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Purification and Characterization of the Anabolic Acetolactate Synthase III from *Serratia marcescens* ATCC 25419

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The anabolic acetolactate synthase III was purified to homogeneity from *Serratia marcescens* using DEAE-Sepharose, Phenyl-Sepharose, and hydroxylapatite column chromatography. The native molecular weight of the enzyme was approximately 165 kDa. The enzyme is composed of two large and two small subunits with molecular weights of 64 and 15 kDa, respectively. The N-terminal sequence of the large and small subunit of the enzyme was Ser-Ala-Thr-Pro-Gln-Pro-Ser-Thr-Arg-Phe-Thr-Cys-Ala-Gln-Leu-Ile-Ala-His-Leu and Met-Leu-Gln-Pro-Gln-Asp-Lys-Pro-Gln-Val-Ile-Leu-Glu-Leu-Ala-Val-Arg-Asn-His-Pro-Gly-Val-Met-Ser-His-Val, respectively. The optimum pH and pI value were 7.5 and 5.5, respectively. The IC_{50} values were 20 μM and 14 μM for valine and herbicide SU7, respectively. The substrate specificity ratio, R value, was determined to be approximately 40, which suggests that this enzyme prefers the formation of α-aceto-α-hydroxybutyrate leading to the synthesis of isoleucine.

Keywords: *Serratia marcescens*, Anabolic ALS III, Purification

Introduction

Acetolactate synthase (ALS, E.C. 4.1.3.18), also known as acetohydroxy acid synthase, is the first common enzyme of the biosynthetic pathway of branched chain amino acids (Umbarger, 1978). The enzyme, found in bacteria, yeast, fungi, and plants, catalyzes the condensation of two moles of pyruvate, or one mole of pyruvate and one mole of α-ketobutyrate, into acetolactate and α-aceto-α-hydroxybutyrate, respectively. Anabolic ALS isozymes require flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), and a divalent cation, such as Mg^{2+} or Mn^{2+} (Schloss and Van Dyk, 1988). Enteric bacterial anabolic ALS isozymes are composed of two kinds of subunits: a large subunit with a molecular weight of approximately 60 kDa, and a small subunit with a molecular weight of 10~17 kDa (Eoyang and Silverman, 1984; Lago *et al.*, 1985; Schloss and Van Dyk, 1988). In eukaryotes, the difficulty of purifying the enzyme leaves open the question of whether there is a small subunit. However, recent evidence suggests that this subunit does exist (Cullin *et al.*, 1996; Duggleby, 1997). *Escherichia coli (E. coli)* K-12 has the genetic potential for three ALS isozymes: ALS I, the product of the *ilvBN* operon; ALS II, the product of the *ilvGM* genes; and ALS III, the product of the *ilvIH* operon. While ALS I and III are normally expressed in the wild-type strain, ALS II is cryptic in *E. coli* K-12, but in *Salmonella typhimurium* ALS III is cryptic (DeFelice *et al.*, 1982; Squires, *et al.*, 1983; Eoyang and Silverman, 1984; Lago *et al.*, 1985; Schloss and Van Dyk, 1988). The *ilvBN* and *ilvGM* operons are repressed in cells grown in media containing excess branched chain amino acids, and are derepressed when leucine and valine (for *ilvBN*), or leucine, valine and isoleucine (for *ilvGM*), are in short supply (Nargang *et al.*, 1980; Frieden *et al.*, 1982; Hauser and Hatfield, 1983; Adams *et al.*, 1985). On the other hand, the expression of *ilvIH* genes is negatively affected by leucine only (DeFelice and Levinthal, 1977).

Although intensive studies have been carried out with the anabolic ALS isozymes from enteric bacteria and plants, anabolic ALS has been purified from recombinant strains of *E. coli*, *S. typhimurium*, and *Saccharomyces cerevisiae* (Barak *et al.*, 1988; Poulsen and Stougaard, 1989; Duggleby, 1997). Some anabolic enzymes have been purified from the wild-type strains in limited bacterial sources, such as *Pseudomonas aeruginosa* and *Serratia marcescens* (Arfin and Koziell, 1973; Yang and Kim, 1993). We previously reported the purification and enzymatic characteristics of the valine-sensitive acetolactate synthase from *S. marcescens* that were grown aerobically in a BHI medium (Yang and Kim, 1993). In this report, we describe the purification and enzymatic properties of anabolic ALS III from *S. marcescens* that was grown anaerobically in a minimal medium containing glucose as a carbon source.

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Materials and Methods

Materials DEAE-Sepharose, Phenyl-Sepharose, Superose 6 H/R, and protein molecular weight markers were purchased from the Pharmacia Co. (Uppsala, Sweden). Cocarboxylase (TPP), flavin adenine dinucleotide (FAD), dithiothreitol (DTT), sodium pyruvate, creatine, acrylamide, valine, isoleucine, leucine, N,N'-methylene-bisacrylamide, and Trizma base were purchased from the Sigma Chemical Co. (St. Louis, USA). Hydroxylapatite was obtained from Bio Rad (California, USA), and α-naphthol was purchased from the Kanto Chemical Co. (Tokyo, Japan).

Bacterial strain and culture condition The bacterial strain used in this study was *Serratia marcescens* ATCC 25419. Cells (8 L) were grown anaerobically for 24 h in a minimal medium at 37°C without shaking. The cells were harvested by centrifugation (15,000 × g, 30 min). The minimal medium contained 22 mM KH_2PO_4, 51 mM K_2HPO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$ and 0.5% glucose.

Enzyme assay and protein determination The activity of ALS was assayed using the method of McEwen and Silverman (1980). A one ml reaction mixture, containing 0.1 M potassium phosphate buffer (pH 7.5), 10 μM $MgCl_2$, 20 mM sodium pyruvate, 0.1 mM TPP, 10M FAD, 10% glycerol, and appropriate amounts of the enzyme, was incubated for 30 min at 37°C. The enzyme reaction was stopped by adding 5 μl of 50% (v/v) sulfuric acid following the addition of 200 μl of 0.5% creatine, and 200 μl of 5% α-naphthol in 2.5 N NaOH solution. It was further incubated for 30 min at 37°C, and the optical density measured at 540 nm. One unit represents the formation of 1 mole acetolactate/min, and the specific activity is expressed as U/mg protein at the standard condition. The amount of protein was determined by the Lowry method (Lowry *et al.*, 1951). The IC_{50} value means the concentration of the inhibitor required for 50% inhibition at standard conditions. The data were fit to the equation

$$\% \text{ Activity} = 100/(1+ [I]/IC_{50})$$

where % activity equals the remaining activity in the presence of the inhibitor as percent of the untreated control, and [I] equals the inhibitor concentration. IC_{50} was calculated by the non-linear least-squares and the Simplex method for error minimization (Chang *et al.*, 1997).

Polyacrylamide gel electrophoresis Nondenaturing PAGE of the native enzyme in a 8% acrylamide gel was performed according to the method of Laemmli (1970). The gel system consisted of 8% acrylamide resolving gel (pH 8.8) and 4% stacking gel (pH 6.5). The SDS-PAGE consisted of 12.5% acrylamide resolving gel and 4% stacking gel. The protein was stained with 0.125% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid, and destained with 15% methanol and 15% acetic acid.

Preparation of the crude extracts Approximately 15 g of the wet cells were resuspended in a standard buffer (20 mM potassium phosphate buffer [pH 7.0] containing 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, 10 mM $MgCl_2$, 0.1 mM sodium pyruvate, and 10% glycerol) that was subjected to ultrasonic treatment for a total of 5 min on ice, then centrifuged at 15,000 × g for 60 min. The supernatants were pooled, and this crude extract was used for purification.

Enzyme purification In order to purify the active ALS III, pyruvate was added to the standard buffer at a final concentration of 0.1 mM, unless otherwise noted. The crude extract was applied to a DEAE-Sepharose column (2.5 × 10 cm), which had been equilibrated with the standard buffer containing 100 mM potassium phosphate. The column was washed with the same buffer until the absorbance of flow-through at 280 nm fell to near zero. The bound proteins were eluted with the standard buffer containing 150 mM potassium phosphate. The active fractions were pooled and applied to a Phenyl-Sepharose column (1.0 × 10 cm), which had been equilibrated with the standard buffer containing 100 mM potassium phosphate. The column was then washed with the standard buffer containing 50 mM potassium phosphate, and then eluted with the standard buffer containing 10 mM potassium phosphate. The active fractions were pooled and applied to a hydroxylapatite column (2.5 × 5 cm), which had been equilibrated with the standard buffer containing 10 mM potassium phosphate. The column was washed with the same buffer until the absorbance of the effluent at 280 nm fell to near zero. The bound proteins were eluted with the standard buffer containing 30 mM potassium phosphate. The active fractions were pooled, concentrated with the Amicon Centriprep-30, and stored at -20°C.

Molecular weight determination The molecular weight of the wild-type anabolic ALS III was measured by gel filtration on FPLC-Superose 6 HR following Andrew's method (Andrew, 1965). The Supersoe 6HR column (1.0 × 30 cm) was equilibrated with the standard buffer at a flow rate of 0.5 ml/min. The standard proteins used were apoferritin (443 kDa), α-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). To determine the subunit molecular weight, 12.5% SDS-PAGE was performed with a Pharmacia LMW-SDS marker consisting of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Isoelectrofocusing The isoelectrofocusing of the purified protein was carried out using the Novex Pre-Cast vertical IEF Gel system (pH range of 3.0 to 10.0). Electrophoresis was performed with ampholytes of pH ranges of 3.0 to 10.0.

N-terminal sequence analysis The N-terminal sequence analysis was carried out by automated Edman degradation with a microsequencing sequencer (Precise Protein Sequencing System, Applied Biosystems).

Substrate specificity To analyze the substrate specificity of the purified enzyme, the gas chromatographic method was employed as described by Gollop *et al.* (1987). 2,3-diketone, the oxidative decarboxylation products of corresponding acetohydroxy acid, were analyzed by HP-20 column (25 m × 0.2 mm × 0.2 μm) at 50°C with a flow rate of helium of 1 ml/min. The injector and detector were held at 220 and 250°C, respectively, using the Hewlett Packard 6890 II Gas Chromatograph. The substrate specificity

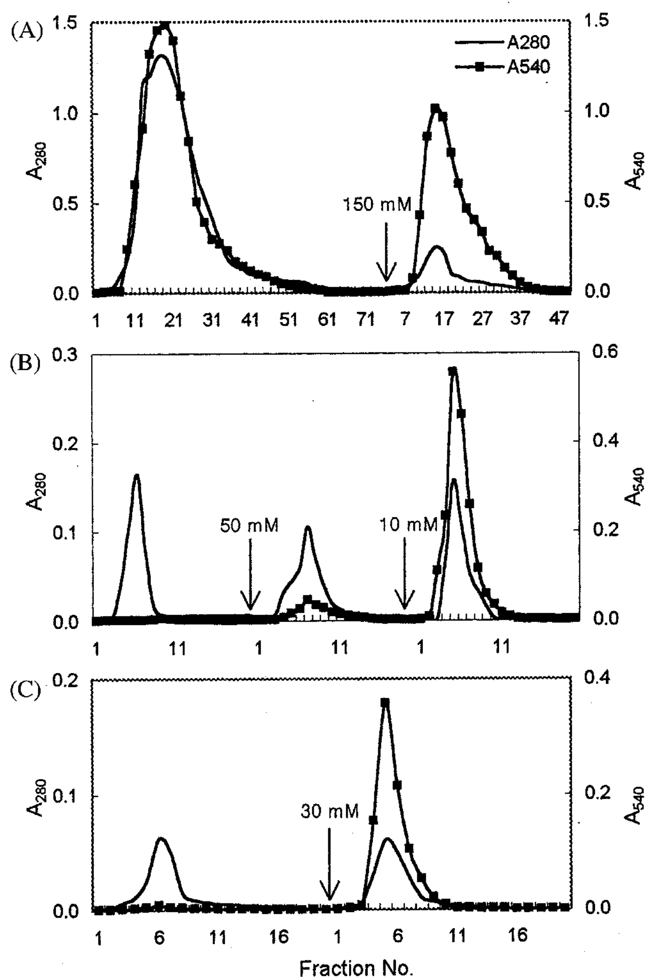

Fig. 1. The elution profile of ALS III. (A) DEAE-Sepharose chromatography; (B) Phenyl-Sepharose chromatography; (C) hydroxylapatite chromatography. Protein; ■, enzyme activity.

ratio, R value, was determined according to the following equation: V [acetohydroxybutyrate]/V[acetolactate] = R · [α-ketobutyrate]/[pyruvate].

Results

Purification of anabolic ALS III Throughout the purification procedure, the temperature was maintained between 0 and 4°C unless noted otherwise. The crude extract was applied to a DEAE-Sepharose column at a flow rate of 50 ml/h. Fig. 1A shows the elution profile of the anabolic ALS III from the DEAE-Sepharose column. The ALS activity in the flow-through fraction was the catabolic ALS, because this ALS activity was resistant to valine and herbicides, and has an optimal pH of 5.5 (Joo and Kim, 1998). The anabolic ALS III activity was eluted with 150 mM potassium phosphate (Fig. 1A). The recovery of the anabolic ALS III in this step was defined as 100%, because most of the catabolic ALS activities were removed. The active fractions were pooled and applied to a Phenyl-Sepharose column at a flow rate of 20 ml/h. The anabolic ALS III was bound to Phenyl-Sepharose at 100 mM potassium phosphate. It was then washed further with the standard buffer that contained 50 mM potassium phosphate. In this procedure, the catabolic ALS was removed, if contaminated, and most of the anabolic ALS III activity was eluted with the standard buffer that contained 100 mM potassium phosphate (Fig. 1B). This step resulted in a 2.4-fold purification with the specific activity of the 2.75 unit/mg protein (Table 1). The active fractions were pooled and applied to a hydroxylapatite column equilibrated with the standard buffer that contained 10 mM potassium phosphate at a flow rate of 20 ml/hr. After washing with the same buffer, the bound enzyme was eluted with the standard buffer that contained 30 mM potassium phosphate (Fig. 1C). This step resulted in a 14.5-fold purification with the specific activity of the 16.36 unit/mg protein (Table 1). The final step resulted in a 1.6% recovery of the total ALS III activity. The low recovery of the anabolic ALS III might be due to the unstability of the enzyme. A Hill slope of 1.5 was obtained, and *Km* and *Vmax* values were determined to be 3.2 mM and 0.54 μmole/min, respectively (Fig. 2).

The physical properties of the anabolic ALS III The homogeneity of the *Serratia* anabolic ALS III was established by 8% nondenaturing PAGE, and demonstrated a single band (Fig. 3A). An analysis of the native molecular weight of the anabolic ALS III by FPLC-Superose 6HR suggested a molecular weight of approximately 165 kDa. An analysis of SDS-PAGE showed the presence of two polypeptides with molecular weights of 64 and 15 kDa (Fig. 3B). These results indicated that the anabolic ALS III was composed of two large and two small subunits. The amino terminal sequence of the purified anabolic ALS III from *S. marcescens* was also determined. From the automated amino acid sequence analysis, the N-terminal sequence of the large and small

Table 1. Purification table of anabolic ALS III.

Step	Total protein (mg)	Total activity (Unit)	specific activity (U/mg)	Yield (%)	Purification fold
Crude	1789.1	826.4	0.46	-	-
DEAE-Sepharose	97.5	109.9	1.13	100.0[1)]	1.0
Phenyl-Sepharose	3.6	19.9	2.75	18.1	2.4
Hydroxylapatite	0.11	1.8	16.36	[1)]1.6	14.5

[1)]The yield of ALS III in the DEAE-Sepharose step was defined as 100%, because most of the catabolic ALS was removed.

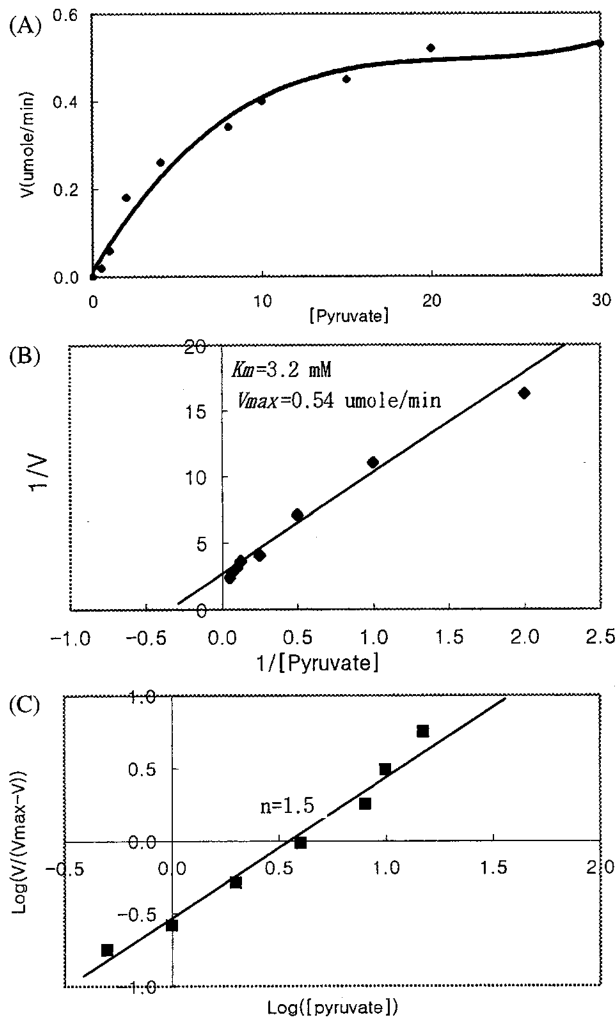

Fig. 2. Substrate saturation curve, modified double reciprocal plot and Hill plot of the purified anabolic ALS III. (A) Substrate saturation curve of velocity versus various pyruvate concentrations in the assay buffer. (B) Modified double reciprocal plot of the velocity versus various pyruvate concentrations. (C) Hill plot of the velocity versus various pyruvate concentrations.

subunits of the purified enzyme were determined as: Ser-Ala-Thr-Pro-Gln-Pro-Ser-Thr-Arg-Phe-Thr-Cys-Ala-Gln-Leu-Ile-Ala-His-Leu and Met-Leu-Gln-Pro-Gln-Asp-Lys-Pro-Gln-Val-Ile-Leu-Glu-Leu-Ala-Val-Arg-Asn-His-Pro-Gly-Val-Met-Ser-His-Val, respectively.

Enzymatic properties of ALS III (1) Optimal pH, thermal stability, and pI value. The effects of pH on the enzyme activity were investigated. The pH optimum of the enzyme was around 7.5, and 50% of the activity decreased at pH below 6.5 and above 8.0. Below pH 4.5, or above pH 9.0, the purified enzyme was almost inactive. The temperature profile of the purified enzyme was studied from 25°C to 80°C. Anabolic ALS III was relatively stable up to 45°C; however, the enzyme activity was rapidly decreased with preincubation above 50°C. The pI value of the enzyme was determined to be approximately 5.5°C.

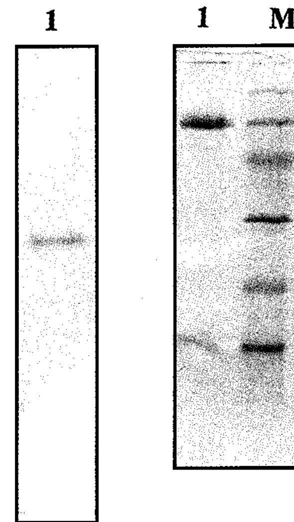

Fig. 3. Nondenaturing PAGE and SDS-PAGE of ALS III that was purified from *S. marcescens*, described in Materials and Methods. Lane M, molecular weight marker; lane 1, crude extract; lane 2, purified ALS III.

Table 2. Effect of the cofactors on ALS III.

Omission from column buffer	Remaining activity (%)
None	100.0
FAD	75.3
TPP	35.6
DTT	58.4
Mg^{2+}	74.9
Glycerol	24.0
All	8.0

(2) Effect of cofactors on enzymatic activity. For *Serratia* anabolic ALS III, the removal of FAD resulted in the loss of approximately 25% of the enzyme activity. This indicates that FAD was not essential for the enzyme activity, as reported previously in *E. coli* ALS III (Barak *et al.*, 1988). The omission of DTT and glycerol from the standard buffer caused the loss of enzymatic activity at approximately 50% and 75%, respectively. This indicates that DTT and glycerol were stabilizers of the enzyme (Table 2).

(3) Inhibition of ALS III with end products. Previous studies of *E. coli* and *S. typhimurium* ALS isozymes demonstrated that ALS I and ALS III are inhibited by valine; whereas, ALS II is resistant to valine (DeFelice *et al.*, 1982; Wek *et al.*, 1985). *Serratia* ALS III can be inhibited by valine, leucine, and isoleucine. However, there is different sensitivity to each amino acid, which was inhibited approximately at 50% at 20 μM valine, 10 mM leucine and 5 mM leucine, respectively (Table 3). The sensitivity to valine of the enzyme was similar to that of *E. coli* ALS III.

(4) Inhibition of ALS III with herbicides. *Serratia* ALS III

Table 3. Differences among *Serratia* ALS I, ALS III and *E. coli* ALS III.

	ALS I[1)]	ALS III	*E. coli* ALS III[2)]
Small subunit	35 kDa	15 kDa	17 kDa
Removal of FAD	70% loss	25% loss	20% loss
optimum pH	7.5	7.5	8.5
R value	near 0	40	40
Expression	BHI	minimal	recombinant
IC_{50} for valine	1 mM	0.02 mM	0.02 mM
IC_{50} for leucine	1 mM	10 mM	40 mM
IC_{50} for isoleucine	1 mM	5 mM	3 mM

[1)]Yang and Kim, 1993; [2)]Barak *et al.*, 1990

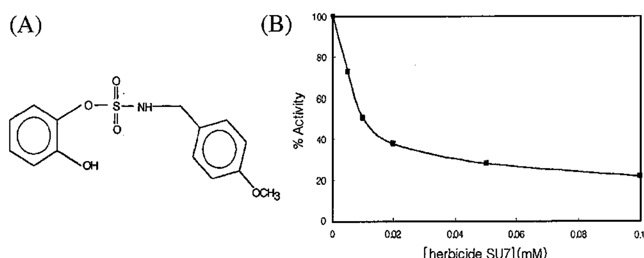

Fig. 4. Inhibition of the purified ALS III by a herbicide SU7. (A) Structure of herbicide SU7. (B) The inhibitor concentrations were varied from 0.005 mM to 0.1 mM, and other assay conditions were as described in Materials and Methods.

was extremely sensitive to the sulfonylurea herbicide SU7 with an IC_{50} value of 14 μM (Fig. 4). However, it was less sensitive to sulfometuron methyl and imazapyr than to herbicide SU7 (data not shown).

(5) Substrate specificity. The substrate specificity of ALS III was examined by determining the amounts of acetolactate and α-aceto-α-hydroxybutyrate that is formed in the presence of pyruvate and α-ketobutyrate. 2,3-diketone, the oxidative decarboxylation products of the corresponding acetohydroxy acid, were analyzed by the gas chromatographic method. In the standard assay condition that contained 20 mM pyruvate and 20 mM α-ketobutyrate, the substrate specificity, R value, was calculated to be approximately 40. This means that *Serratia* ALS III has high specificity to α-ketobutyrate, as reported in *E. coli* ALS II and ALS III (Table 3) (Gollop *et al.*, 1990).

Discussion

ALS has attracted attention, since it is the site of the action of several new classes of herbicides, such as sulfonylurea and imidazolinone (LaRossa and Schloss, 1984). There exists three different ALS isozymes; II III, and I in *E. coli* and *S. typhimurium*. These isozymes are composed of two large 60 kDa and two small 9-17 kDa subunits (Schloss and Van Dyk, 1988). These isozymes differ in their affinity for cofactors, allosteric inhibition by the end-products, pH optima, and substrate specificity (Grimminger and Umbarger, 1979; Gollop, 1983). In *S. marcescens*, we identified the presence of at least two anabolic ALS isozymes. One is ALS III (in this work), and the other is valine-sensitive ALS I (in a previous work) (Yang and Kim, 1993). It was reported that ALS III is very labile, so the characterization of this enzyme has been carried out with partially purified preparations from mutants which synthesize ALS III only, or with purified recombinant ALS III from the strain harboring plasmid carrying entire *ilvIH* operon (Barak *et al.*, 1988). In this work, we purified anabolic ALS III to homogeneity from *S. marcescens*. Omission of pyruvate during the purification procedure failed to purify the active anabolic ALS III. This suggests that pyruvate acts as a stabilizer of the enzyme. The purified anabolic ALS III was composed of two polypeptides with molecular weights of 64 and 15 kDa in $\alpha_2\beta_2$ structure. The enzyme had an R value of 40, and an IC_{50} value of 20 μM for valine. FAD was not essential for the enzyme activity. These enzymatic characters, described previously, were similar to those of *E. coli* anabolic ALS III, rather than the ALS I and ALS II isozyme (Barak *et al.*, 1990).

It was reported that organisms seem to produce at least one ALS isozyme with a high affinity for α-ketobutyrate that is, ALS isozymes with a high R value. In *E. coli*, the R values are 2.0, 65, and 40 for ALS isozyme I, II, and III, respectively (Gollop *et al.*, 1990). Barak *et al.* suggested that ALS II, or III alone, should suffice for the production of the branched chain amino acids in *E. coli* that grow on glucose (Barak *et al.*, 1990). On the other hand, anabolic ALS isozymes with a low R value, such as ALS I, were a special adaptation for poor carbon sources, such as acetate or oleate (Dailey and Cronan, 1986; Dailey et al., 1987). In the present study, catabolic ALS (R = 0) and anabolic ALS III (R = 40) were expressed, but anabolic ALS I and ALS II were undetected in *S. marcescens* that were grown anaerobically in a minimal medium, which contained glucose as a sole carbon source. On the basis of the R value, ALS III prefers the formation of α-aceto-α-hydroxybutyrate; whereas, catabolic ALS catalyzes only the formation of acetolactate. In this regard, catabolic ALS plays a role in supplying acetolactate as an intermediate of valine and leucine (Joo and Kim, 1998; Joo and Kim, 1999). However, ALS III may be involved in producing α-aceto-α-hydroxybutyrate as an intermediate of isoleucine in this growing condition.

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