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Purification and Characterization of the Catabolic α-Acetolactate Synthase from *Serratia marcescens*

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The catabolic α-acetolactate synthase was purified to homogeneity from *Serratia marcescens* ATCC 25419 using ammonium sulfate fractionation, DEAE-Sepharose, Phenyl-Sepharose, and Hydroxylapatite column chromatography. The native molecular weight of the enzyme was approximately 150 kDa and composed of two identical subunits with molecular weights of 64 kDa each. The N-terminal amino acid sequence of the enzyme was determined to be Ala-Gln-Glu-Lys-Thr-Gly-Asn-Asp-Trp-Gln-His-Gly-Ala-Asp-Leu-Val-Val-Lys-Asn-Leu. It was not inhibited by the branched chain amino acids and sulfometuron methyl herbicide. The optimum pH of the enzyme was around pH 5.5 and the pI value was 6.1. The catabolic α-acetolactate synthase showed weak immunological relationships with recombinant tobacco ALS, barley ALS, and the valine-sensitive ALS isozyme from *Serratia marcescens*.

Keywords: Branched chain amino acids, Catabolic α-acetolactate synthase, Inhibition.

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 valine, leucine and isoleucine (Umbarger, 1978). The enzyme has been found in enteric bacteria, yeast, fungi, and in chloroplasts of higher plants and 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.

There are two different enzymes that can catalyze the formation of acetolactate in enteric bacteria (Hapern and Umbarger, 1959). One is the biosynthetic acetolactate synthase (ALS), which catalyses the formation of α-acetolactate and α-acetohydroxybutyrate, the intermediates in the biosynthesis of valine and isoleucine, respectively. The second enzyme is the catabolic α-acetolactate synthase (α-ALS), also known as pH 6 acetolactate-forming enzyme, which catalyses the formation of α-acetolactate from pyruvate.

Bacterial biosynthetic ALS isozymes are composed of two dissimilar polypeptides of approximately 60~62 kDa and 10~17 kDa (Eoyang and Silverman, 1984; Lago *et al.*, 1985; Schloss *et al.*, 1985). The requirement of FAD for full activity has been shown for ALS from both bacteria and plants (Muhitch *et al.*, 1987), an unusual feature because there is no oxidation–reduction involved in this reaction. Futhermore, the enzyme activity is unaffected by reduction of the endogenous FAD, or by its replacement with flavin analogs of higher or lower redox potentials (Schloss *et al.*, 1988). In the plant enzyme, FAD has a structural function and maintains the oligomeric enzyme structure (Singh and Schmitt, 1989; Durner and Böger, 1990).

In some bacteria, pyruvate can be converted into the neutral compound 2,3-butanediol via the production of α-acetolactate (Johnson *et al.*, 1975). Three enzymes are involved in the 2,3-butanediol pathway: catabolic α-acetolactate synthase (α-ALS), α-acetolactate decarboxylase and acetoin reductase. The catabolic α-ALS is involved in the formation of acetolactate from two moles of pyruvate (Störmer, 1975; Blomqbist *et al.*, 1993). The catabolic α-ALS is not a flavoprotein and is activated by acetate (Störmer, 1968a, b; Störmer, 1977). It has been reported that the synthesis of this enzyme is induced by growth of the cells using minimal medium plus acetate in *Bacillus subtilis* and *Aerobacter aerogenes* (Störmer, 1967; Huseby *et al.*, 1971; Holtzclaw and Chapman, 1975). The catabolic α-ALS was isolated from *Aerobacter aerogenes*,

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Bacillus subtilis and *Serratia marcescens* (Störmer, 1967; Störmer, 1970; Holtzclaw and Chapman, 1975). The molecular weight of the catabolic α-ALS is 220 kDa for *Aerobacter aerogenes* as determined by gel filtration and composed of four identical subunits with molecular weights of approximately 58 kDa (Huseby *et al.*, 1971), 224 kDa for *Serratia marcescens* as determined by analytical ultracentrifugation (Störmer, 1970), and 250 kDa for *Bacillus subtilis* as determined by gel filtration (Holtzclaw and Chapman, 1975).

Although intensive studies have been carried out with biosynthetic ALS isozymes from enteric bacteria and plants, there is relatively little information available for the catabolic α-ALS from enteric bacteria. In this report, we describe the purification, some enzymatic properties, and N-terminal sequence and immunological properties of the catabolic α-ALS from *Serratia marcescens*.

Materials and Methods

Materials DEAE-Sepharose, Phenyl-Sepharose, Superose 12 H/R and protein molecular weight marker were purchased from Pharmacia Co. (Uppsala, Sweden); Cocarboxylase (TPP), flavin adenine dinucleotide (FAD), dithiothreitol (DTT), pyruvic acid, creatine, acrylamide, valine, isoleucine, leucine, N,N′-methylene-bisacrylamide, Tween 20, 4-chloro-1-naphthol and Trizma base were purchased from Sigma Chemical Co. (St. Louis, USA); Hydroxylapatite was obtained from Bio Rad (California, USA); Brain heart infusion was purchased from Difco Lab (Detroit, USA); α-naphthol was purchased from Kanto Chemical Co. (Japan, Tokyo); HRP-conjugated Goat anti rabbit IgG was purchased from Gibco BRL Co. (Gaithersburg, USA). All other reagents used were reagent grade. Partially purified barley ALS and recombinant tobacco ALS were obtained from Dr. Jung-Do Choi and Dr. Soo-Ik Chang, respectively (Chungbuk National University, Cheongju, Korea).

Methods

Bacterial strain and culture condition The bacterial strain used in this study was *Serratia marcescens* ATCC 25419. Cells (3 l) were grown aerobically for 24 h in 5 l culture flasks containing brain heart infusion or minimal medium at 37°C with 60 rpm on a reciprocal shaker. The cells were harvested by centrifugation (15000 × *g*, 30 min). BHI medium contained 39 g BHI broth per liter and minimal medium contained 22 mM potassium phosphate monobasic, 51 mM potassium phosphate dibasic, 8 mM ammonium sulfate, 0.4 mM magnesium sulfate and 0.5% glucose.

Preparation of crude extracts About 15 g of wet cells were resuspended in column buffer (20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM TPP, 0.5 mM DTT, 10 uM FAD, 2 mM $MgCl_2$, and 10% glycerol). The cell suspension was then subjected to ultrasonic treatment for 30 s, 15 min intervals on ice, then centrifuged at 15,000 × *g*, for 40 min. The supernatants were pooled, and this crude extract was used for purification.

Enzyme assay and protein determination The activity of catabolic α-ALS was assayed using the method of McEwen and Silverman (1980). From the reaction mixture containing 0.1 M sodium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 20 mM pyruvic acid, 80 μg TPP, 20 μg FAD, 10% glycerol and enzyme, 1 ml was incubated for 30 min at 37°C. The enzyme reaction was stopped by adding 5 μl of 50% (v/v) sulfuric acid. After the addition of 200 ml of 0.5% creatine and 200 ml of 5% α-naphthol in 2.5 N NaOH solution, the reaction mixture was further incubated for 30 min at 37°C. After centrifugation at 15,000 × *g* for 5 min, the absorbance of supernatant of the reaction mixture was measured at 540 nm. One unit of enzymatic activity represents the formation of 1 μmol acetolactate/min, and the specific activity is expressed as U/mg of protein at standard condition. The amount of protein was determined by the Lowry method (1951).

Polyacrylamide gel electrophoresis Nondenaturing polyacrylamide gel electrophoresis of native enzyme in 9% acrylamide gel and the denaturing polyacrylamide gel in 15% was performed according to the method of Laemmli (1970). Protein bands were stained with 0.125% Coomassie brilliant blue R-250 and destained with 10% acetic acid/15% methanol.

Enzyme purification Crude extracts were subjected to ammonium sulfate fractionation to enrich ALS activity and precipitated with 50~80% ammonium sulfate. The resulting precipitate was collected by centrifugation at 15,000 rpm for 20 min, and dissolved in a minimal volume of column buffer (20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, 2 mM $MgCl_2$, and 10% glycerol). The solution was dialyzed twice for 16 h against 100 volumes of the same buffer. The ammonium sulfate fraction was applied to a DEAE-Sepharose, fast flow column (2.5 × 15 cm), which had been equilibrated with the column buffer. The column was then washed with the same buffer until the absorbance of effluent at 280 nm fell to near zero and the bound proteins were eluted with 600 ml linear gradient of the column buffer (0.02~0.2 M) at a flow rate of 50 ml/h. Active fractions were pooled and the enzyme solution was applied to a Phenyl-Sepharose column (1.5 × 8 cm), which had been equilibrated with the 0.8 M potassium phosphate buffer (pH 7.0) containing 0.1 mM TPP, 0.5 mM DTT, 10 μM FAD, 2 mM $MgCl_2$, and 10% glycerol. The column was then washed with the same buffer until the absorbance of effluent at 280 nm fell to near zero. After washing the column, the bound proteins were eluted with column buffer at a flow rate of 10 ml/h. Active fractions were pooled and applied to a hydroxylapatite column (2.5 × 8 cm), which had been equilibrated with the column buffer and then washed with the same buffer until the absorbance of effluent at 280 nm fell to near zero. The bound proteins were eluted with 200 ml linear gradient of the column buffer (0.02~0.4 M) at a flow rate of 20 ml/h. Active fractions were pooled and stored at −20°C.

Molecular weight determination The molecular weight of native catabolic α-ALS was measured by gel filtration on FPLC-Superose 12 H/R preparative chromatography following Andrews' method (1965). The standard proteins used in this procedure 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. 15% SDS-PAGE was performed with standard molecular weight markers and Pharmacia LMW-SDS marker.

Isoelectrofocusing The isoelectrofocusing of the purified protein was carried out using the Novex Pre-Cast vertical IEF Gel system. Electrophoresis was performed with Ampholites of pH range of 3.0–10.0.

***N*-terminal sequencing** N-terminal amino acid analysis of the catabolic α-ALS was performed on a gas phase sequencer (Applied Biosystems Inc.) using automated Edman degradation.

Preparation of antibodies Emulsified enzyme (100 μg) in Freund's complete adjuvant was injected into rabbit intradermally. Two weeks later, emulsified enzyme in Freund's incomplete adjuvant was injected intradermally. Booster injection was given with only enzyme solution at 10-d intervals. After boosting, blood was collected and the antiserum was recovered by centrifugation. Antiserum was precipitated with 40% ammonium sulfate and the resulting precipitates were collected by centrifugation at 15,000 rpm for 20 min. The IgG fraction was finally purified by Protein A-Sepharose affinity chromatography.

Western blot analysis After SDS-PAGE and native PAGE, the proteins were transferred electrically to 0.45 μm nitrocellulose (NC) membranes using a Novex XCell Blot Module at a constant current of 150 mA for 30 min. Following transfer, NC membrane was blocked with 3% BSA for 20 min and washed briefly with PBS containing 0.1% Tween 20 (PBST). The membrane was then incubated for 12~16 h at room temperature with rabbit anti α-ALS (5000 : 1 diluted in PBS containing 0.1% Tween 20) to prevent nonspecific binding and washed with PBST for 15 min, three times. After washing, NC membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1000 : 1 diluted in PBST) for 3 h at room temperature, washed with PBST and developed with 4-chloro-1-naphthol in 100 mM sodium acetate buffer (pH 5.0).

Results and Discussion

Purification of the catabolic α-acetolactate synthase *Ammonium sulfate fractionation:* Solid ammonium sulfate was added to the cell lysate until 50% (0.292 g/ml) saturation was achieved. The pellet was discarded after centrifugation at 15,000 × *g*, for 30 min and the supernatant was pooled. Additional solid ammonium sulfate was added to the supernatant until 80% (0.179 g/ml) saturation was achieved. The resulting precipitate was collected by centrifugation at 15,000 × *g* for 30 min and dissolved in a minimal volume of the column buffer. The solution was twice dialyzed for 12 h against 100 volumes of the same buffer.

DEAE-Sepharose ion exchange chromatography: The 50~80% ammonium sulfate fraction was applied to a DEAE-Sepharose column (2.5 × 18 cm). Figure 1A shows the elution profile of the catabolic α-ALS from the DEAE-Sepharose. The activity was eluted around 80 mM phosphate in 0.02~0.2 M phosphate linear gradient. This step resulted in 7.2-fold purification with 40.3% recovery of total activity. The second peak of ALS activity that eluted at 0.15 M potassium phosphate gradient was ALS II isozyme, which is less valine sensitive than ALS I (Kim and Kim, 1992).

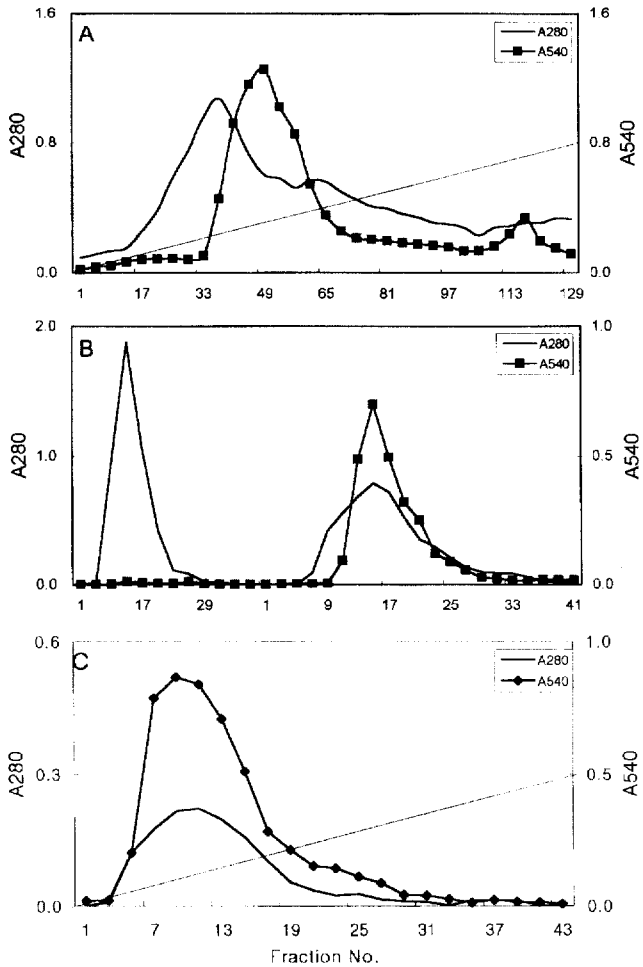

Fig. 1. The elution profile of the catabolic α-ALS. (A) DEAE-Sepharose column chromatography. The slope is 0.02–0.2 M potassium phosphate gradient. The volume of each fraction was 8 ml.; (B) Phenyl-Sepharose column chromatography. The volume of each fraction was 3 ml.; (C) hydroxylapatite column chromatography. The slope is 0.02–0.4 M potassium phosphate gradient. The volume of each fraction was 5 ml. ——, Protein; —■—, enzyme activity

Phenyl-Sepharose chromatography: Chromatography on Phenyl-Sepharose was based on the hydrophobicity of the enzyme. In this step, the catabolic α-ALS was bound to the Phenyl-Sepharose column (1.0 × 10 cm) at 0.8 M potassium phosphate, and eluted with the column buffer. Figure 1B shows the elution profile of the Phenyl-Sepharose chromatography. This step resulted in approximately twofold purification and the recovery was 27.2%.

Table 1. Summary of the purification procedures of the catabolic α-ALS from *Serratia marcescens* ATCC 25419.

	protein (mg)	activity (U)	specific activity (U/mg)	Yield (%)	purification factor
Crude	1375.0	118.8	0.09	100.0	1.0
Ammonium sulfate	707.2	77.0	0.11	64.9	1.3
DEAE-Sepharose	75.4	47.8	0.63	40.3	7.3
Phenyl-Sepharose	33.0	32.3	0.98	27.2	11.3
Hydroxylapatite	3.6	22.5	6.25	18.9	72.4

Hydroxylapatite chromatography: The third column chromatography used was the hydroxylapatite column (2.5 × 10 cm), which had been pre-equilibrated with the column buffer. Figure 1C shows that the bound enzyme was eluted around 0.2 M phosphate gradient in 0.02~0.4 M phosphate linear gradient. The final hydroxylapatite chromatography step resulted in approximately 72-fold purification with 18.9% recovery of the total activity (Table 1).

Criteria for purity and molecular size The catabolic α-ALS purified from *Serratia marcescens* ATCC 25419 was homogeneous on native and SDS-PAGE (Figs. 2 and 3). Analysis of the native molecular weight of the purified catabolic α-ALS by FPLC-Superose 12 H/R suggested the molecular weight of approximately 150 kDa. SDS-PAGE analysis indicated that the enzyme was composed of only a single polypeptide of 64 kDa (Fig. 3). Taken together, these data suggest that the catabolic α-ALS purified from *Serratia marcescens* is composed of two identical subunits unlike the previous report (Störmer, 1970). The subunit composition of the catabolic α-ALS from *Serratia marcescens* was somewhat different from the catabolic α-ALS purified from *Aerobacter aerogenes* and the anabolic ALS isozymes of enteric bacteria. That is to say, the former was a tetramer consisting of identical subunits, the latter was a tetramer consisting of two large subunits and two small subunits (Eoyang and Silverman, 1984; Lago *et al.*, 1985). The enzymatic properties of the catabolic α-ALS such as activation by acetate, nonflavoprotein nature and lack of small subunit were similar between *Serratia marcescens* and *Bacillus subtilis* except subunit composition and the pH optimum.

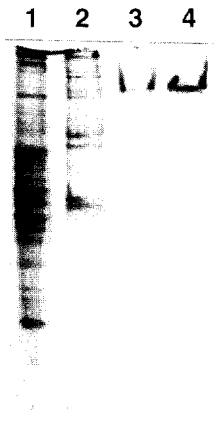

Fig. 2. Native PAGE of the catabolic α-ALS during purification step. Lane 1, 50–80% ammonium sulfate fraction; Lane 2, DEAE eluent; Lane 3, Phenyl-Sepharose eluent; Lane 4: hydroxylapatite eluent.

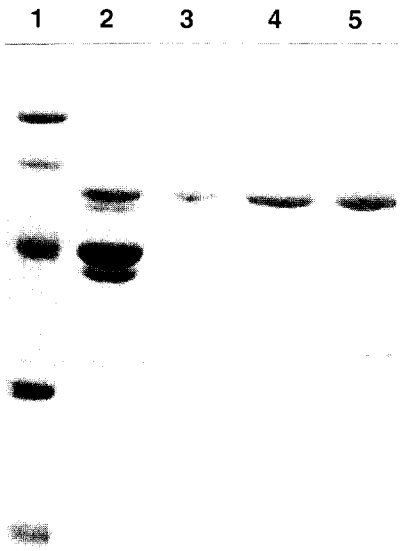

Fig. 3. SDS-PAGE of the catabolic α-ALS during purification step. Lane 1, marker (97, 66, 43, 30, 20.1, 14.4 kDa); Lane 2, DEAE eluent; Lane 3, Phenyl-Sepharose eluent; Lane 4, 5 hydroxylapatite eluent.

Biosynthesis of the catabolic α-acetolactate synthase *Serratia marcescens* was grown in BHI or minimal medium at 37°C for overnight with 60 rpm on a reciprocal shaker. Both total activity and the specific activity of the catabolic α-ALS grown in minimal media were higher than those grown in BHI media (Table 2). In addition, the expressed catabolic α-ALS in minimal media was much higher than anabolic ALS isozymes. When *Serratia marcescens* was grown in minimal media, there was very little or no valine-sensitive ALS I isozyme (data not shown). And, the activity of ALS II isozyme which is less valine-sensitive than ALS I (Kim and Kim, 1992) was

Table 2. Expression level of the catabolic α-ALS in BHI and minimal medium.

Medium	total U	total protein (mg)	specific activity (U/mg)
BHI	332.5	1045.5	0.32
Minimal	412.0	834.5	0.49

below 5% of the total ALS activity (Fig. 1A, peak 2). In this respect, the α-acetolatate produced by the catabolic α-ALS could be used in the biosynthesis of valine and leucine because of the low level of the anabolic ALS isozymes in minimal media. It was reported that the catabolic α-ALS might play a role in preventing intracellular acidification by changing the metabolism from acid production to the formation of neutral compounds such as 2,3-butanediol (Störmer, 1968a; Broth, 1985). Thus, it is more likely that the catabolic α-ALS plays a role in not only preventing intracellular acidification but also supplying α-acetolactate as a intermediate of branched chain amino acids biosynthesis.

N-terminal sequencing of the catabolic α-acetolactate synthase From the N-terminal amino acid sequence analysis, the catabolic α-ALS was shown to have a single N-terminal sequence of Ala-Gln-Glu-Lys-Thr-Gly-Asn-Asp-Trp-Gln-His-Gly-Ala-Asp-Leu-Val-Val-Lys-Asn-Leu (Table 3). Sequence homologies of the catabolic α-ALS with *Serratia marcescens* ALS I and *Enterobacter aerogenes* catabolic α-ALS approximately were observed to be 33% and 50%, respectively, in the N-terminus region (Blomqbist, 1993; Yang and Kim, 1993).

Characteristics of the catabolic a-acetolactate synthase The optimum pH of the catabolic α-ALS was detemined to be around 5.5 and is the same as the pH optimum of the catabolic α-ALS from *Aerobacter aerogenes* (Muhitch *et al.*, 1987). However, the pH optimum of *Bacillus subtilis* catabolic α-ALS was reported to be 7.0 (Holtzclaw and Chapman, 1975). The pI value of the *Serratia* catabolic α-ALS was 6.1. The heat stability of the enzyme was determined by preincubating the enzyme solution at 37, 45, 50, 55, 60, 70, and 80°C for various time intervals and measuring the remaining activity. The enzyme was stable at 60°C for 30 min preincubation period (data not shown). The effect of increasing pyruvate concentration on the enzyme activity revealed that the rate of acetoin synthesis follows a typical Michaelis–Menten substrate saturation pattern. The enzyme was not inhibited by the end-products, such as valine, leucine and isoleucine. The Hill slope of 1.0 suggests that the catabolic α-ALS is not an allosteric enzyme. The double reciprocal plot of 1/V versus 1/[S] yielded K_m and V_{max} values of 22.59 mM and 4.19 umole/min, respectively (Table 3). FAD and Mg^{2+} ion were reported to be essential for the enzymatic activity of *E.coli* ALS I, ALS II and *Serratia marcescens* ALS I (Grimminger and Umbarger, 1979; Barak *et al.*, 1988; Yang and Kim, 1993). As shown in Table 4, the purified catabolic α-ALS does not require FAD for its activity and the ultraviolet absorption spectrum analysis of the native enzyme (0.8 mg per ml in 50 mM potassium phosphate buffer, pH 7.0) did not show visible absorption spectrum characteristrics of bound FAD (data not shown).

Table 4. Effect of cofactors on the catabolic α-ALS activity.

Omission from column buffer	Relative activity (%)
None	100.0
FAD	122.3
TPP	68.8
DTT	84.2
Mg^{2+}	74.6
All	26.0

Table 3. Comparision of the characteristics between ALS I and the catabolic α-ALS from *Serratia marcescens*.

	ALS I[a]	catabolic α-ALS
molecular weight	207 kDa	150 kDa
subunit composition	$\alpha_2\beta_2$	α_2
pI	5.2	6.05
optimal pH	7.5	5.5
K_m	14.4 mM	22.6 mM
V_{max}	0.0024 umole/min	4.19 umole/min
Hill coefficient	2.0	1.0
N-terminal sequence	ANNGTAGQWARQLDL[b]	AQEKTGNDWQHGADLVVK
50% Inhibition by valine	0.1 mM	no
50% Inhibition by leucine	1.0 mM	no
50% Inhibition by isoleucine	1.0 mM	no

[a] Yang and Kim (1993).
[b] Bold character is the sequence homology between ALS I and catabolic α-ALS.

In addition, the addition of FAD to the enzyme assay mixture did not stimulate the formation of acetolactate. These data indicate that the purified catabolic α-ALS from *Serratia marcescens* is not a flavoprotein.

Immunological relationship between catabolic α-ALS and anabolic ALS isozymes from various sources Figure 4 shows immunological relationships between catabolic α-ALS and anabolic ALS isozymes from other sources. The antibody against the catabolic α-ALS recognized approximately 64 kDa band corresponding to the large subunit of the catabolic α-ALS in SDS-PAGE (Fig. 4, lane 2). The antibody also reacted weakly with the recombinant tobacco ALS (lane 4), barley ALS (lane 5), and ALS II isozyme from *Serratia marcescens* (lane 3). From these results, we conclude that the catabolic ALS and anabolic ALS isozymes share common epitopes, probably in the active site region because they catalyze the same reaction.

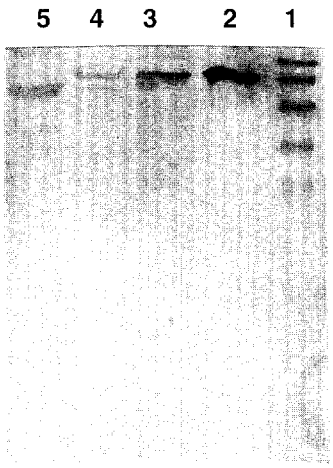

Fig. 4. Immunological relationship between catabolic α-ALS and anabolic ALS from other sources. Lane 1, marker; Lane 2, catabolic α-ALS; Lane 3, anabolic ALS isozyme from *Serratia marcescens*; lane 4, recombinant tobacco ALS; lane 5, barley ALS.

Insensitivity of feedback regulation of the catabolic α-acetolactate synthase The striking differences between the valine-sensitive ALS I and the catabolic α-ALS from *Serratia marcescens* are the sensitivities to the branched chain amino acids and sulfometuron methyl (Table 5). For the valine-sensitive ALS I, 0.1 mM valine inhibited the enzyme activity approximately 50% (Yang and Kim, 1993). In the cases of leucine and isoleucine, 50% of the enzyme activity was lost at 1 mM of each amino acid (Yang and Kim, 1993). It was reported that ALS I was inhibited by sulfometuron methyl herbicide (Yang and Kim, 1997). However, the catabolic α-ALS was not inhibited by branched chain amino acids and sulfometuron methyl (Table 5). Most enteric bacterial ALS isozymes are composed of two different subunits having molecular weights of approximately 60–62 kDa and 10–17 kDa, respectively (Eoyang and Silverman, 1984; Lago *et al.*, 1985; Schloss, 1985). And, it is generally accepted that the 60 kDa large subunit is the catalytic subunit, while the small subunits are involved in feedback regulation of the enzymes by branched chain amino acids (Eoyang and Silverman, 1986; Lu and Umbarger, 1987). This assumption is supported by the truncation of the small subunit gene which results in feedback resistance (Squires *et al.*, 1981). We showed in the present study that the catabolic α-ALS from *Serratia marcescens* is composed of only large subunits and is resistant to the branched chain amino acids (Table 5). Thus, the resistance of feedback regulation against the branched amino acids might be due to the absence of small subunits in the catabolic α-ALS.

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Table 5. Effect of branched chain amino acids and sulfometuron methyl on the activity of ALS I and catabolic α-acetolactate synthase.

Component	Concentration (mM)	Relative activity (%)	
		ALS I	catabolic α-ALS
None	–	100	100.0
Valine	0.1	50	97.9
	1.0	ND	94.6
Leucine	0.4	75	98.5
	1.0	42	94.2
Isoleucine	0.4	70	97.5
	1.0	52	95.2
sulfometuron methyl	0.5	80	101.5
	1.0	50	98.3

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