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Studies on the active site of the *Arabidopsis thaliana* S-Adenosylmethionine Decarboxylase: Lys[81] residue involvement in catalytic activity

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The *Arabidopsis thaliana* S-Adenosylmethionine decarboxylase (AdoMetDC) cDNA (GenBank™ U63633) was cloned, then the AdoMetDC protein was expressed and purified. The purified AdoMetDC was inactivated by salicylaldehyde in a pseudo first- order kinetics. The second-order rate constant for inactivation was 126 $M^{-1}\cdot min^{-1}$ with the slope of n=0.73, suggesting that inactivation is the result of the reaction of one lysine residue in the active site of AdoMetDC. Site-specific mutagenesis was performed on the AdoMetDC to introduce mutations in conserved lysine[81] residues. These were chosen by examination of the conserved sequence and proved to be involved in enzymatic activity by chemical modification. Changing Lys[81] to alanine showed an altered optimal pH. The substrate also provided protection against inactivation by salicylaldehyde. Considering these results, we suggest that the lysine[81] residue may be involved in catalytic activity.

Keywords: *Arabidopsis thaliana*, S-adenosylmethionine decarboxylase, Salicylaldehyde, Site-directed mutagenesis.

Introduction

S-Adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50) is a key enzyme in polyamine biosynthesis. Its product, decarboxylated AdoMet, serves as an aminopropyl donor in the biosynthesis of spermidine and spermine (Tabor and Tabor, 1984). Under physiological conditions, the decarboxylated AdoMet is a limiting factor in polyamine synthesis. Although ubiquitous in eukaryotic cells, AdoMetDC constitutes only a minor fraction of intracellular proteins. This is partly due to its very short half-life, but also to the fact that AdoMetDC expression is regulated at multiple levels, transcriptional, translational, as well as posttranslational (Tabor and Tabor, 1984; Heby and Persson, 1990; Marie *et al.*, 1992; Pegg and McCann, 1992). In plants, the enzyme has been cloned and purified from wheat (Stanley *et al.*, 1989), maize (GenBank™ Y07767), rice (GenBank™ Y07766), potato (Mad Arif *et al.*, 1994), tomato (GenBank™ Y07768), periwinkle (Schroder and Schroder, 1995), cabbage (GenBank™ X95729), carnation (Lee *et al.*, 1997) and *Arabidopsis* (GenBank™ U63633, Park and Cho, 1999), and is known to contain a covalently bound pyruvate prosthetic group. All of the known sequences except that from *Escherichia coli* shows a significant degree of similarity with about 50 fully conserved residues in the core region of 300 amino acids, which include the sequence for proenzyme cleavage and the sequence (KTCTG) containing an essential active site cysteine residue (Stanley and Pegg, 1991; Stanley *et al.*, 1994; Xiong *et al.*, 1997). Studies revealed that Glu[8], Glu[11], and Cys[82] are essential for catalytic activity in human AdoMetDC (Stanley and Pegg, 1991; Stanley *et al.*, 1994; Dresselhaus *et al.*, 1996; Xiong *et al.*, 1997; Xiong *et al.*, 1999). Extensive studies have been done on the enzyme active site, but most of them studied cysteine residues.

Recently, we overexpressed and characterized the *Arabidopsis thaliana* AdoMetDC. We reported that incubation of *Arabidopsis thaliana* AdoMetDC with PLP and salicylaldehyde inactivated the enzyme (Park and Cho, 1999). Detailed studies, however, have not yet been undertaken.

This paper describes the investigation of chemical modification on AdoMetDC from *Arabidopsis thaliana* using salicylaldehyde.

Materials and Methods

Materials Restriction enzymes and T4 DNA ligase, were purchased from Promega. Isopropyl-1-thio-β-D-galactopyranoside and ampicillin were purchased from Sigma (St. Louis, USA). Pfu DNA polymerase and *Arabidopsis thaliana* cDNA library were purchased from Stratagene (La Jolla, USA). DNA sequencing was performed with the Sequenase 2.0 system from United States Biochemical (Cleveland, USA). The pGEX-2T vector, glutathione, glutathione Sepharose 4B, thrombin and S-adenosyl-[carboxy^{14}C]methionine were obtained from Amersham Pharmacia Biotech (Richmond, USA). All of the

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Table 1. Oligonucleotides for site-directed mutagenesis of AdoMetDC.

5`	CGGGATCCTGTTCGCTCACACAACAAGG	Sense
5`	CCCGGGCTAGATTCCCTCGTCCTTCT	Antisense
5`	^{762}CAAAGTCATCATCAAGACTTGCGGTACCAC	K81WT
5`	CAAAGTCATCATC**GC**GACTTGCGGTACCAC	K81A (S)
5`	GTGGTACCGCAAGTC**GC**GATGATGACTTTG	K81A (AS)

Nucleotides that were exchanged in order to obtain the desired mutation are underlined and in bold letter. Numbers in superscript indicate the position of nucleotides in the coding region of the gene. Restriction sites are underlined (Sense; *BamH*I, Antisense; *Sma*I). The mutation at a specific residue number is indicated by one-letter amino acid abbreviation. The first letter is the wild type residue, and the last letter is the amino acid which it is changed by the mutated codon. WT, wild type; S, sense; AS, antisense

oligonucleotides were acquired from Koma Biotech (Seoul, Korea). The remaining reagents were obtained from commercial sources.

Cloning of AdoMetDC cDNA and Site-Directed Mutagenesis The AdoMetDC cDNA was amplified by PCR using *Arabidopsis thaliana* cDNA library. The oligonucleotides contained *BamH*I and *Sma*I sites, respectively, in order to facilitate cloning (Sambrook *et al.*, 1989). The PCR product was digested with *Bam*HI and *Sma*I and ligated to the *BamH*I-*Sma*I backbone fragment of pGEX-2T. It contains T7lac promoter and Glutathione-S-transferase preceding the N-terminus of the recombinant protein. The *E.coli* strain BL 21 (DE3) was transformed with the ligation product. Ampicillin-resistant transformants were selected and plasmid DNA was purified from individual candidates. The identity of the cloned cDNA, and the fidelity of the PCR, was confirmed by DNA sequencing of the plasmid inserts by the dideoxy termination method (Sanger *et al.*,

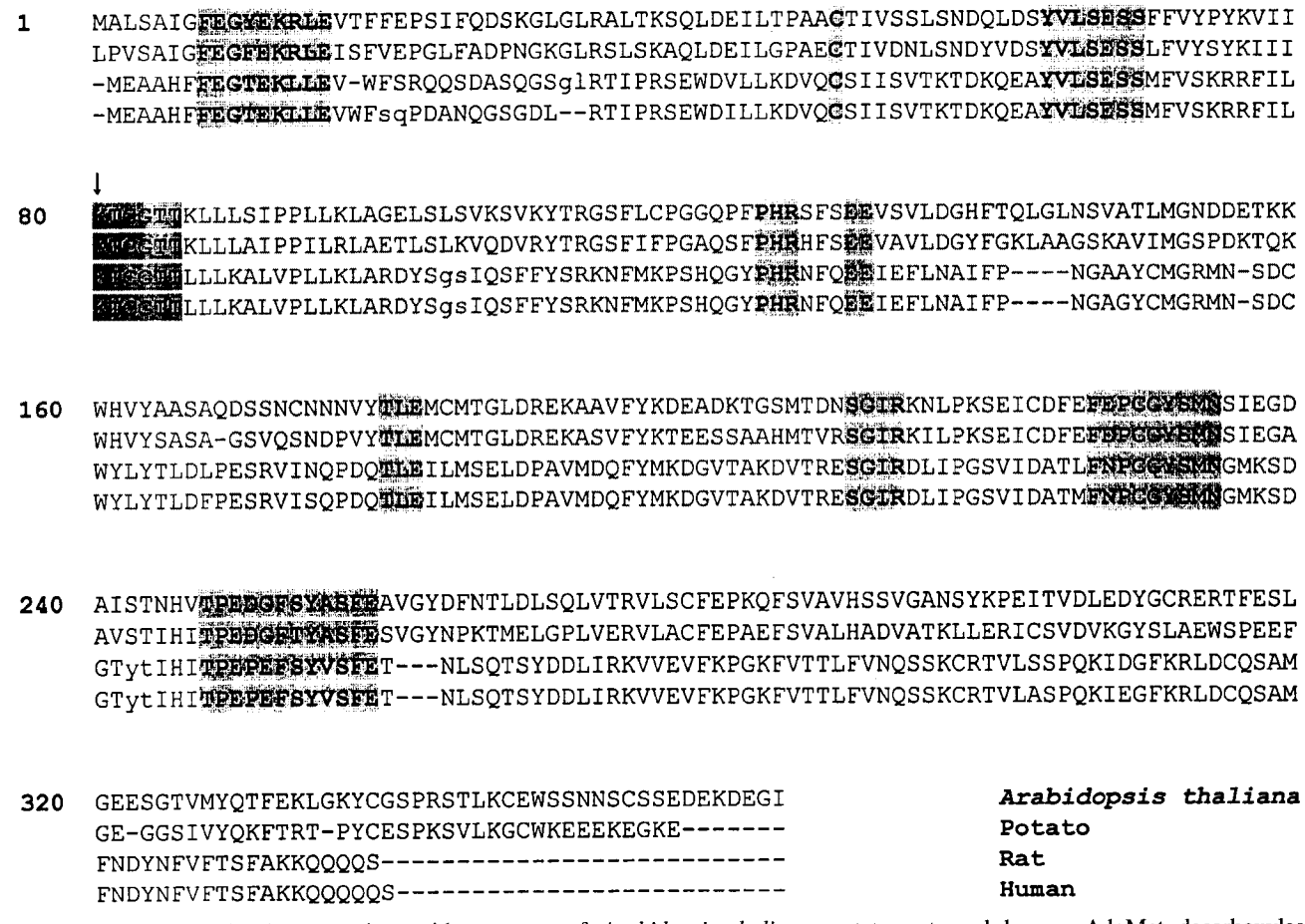

Fig. 1. Comparison of primary amino acid sequence of *Arabidopsis thaliana*, potato, rat, and human AdoMet decarboxylase. Sequences were deduced from the papers describing the *Arabidopsis thaliana* (GenBank™ U63633), potato (Mad Arif *et al.*, 1994), rat (GenBank™ M34464), and human (GenBank™ M21154). Regions of primary sequence similarity were shown in the shaded box. The arrows indicate the amino acid residues in the *Arabidopsis thaliana* sequence, which were mutated in the present experiments, as detailed under Materials and Methods.

1977). Mutagenesis of the AdoMetDC coding sequence was performed by sequential PCR (Dieffenbach and Dveksler, 1995). Two oligonucleotides were designed to replace active site lysine residue potentially involved in AdoMetDC activity, which was conserved in potato, rat and human S-adenosylmethionine decarboxylase (Table 1, Fig. 1). The putative active site lysine[81] was also replaced by alanine. Terminal sense, and antisense primers, was designed to hybridize with the N-terminal region and the C-terminal region, respectively, with the introduction of restriction sites (*BamH*I in primer sense and *Sma*I in primer antisense) to facilitate ligation and cloning. For each mutation a set of internal primers were designed to hybridize with the regions flanking the mutation site. The internal antisense primer has a mutated codon sequence, and more than half of the sequence of this primer is complementary to the internal sense primer. In the first reaction, either terminal sense primer and internal antisense primer, or internal sense primer and terminal antisense primer were used as a primer set. A recombinant pGEX-2T plasmid, containing *Arabidopsis thaliana* AdoMetDC cDNA sequence, was used as a template. In the second round, a terminal sense primer and a terminal antisense primer were used as a primer set, and two PCR products from the first round reactions were used as templates to produce a single annealed PCR product. The final PCR product was digested with BamHI and SmaI and ligated to the pGEX-2T vector linearized with BamHI and SmaI and dephosphorylated by calf intestinal alkaline phosphatase. This ligation mixture was used to transform *E.coli* BL 21 (DE3). Verification of all of the mutants was analyzed by performing DNA sequencing of the plasmid inserts by the dideoxy termination method (Sanger *et al.*, 1977).

Expression and Purification of Wild Type and Mutant AdoMetDC *E.coli* BL 21 (DE3) was transformed with the pGEX2T-AdoMetDC plasmid and transformants were tested for AdoMetDC expression upon induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG). *E.coli* BL 21 (DE3) cells carrying the expression plasmid (containing wild type or mutant AdoMetDC) were grown overnight at 37°C in LB medium (containing 50 μg/ml ampicillin) (Sambrook *et al.*, 1989). The cells were diluted 100-fold in the same medium and allowed to grow until A_{600} reached 0.5. To induce the expression, 1 mM IPTG was added to the culture. The cells were harvested 4 h after induction by centrifugation (5000 x g; 10 min) and sonicated in Phosphate-buffered saline (PBS). The cell lysate was centrifuged at 13,000 x g for 20 min. The resulting supernatant was used for purification on Glutathione-S-transferase Sepharose 4B resin in a batch procedure according to the manufacturers recommendation. The mutated or wild type recombinant AdoMetDC was recovered from fusion protein by thrombin cleavage and then purified according to the manufacturers recommendation.

Chemical Modification of AdoMetDC with Salicylaldehyde The wild type AdoMetDC was incubated with lysyl group modification reagents, salicylaldehyde, under appropriate conditions (Means and Robert, 1971; Lee and Cho, 1993a; Lee and Cho, 1993b; Park and Cho, 1995). Modification reactions were initiated by adding the reagent to the enzyme solution and then incubating for 10 min at 25°C. In order to protect the AdoMetDC from the modification reagent, salicylaldehyde, the enzyme was preincubated at 25°C for 10 min with salicylaldehyde and substrate. Aliquots of the reaction mixture, containing AdoMetDC, were taken at indicated time intervals and assayed. The control enzyme was subjected to the same treatment without the addition of modification reagent.

AdoMetDC Activity Assay AdoMetDC activity was assayed at 37°C for 60 min by the liberation of $^{14}CO_2$ from S-adenosyl-[carboxy^{14}C]methionine as substrate (Yang and Cho, 1991). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmole $^{14}CO_2$ per h. AdoMetDC activity was a linear function of both incubation time and concentration under these conditions.

Results and Discussion

Expression and Purification of Wild Type and Mutant AdoMetDCs The cDNA encoding AdoMetDC was amplified by PCR from the *Arabidopsis thaliana* cDNA library. The 1.1 kb PCR product was cloned into an expression vector-pGEX-2T- for expression in *E.coli* BL 21 (DE3). Mutagenesis of the AdoMetDC coding sequence was performed by sequential PCR. Two oligonucleotides were designed to replace the lysine residues. The possible candidate residues for mutagenesis were chosen by examination of the sequences that were conserved in the four known eukaryotic AdoMetDC (Table 1, Fig. 1). The putative active site lysine[81] was also replaced by alanine. Utilizing the pGEX-2T/ Glutathione-S-transferase (GST) fusion vector, we obtained high levels of expression of AdoMetDC in essentially soluble form. After lysis of the cells, and clarification by centrifugation, the GST-AdoMetDC fusion protein can be purified to near homogeneity by using a GST-Sepharose 4B affinity column. Treatment of the GST-AdoMetDC fusion protein with thrombin, and subsequent GST-sepharose 4B

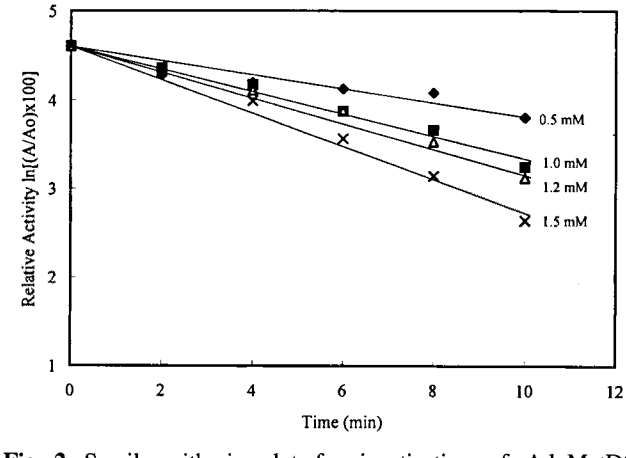

Fig. 2. Semilogarithmic plot for inactivation of AdoMetDC activity by salicylaldehyde. The purified enzyme (1mg) was incubated with various concentrations of salicylaldehyde in phosphate-buffered saline (PBS) at 25°C. (Ao, original enzyme activity; A, enzyme activity at time indicated).

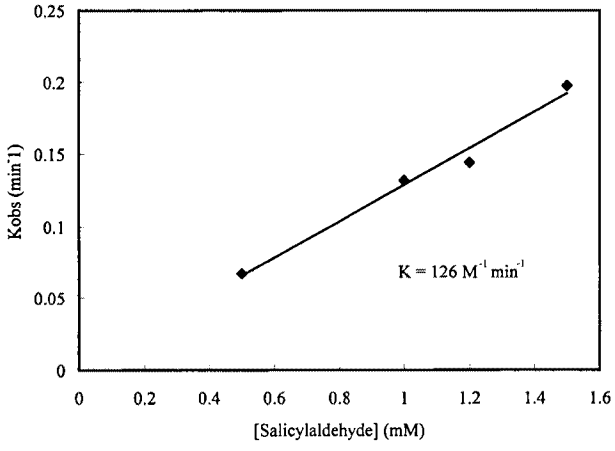

Fig. 3. Plot of apparent first-order rate constant for inactivation (Kobs) obtained at various concentration (mM) of salicylaldehyde against concentration of reagent.

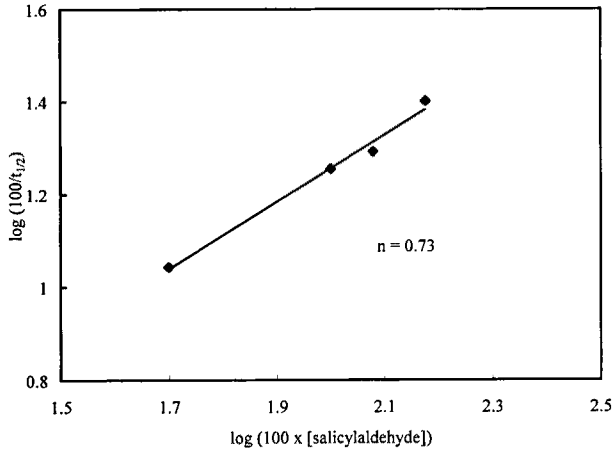

Fig. 4. Double-logarithmic plot of the half times of inactivation against salicylaldehyde concentration.

affinity column, yielded significant quantities of homogeneous AdoMetDC protein (Park and Cho, 1999).

The effect of lysine group modification on *Arabidopsis thaliana* AdoMetDC activity Previous experiments from this laboratory reported that the addition of lysyl group modifying reagents, PLP and salicylaldehyde, to *Arabidopsis thaliana* AdoMetDC causes inactivation of the enzyme (Park and Cho, 1999). For detailed studies on the lysine residue, the enzyme was incubated with salicylaldehyde. Fig. 2 shows the time course for inactivation of AdoMetDC incubated with salicylaldehyde. AdoMetDC results in the rapid loss of activity. Loss of activity followed pseudo first-order kinetics. The second-order rate constant for inactivation was 126 M^{-1} · min^{-1}. This was determined by the rate of inactivation at 0.5, 1.0, 1.2 and 1.5 mM salicylaldehyde (Fig. 3). The reaction order, with respect to salicylaldehyde, was determined from a plot of log ($1/t_{1/2}$) against log (100×[salicylaldehyde]). When the data of Fig. 3 was plotted (as indicated above), the slope

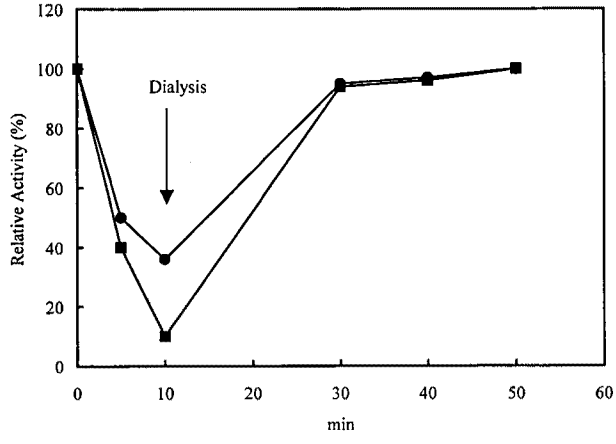

Fig. 5. Reactivation of the salicylaldehyde-inactivated wild type AdoMetDC by dialysis. The purified enzyme was incubated with 10 mM salicylaldehyde in the presence(●) or absence(■) of AdoMet at 25°C for 10 min. By dialyzing the salicylaldehyde-inactivated enzyme, reactivation of the AdoMetDC was performed. At various times, aliquots (1μg) were removed and assayed for AdoMetDC activity at 37°C for 1 h.

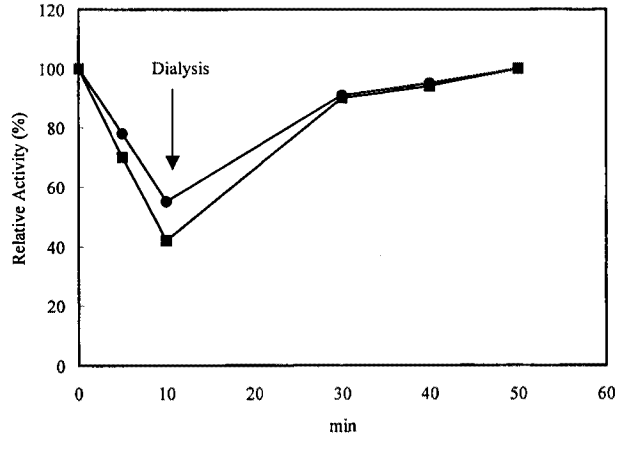

Fig. 6. Reactivation of the salicylaldehyde-inactivated K81A mutant AdoMetDC by dialysis. The purified enzyme was incubated with 10 mM salicylaldehyde in the presence (●) or absence (■) of AdoMet at 25°C for 10 min. By dialyzing the salicylaldehyde-inactivated enzyme, reactivation of the AdoMetDC was performed. At various times, aliquots (1μg) were removed and assayed for AdoMetDC activity at 37°C for 1 h.

was n=0.73 (Fig. 4). This suggests that inactivation is the result of the reaction of one lysine residue in active site of AdoMetDC. Furthermore, according to previous reports, the spectrum of the K81A mutant is similar to that of wild type. This indicates that neither the substitution itself, nor the way the mutant was synthesized in *E. coli,* had imposed alterations of backbone secondary structure elements (Park and Cho, 1999).

The inactivation of wild type, and mutant AdoMetDC by salicylaldehyde, was reversed upon dialysis of modified

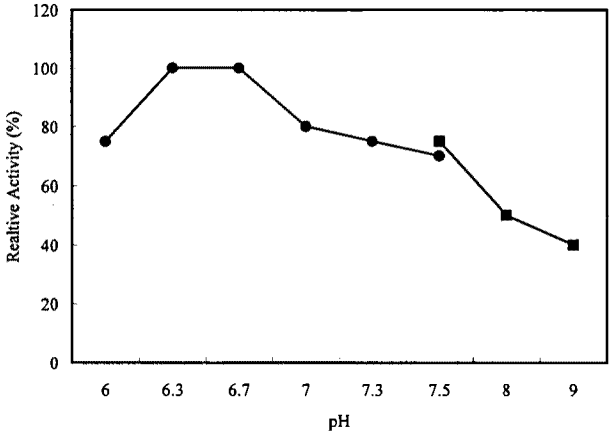

Fig. 7. Optimal pH of wild type AdoMetDC. Wild type AdoMetDC were purified as described under Materials and Methods. Activity was assayed in buffers from pH 6.0 to 9.0, using each buffer system in its effective range (potassium phosphate buffer: 6-7.5 (●), Tris buffer: 7-9 (■)).

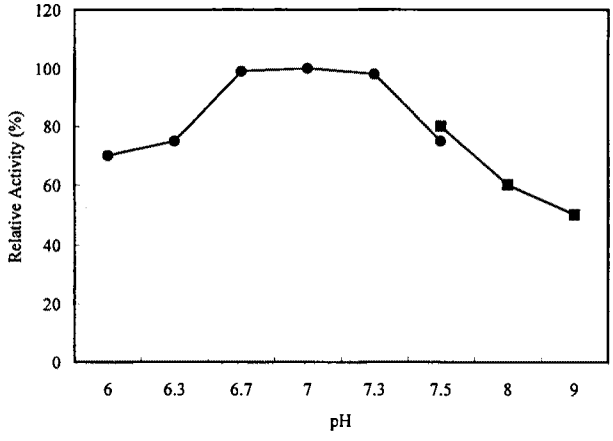

Fig. 8. Optimal pH of K81A mutant AdoMetDC. K81A mutant AdoMetDC were purified as described under Materials and Methods. Activity was assayed in buffers from pH 6.0 to 9.0, using each buffer system in its effective range (potassium phosphate buffer: 6-7.5 (●), Tris buffer: 7-9 (■)).

enzyme (Fig. 5, Fig. 6). Then, the enzyme activity was restored to more than 95% of the control AdoMetDC (without salicylaldehyde). Additionally, the substrate provides protection against inactivation by salicylaldehyde. This result implies that the inactivation of AdoMetDC can be attributed, not only to change in the conformation of the enzyme; but also to modification of the active site amino acid residue. Therefore, this result suggests that lysine residue is localized on the active site, which was further supported by the fact that wild type AdoMetDC was much more inhibited than K81A mutant (Fig. 5, Fig. 6). According to human AdoMetDC crystal structure, Glu^{8}, Glu^{11}, Ser^{68}, Cys^{82}, Ser^{229} and His^{243} residues (facing the pyruvoyl-containing pocket of active site) appear large enough to accommodate the substrate without requiring conformational change to the enzyme structure (Ekstrom *et al.*, 1999). The Glu^{11}, Lys^{80}, Asp^{174}, Glu^{178} and Glu^{256} residues also may be a potential site for interaction with positively charged putrescine molecules (Ekstrom *et al.*, 1999). However, *Arabidopsis thaliana* AdoMetDC was putrescine-insensitive (Park and Cho, 1999).

The effect of Lys81 mutagenesis on optimal pH of AdoMetDC Unexpectedly, K81A mutagenesis of *Arabidopsis thaliana* AdoMetDC increased substrate specificity toward lysine (Park and Cho, 1999). The K81A mutant showed a 6-fold higher substrate specificity with lysine. Wild type, and K81A mutant AdoMetDC, was assayed against lysine over a pH range from 6.0 to 9.0, using two different buffer systems, potassium phosphate and Tris buffer. Both wild type and K81A AdoMetDC showed broad optimum pH. Activity of wild type AdoMetDC toward lysine was almost the same between pH 6.3 and 6.7; whereas, K81A mutant showed broad pH optimum between 6.7 and 7.3 (Fig. 7, Fig. 8). Optimal pH of wild type and K81A mutant AdoMetDC towards AdoMet was 6.8 to 7.2 and 7.2 to 7.8, respectively (Park and Cho, 1999). Therefore, present results support our previous research results that Lys^{81} residue may be involved in the catalytic activity of *Arabidopsis thaliana* AdoMetDC.

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