The cells(5 ℓ of BHI media supplemented with 0.05 g of pyridoxine HCl) were grown anaerobically(no-shaking, standing condition) for 24 hr in a 5 ℓ culture flask at 37℃ in an incubator. The cells were harvested by centrifugation(10,000 × g, 30 min) when the optical density of the culture at 660 ㎚ was 1.00. Thereafter, the cells were stored at −70℃.

3. Preparation of Crude Extracts

Approximately 15 g of wet cells, stored in a freezer, were thawed and resuspended in 2.5 volumes of a 50 mM potassium phosphate buffer(pH 8.0) containing 3 mM AMP, 2 mM DTT, and 1 mM PMSF. The cell suspension was then subjected to ultrasonic treatment(Sonic and Materials, Inc.) for 3 min intervals for a total of 15 min, then centrifuged at 15,000 × g for 30 min. The supernatants were pooled, and the crude extract(976.8 ㎎ protein/37 ㎖) used for purification.

4. Enzyme Purification

Throughout the purification procedure the temperature was maintained between 0 and 4℃. Solid streptomycin sulfate was added slowly, to 37 ㎖ of the crude extract while stirring until a 3%(1.11 g/㎖) saturation was achieved. The pellet was discarded after centrifugation at 15,000 × g for 30 min, and the supernatants were pooled. The supernatant fluid obtained from the 3% streptomycin sulfate treatment was dialyzed twice for 16 hr against 100 volumes of a standard buffer(50 mM potassium phosphate pH 6.8, 1 mM AMP, 1 mM DTT, and 1 mM PMSF). The dialyzed material was applied to a Sephadex G-25 column (3.2 × 18 ㎝), which had been equilibrated with the standard buffer and then eluted with the same buffer at a flow rate of 10 ㎖/hr. The fractions(8 ㎖ each) containing enzyme activity were pooled and lyophilized. The lyophilized sample(721.6 ㎎) obtained from the previous step was dissolved in a minimum volume of the standard buffer and applied to a Sephadex G-200 column(3.4 × 94 ㎝), which was pre-equilibrated with the standard buffer and eluted with the same buffer at a flow rate of 3 ㎖/hr. The fractions(3 ㎖ each) containing enzyme activity were pooled and concentrated by lyophilization. The lyophilized sample was then dissolved in a minimum volume of an AMP-free buffer(50 mM potassium phosphate pH 6.8, 1 mM DTT, and 1 mM PMSF) and dialyzed twice for 16 hr against 100 volumes of the same buffer. The dialyzed sample(109 mg) in the AMP-free buffer was concentrated by ultrafiltration under 3 ㎖ and applied

to a AMP-Sepharose affinity column(1.5 × 6 ㎝) which had been equilibrated with the same buffer. The column was washed with 5 to 10 column volumes of the AMP-free buffer containing 1 mM of both NAD^+ and $NADP^+$ until the eluate had a negligible absorbance at 280 ㎚. The column was then eluted with the 10 mM AMP at a flow rate of 6 ㎖/hr. The fractions(1.2 ㎖ each) containing enzyme activity were pooled and concentrated by ultrafiltration.

5. Enzyme Assay

The activity of the catabolic threonine dehydratase was measured colorimetrically using the modified method of Bhadra and Datta(1978). One ㎖ of the reaction mixture contained 100 mM potassium phosphate(pH 8.0), 50 mM L-threonine, 3 mM AMP, 10 mM L-isoleucine(to inhibit the activity of biosynthetic threonine dehydratase, if present), and the enzyme solution. One unit of enzyme activity represented the formation of 1 ㎛mol of α-ketobutyrate per min. The specific activity was expressed as units per ㎎ of protein. The concentration of α-ketobutyrate was determined based on a molar extinction coefficient of 4,000 $M^{-1} \times cm^{-1}$ at 540 ㎚. The amount of protein was determined using the Lowry method(1951).

6. Molecular Weight Estimation of the Enzyme

The molecular weight of the native enzyme was estimated using a native pore gradient PAGE, as described by Manwell (1977). In this procedure, the electrophoresis was performed using a Phast system on a 5 to 20% acrylamide gradient gel (Pharmacia LKB model 17-0542-01). The standard proteins used in this procedure were thyroglobulin(a, 669,000), ferritin(b, 440,000), catalase(c, 232,000), lactate dehydrogenase(d, 140,000), and BSA (e, 66,000). The proteins were stained with Coomassie brilliant blue R-250. To determine the subunit molecular weight, SDS-PAGE on a 12.5% acrylamide running gel was performed by the Laemmli method(1970). The molecular weight markers were thyroglobulin(a, 97,400), BSA(b, 66,000), ovalbumin(c, 45,000), carbonic anhydrase(d, 31,000), trypsin inhibitor(e, 21,500), and lysozyme(f, 14,400).

7. Sulfhydryl Group and Pyridoxal Phosphate Determination of the Enzyme

The number of free sulfhydryl groups was determined by titration with DTNB, according to the method of Ellman(1959). The enzyme(442 ㎍/100 ㎕, 36.8 ㎛M) was incubated with 10 mM DTNB at 25℃. The absorbance was followed as a function of time at 412 ㎚ and the number of sulfhydryl groups was determined using a molar extinction coefficient of 13,600 $M^{-1} \times cm^{-1}$.

The pyridoxal phosphate(PLP) was determined using the phenylhydrazine reagent, as described by Wada and Snell(1961). 30 ㎕ of conc. H_2SO_4 was added to 200 ㎕ of the enzyme solution(884 ㎍, 36.8 ㎛M). The samples were then centrifuged and after the pellet was discarded, 20 ㎕ of the phenylhydrazine reagent was added to 180 ㎕ of the supernatant. After 10 min at room temperature, the absorbance was determined at 410 ㎚. PLP was used as the standard.

8. Effects of α-Keto Acids and Their Derivatives on the Enzyme Activity

The purified enzyme solution was exhaustively dialyzed at 4℃ against a 100 mM potassium phosphate buffer, pH 8.0, to remove any AMP. The AMP-free enzyme(5 ㎍) was then incubated for 15 min at 37℃ with 25 mM α-ketobutyrate, pyruvate, PEP, glyoxylate, α-ketoglutarate, α-aminobutyrate, α-hydroxybutyrate and oxalate in the presence and absence of 3 mM AMP. The enzyme activities were assayed under standard assay conditions.

9. Effects of ADP and cAMP on the Enzyme Activity

The enzyme from *E. coli* was partially purified by streptomycin sulfate treatment and AMP-Sepharose 4B affinity chromatography as described by Bhadra and Datta(1978). Both AMP-free *S. marcescens* and *E. coli* enzymes(5 ㎍ each), prepared by exhaustive dialysis against 100 mM potassium phosphate buffer, pH 8.0, was incubated for 15 min at 37℃ with different concentrations of ADP and cAMP. The enzyme activities were assayed under standard assay conditions.

RESULTS AND DISCUSSION

1. Purification of *Serratia marcescens* Catabolic Threonine Dehydratase

The crude extract prepared from cells grown in BHI media was treated with 3% streptomycin sulfate and this step resulted in a 1.2 fold purification with 86.2% recovery of the total activity. Gel filtration with Sephadex G-200 of the streptomycin sulfate treated samples showed three distinct protein peaks(Fig. 1A), where the last peak represented fractions containing enzyme activity. The enzyme fractions were pooled and concentrated by

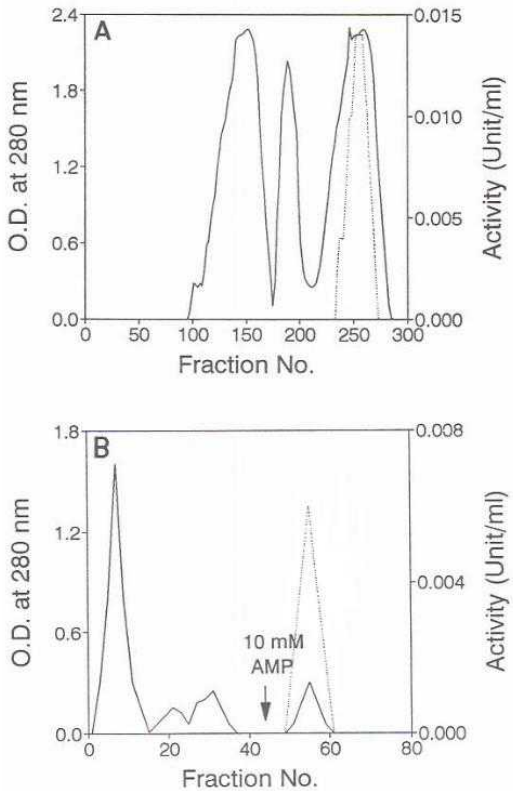

Fig. 1. Sephadex G-200 gel filtration(A) and AMP-Sapharose 4B(B) chromatographic pattern of *Serratia marcescens* catabolic threonine dehydratase. (—): Protein profile, (---): Enzyme activity.

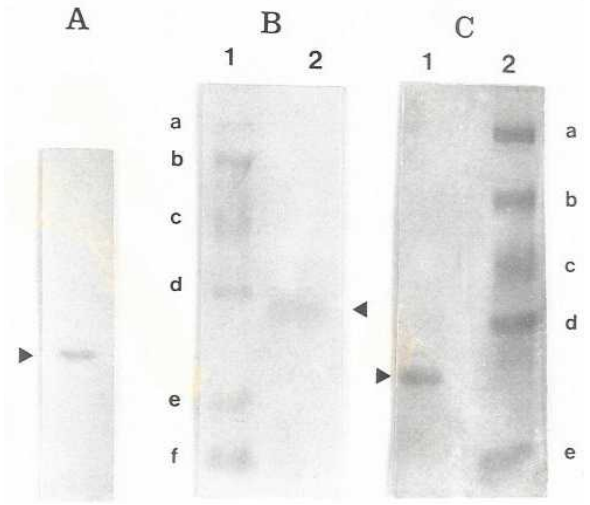

Fig. 2. A. Nondenaturing PAGE of *S. marcescens* catabolic threonine dehydratase(3 μg) on 10% polyacrylamide gel. B. SDS-PAGE of *S. marcescens* catabolic threonine dehydratase on 12.5% polyacrylamide gel. Lane 1: molecular weight markers, Lane 2: purified enzyme(6 μg). **C. Native pore gradient PAGE of *S. marcescens* catabolic threonine dehydratase on 5〜20% polyacrylamide gel.** Lane 1: purified enzyme(6 μg), Lane 2: molecular weight markers. Molecular weight markers are described in Materials and Methods. The position of the catabolic threonine dehydratase is indicated by an arrow.

amicon filtration for further purification. The Shepadex G-200 column step gave a 6.7 fold purification with 73.6% recovery of the total activity. The active fractions obtained from the Sephadex G-200 column step were applied to a AMP-Sepharose affinity column. AMP-Sepharose affinity chromatography revealed only one peak when eluted with 10 mM AMP(Fig. 1B). The affinity column step resulted in a 50 fold overall purification with 15.5% recovery of the original enzyme activity. The purification results are summarized in Table 1. Non-denaturing PAGE of the enzyme revealed a single band and found to be more than 95% pure(Fig. 2A). This purified enzyme solution was then used for all other studies.

2. Molecular Weight

The molecular weight of the native enzyme from *Serratia marcescens*, as estimated by native pore gradient PAGE, was 120,000(Fig. 2B). SDS-PAGE of the enzyme revealed only one protein band(Fig. 2C) compared with the standard curve of molecular weight markers. The enzyme was composed of four identical subunits with subunit molecular weights of 30,000, as estimated by SDS-PAGE, thereby indicating that the enzyme is homo-tetrameric protein. Thus, the native molecular weight and

Table 1. Purification of *Serratia marcescens* catabolic threonine dehydratase

Step	Total volume (mℓ)	Total unit (μmole/min) $\times 10^{-3}$	Total protein (mg)	Specific activity (unit/mg) $\times 10^{-3}$	Purification (-fold)	Recovery (%)
Crude extract	37.0	999	976.8	1.0	1.0	100
3% Streptomycin sulfate	41.0	861	721.6	1.2	1.2	86.2
Sephadex G-200	61.0	735	109.0	6.7	6.7	73.6
AMP-Sepharose 4B	6.2	155	3.1	50.0	50.0	15.5

subunit molecular weight of the catabolic threonine dehydratase from *S. marcescens* would appear to be slightly smaller than the weights of the enzymes isolated from *E. coli* and *S. typhimurium*.

3. Pyridoxal Phosphate Content and Free Sulfhydryl Group Determination

The chemical analysis of the pyridoxal phosphate(PLP) content of the native enzyme from *S. marcescens* revealed 2 moles of PLP per 1 mole of enzyme(Table 2). It has been previously reported that the catabolic enzyme from *E. coli* W has 4 moles of PLP per 1 mole of enzyme(Shizuta et al. 1969), whereas the catabolic and biosynthetic enzyme from *S. typhimurium* has 2 moles of PLP per 1 mole of enzyme(Bhadra & Datta 1978). The purified enzyme solution exhaustively dialyzed against a 100 mM potassium phosphate buffer(pH 8.0) containing 3 mM AMP was titrated with DTNB according to the Ellman procedure (1959). For the native enzyme, 16 moles of free sulfhydryl groups(-SH) per 1 mole of enzyme reacted with DTNB(Table 2). In previous reports, *E. coli* and *S. typhimurium* enzymes have 24 and 16 moles of -SH per 1 mole of enzyme(Bhadra & Datta 1978; Shizuta et al. 1969).

4. Kinetic Constants

The enzyme activity of *S. marcescens* increased with an increase in the concentrations of L-threonine, L-serine, and α-aminobutyrate. The Km values of the enzyme from *S. marcescens* for L-threonine with and without AMP were 7.3 and 92 mM, respectively. The Km values of *E. coli* and *S. typhimurium* enzymes for L-threonine with and without AMP have been reported to be 3 and 70 mM, and 8 and 125 mM, respectively(Bhadra & Datta 1978; Shizuta et al. 1969). The Km values of the enzyme from *S. marcescens* for L-serine and α-aminobutyrate in the presence of AMP were 8.1 and 2.2 mM, respectively. It has been reported that the Km value of the enzyme from *E. coli* for L-serine in the presence of AMP was 4 mM(Hirata et al. 1965). The properties of the catabolic threonine dehydratases from the three enteric bacteria are summarized in Table 2.

5. Effects of α-Keto Acids and Their Derivatives

The enzyme activity of *S. marcescens* was considerably inhibited by most α-keto acids and keto acid derivatives, such as the *E. coli* and *S. typhimurium* enzymes(Table 3). However, α-hydroxybutyrate and oxalate were not inhibitory. Upon incubation of the enzyme(5 μg) at 37℃ for 15 min with 25 mM metabolites, the inhibition values of α-ketobutyrate, pyruvate, glyoxylate, and phosphoenol pyruvate(PEP) in the presence of AMP were 52, 76, 70, and 25%, respectively. Inhibition of the enzyme from *S. marcescens* by α-ketobutyrate, pyruvate, and glyoxylate was also observed in the presence of AMP, as with the *E. coli* K-12 and *S. typhimurium* enzymes(Bhadra & Datta 1978; Park & Datta 1979), yet no inhibition of the enzyme from *E. coli* W by α-ketobutyrate or pyruvate has been observed in the presence

Table 3. Effects of α-keto acids and keto acid derivatives on the activity of *S. marcescens* catabolic threonine dehydratase

Addition(25 mM)	Relative activity(%)[a]	
	− AMP	+ AMP(3 mM)
None	100	100
α-Ketobutyrate	28	48
Pyruvate	14	24
PEP	25	75
Glyoxlate	13	30
α-Ketoglutarate	32	80
α-Aminobutyrate	99	132
α-Hydroxybutyrate	96	108
Oxalate	91	94

[a] The percent enzyme activity is expressed relative to a control sample without the addition of acids or acid derivatives.

Table 2. Comparison of properties of catabolic threonine dehydratases from three enteric bacteria

Properties	*E. coli* W	*S. typhimurium*	*S. marcescens*
Native molecular weight(M_r)	147,000	140,000	120,000
Subunit number(M_r)	4(38,000)	4(36,000)	4(30,000)
PLP/mole protein	4	2	2
Free -SH group/mole protein	24	16	16
Km for L-Thr(without AMP)	70 mM	125 mM	92 mM
Km for L-Thr(with AMP)	3 mM	8 mM	7.3 mM
Stimulation by ADP	No	Yes	Yes

of AMP(Kim & Datta 1982). The inhibition of the enzyme from *S. marcescens* by α-ketoglutarate is interesting because the activities of the enzymes from *E. coli* K-12 and *S. typhimurium* were not inhibited by α-ketoglutarate(Bhadra & Datta 1978; Hobert & Datta 1983).

6. Activation of ADP and cAMP

It has been previously reported that the L-threonine deaminase from *Clostridium tetanomorphum* is activated by ADP(Park & Datta 1979), whereby ADP is bound to stabilize the enzyme activity, by increasing its substrate affinity(Shizuta & Hayaishi 1976). Stimulation of enzyme activity by ADP has also been seen with the enzyme from *S. typhimurium*(Bhadra & Datta. 1978). In this study, ADP and cAMP both increased the activity of the enzyme from *S. marcescens* up to 30 mM, whereas only cAMP increased the activity of the partially purified *E. coli* K-12 enzyme(Fig. 3).

For enzyme properties in comparison with *S. marcescens*, *E. coli*, and *S. typhimurium* enzyme, such as the PLP content, number of free sulfhydryl groups, and existence of ADP binding site, the *S. marcescens* enzyme was more similar to the *S. typhimurium* enzyme than the *E. coli* enzyme. Of the three enteric bacteria, the *E. coli* and *S. typhimurium* enzyme was increased the activity by ADP and cAMP, respectively, but only the *S. marcescens* enzyme was increased the activity by both ADP and cAMP. Therefore, the subtle differences in the properties between enzymes from the three enteric bacteria may represent minor structural differences among these enzymes and warrants further study. Also, the results obtained from this study will help clarify the structure and regulation of the *S. marcescens* catabolic threonine dehydratase, and ultimately, the control of branched chain amino acids in *S. marcescens*.

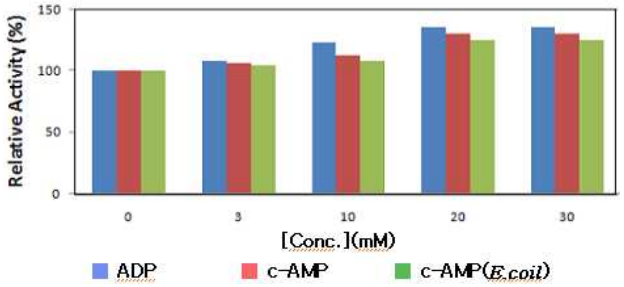

Fig. 3. Activation of *S. marcescens* and *E. coli* catabolic threonine dehydratase by ADP and cAMP. The percent enzyme activity is expressed relative to a control sample without ADP or cAMP.

SUMMARY

The catabolic threonine dehydratase from *Serratia marcescens* ATCC 25419 was purified to homogeniety using Sephadex G-200 gel filtration and AMP-Sepharose 4B affinity chromatography. The molecular weight of the native enzyme was 120,000 by native pore gradient PAGE. The enzyme was composed of four identical subunits with subunit molecular weights of 30,000 by SDS-PAGE. The Km values of the enzyme for L-threonine with and without AMP were 7.3 and 92 mM, respectively. There were 2 moles of pyridoxal phosphate and 16 moles of free -SH groups per 1 mole of enzyme. The enzyme was inhibited by α-keto-butyrate, pyruvate, glyoxylate, and phosphoenol pyruvate(PEP) in the presence of AMP, yet stimulated by cAMP and ADP. For enzyme properties in comparison with *S. marcescens*, *E. coli*, and *S. typhimurium* enzyme, such as the PLP content, number of free sulfhydryl groups, and existence of ADP binding site, the *S. marcescens* enzyme was more similar to the *S. typhimurium* enzyme than the *E. coli* enzyme. Of the three enteric bacteria, the *E. coli* and *S. typhimurium* enzyme was increased the activity by ADP and cAMP, respectively, but only the *S. marcescens* enzyme was increased the activity by both ADP and cAMP. Therefore, the subtle differences in the properties between enzymes from the three enteric bacteria may represent minor structural differences among these enzymes and warrants further study.

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