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Separation and Characterization of Two Forms of Acetolactate Synthase from Etiolated Pea Seedlings

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Acetolactate synthase (ALS) catalyzes the first reaction common to the biosynthesis of L-valine, L-leucine, and L-isoleucine. ALS is the target site of several classes of herbicides, including the sulfonylureas, the imidazolinones, and the triazolopyrimidines. Two forms of ALS (ALS I and ALS II) which have different affinity for Heparin have been separated from etiolated pea seedlings. The substrate saturation curves of both ALS I and ALS II were hyperbolic in contrast to previous reports. The two forms of ALS showed significant differences in their physical and kinetic properties. The values of K_m for ALS I and ALS II were 9.0 mM and 4.8 mM, respectively. The pI values for ALS I and ALS II were determined to be 5.3 and 5.75 by isoelectric focusing, respectively. The native molecular weights of ALS I and ALS II obtained by nondenaturing gel electrophoresis and activity staining were 124 and 244 kDa, respectively. They also exhibited different sensitivity to feedback inhibition by end-product amino acids and inhibition by Cadre, an imidazolinone herbicide.

Keywords: Acetolactate synthase, Herbicide, Pea.

Introduction

Acetolactate synthase (ALS, EC 4.1.3.18: also referred to as acetohydroxy acid synthase) is the common enzyme involved in the biosynthesis of valine, leucine, and isoleucine in bacteria, yeast, and higher plants. ALS can either catalyze the condensation of 2-acetolactate from two molecules of pyruvate in the first step of the valine and leucine synthetic pathway, or the formation of 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate as the second step of isoleucine biosynthesis. The enzyme is a key controlling point for the levels of branched-chain amino acids in both prokaryotes and eukaryotes. The control involves feedback inhibition of enzyme activity by end-product amino acids or repression of the synthesis of the enzyme or both (Miflin and Cave, 1971; Umbarger, 1987; Lee *et al.*, 1991). ALS has raised strong interest since several structurally unrelated classes of herbicides, the sulfonylureas (LaRossa and Schloss, 1984; Ray, 1984), the imidazolinones (Shaner *et al.*, 1984), the triazolopyrimidines (Kleschick, 1984; Lee *et al.*, 1988), the pyrimidyloxy-benzoates (Babczinski and Zelinski, 1991; Choi *et al.*, 1993), and the 4,6-dimethoxypyrimidines (Shim *et al.*, 1995) have shown to specifically inhibit the enzyme.

In bacteria, three ALS isozymes differing in substrate preference and feedback regulation have been identified and purified (Grimminger and Umbarger, 1979; Schloss *et al.*, 1985). Each of the isozymes is composed of two catalytic subunits of about 60 kDa and two smaller, regulatory subunits (9 to 17 kDa) (Grimminger and Umbarger, 1979; Eoyang and Silverman, 1984; Schloss *et al.*, 1985). The low abundance and labile nature of plant ALS have severely hampered the purification and biochemical characterization of this enzyme. Although recently ALS was purified to homogeneity from barley, only limited studies of its characterization were carried out due to very low yield of the purification (Chong *et al.*, 1997). There has been speculation on the presence of different isozymes in plants based on differential sensitivity of the enzyme to inhibition by the branched-chain amino acids at different pH values (Miflin, 1971; Davis *et al.*, 1977). Singh *et al.* (1988) reported that ALS from Black Mexican Sweet Corn cell cultures yielded two active peaks with different enzymatic properties by Mono-Q chromatography. Recently, Bekkaoui *et al.* (1991) reported the separation of two forms of ALS from *Brassica napus*, which had equal native molecular weight, but exhibited different properties with respect to subunit

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structure and sensitivity to feedback inhibition by branched-chain amino acids.

In this report, we present evidence for two different forms of ALS from etiolated pea seedlings, based on differences in the Heparin chromatographic separation and physical and kinetic properties of ALS.

Materials and Methods

Materials Pea seeds were sown in vermiculate. The shoots were grown in a dark room at 25°C for 7 days and then harvested. DEAE-Sephacel, hydroxylapatite, and Heparin were purchased from Bio-Rad Laboratories (Hercules, USA). Sodium pyruvate, thiamine pyrophosphate (TPP), Sephadex G-25, polyethylene glycol 8000 (PEG 8000), flavin adenine dinucleotide (FAD), and α-naphthol were obtained from Sigma Chemical Co. (St. Louis, USA). A series of isoelectric focusing agents were from NOVEX (St. Roselle, USA). Cadre, an imidazolinone herbicide, was given by Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Taejon, Korea). All other chemicals were of the highest commercial grade available.

Partial purification of acetolactate synthase from pea All procedures were performed at 4°C, with light exposure minimized during homogenization, chromatography, and other experiments. Usually, 100 g of etiolated pea seedling shoots were homogenized in a blender for 240 s (8 × 30 s) in 800 ml of standard buffer (100 mM phosphate, pH 7.5, 15% (v/v) glycerol, 1 mM EDTA, 1 mM DTT) containing 50 μM FAD, 5 mM $MgCl_2$, and 1 mM pyruvate. The homogenate was centrifuged at 14,000 × g for 20 min. The supernatant was treated to 9% saturation of PEG 8000 and the precipitate was removed by centrifugation at 15,000 × g for 30 min. The supernatant was additionally treated to 14.5% saturation of PEG 8000, and centrifuged for 30 min at 14,500 × g. The precipitate was resuspended with a minimum volume of standard buffer, and the solution was loaded onto a DEAE-Sephacel ion-exchange column (2.5 × 20 cm). The proteins unbound to the column were washed with standard buffer, and then ALS was eluted by a linear gradient of 0 to 350 mM NaCl. Fractions containing ALS activity were pooled and applied onto a hydroxylapatite column (2.5 × 15 cm). The column was washed with standard buffer until the absorbance of eluent at 280 nm was near 0. The proteins bound to the hydroxylapatite were eluted by a phosphate gradient of 25 to 300 mM. Active fractions were pooled and concentrated by ultrafiltration using Amicon-Cell with PM-30 membrane. The concentrated sample was applied onto a Sephadex G-200 gel filtration column (1.6 × 51 cm) equilibrated with Tris buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 15% (v/v) glycerol). Active fractions were pooled and concentrated by ultrafiltration as described previously. The sample was applied to a Heparin-affinity column (2.5 × 10 cm) equilibrated with Tris buffer. Elution of Heparin-bound proteins was carried out with Tris buffer containing 500 mM NaCl. The fractions containing ALS activity were then stored in liquid nitrogen.

Enzyme assay Enzyme activity was measured according to the method of Westerfeld (1945), as modified by Ray (1984). The assay mixture contained 20 mM potassium phosphate (pH 7.0), 0.5 mM TPP, 10 μM FAD, 20 mM pyruvate, 20 mM $MgCl_2$, plus the enzyme in a final volume of 200 μl. After incubation at 37°C for 60 min, the enzyme reaction was stopped by the addition of 20 μl of 6 N H_2SO_4. Acetolactate, the product of the enzyme reaction, was allowed to decarboxylate at 60°C for 15 min. The acetoin formed by acidification was incubated with 200 μl of 0.5% creatine, and 200 μl of 5% α-naphthol at 60°C for 15 min. The absorbance of the reaction mixture was then measured at 525 nm. The concentration of protein was determined by the method of Bradford (1976).

Gel electrophoresis SDS-PAGE was performed on 11% acrylamide gel containing 0.1% SDS. Nondenaturing gel electrophoresis was carried out according to the method of Laemmli (1970) without SDS in 7.5% acylamide separating gel and 4% stacking gel. Native isoelectric focusing (native IEF) was carried out in a 5% acrylamide gel in which the pH gradient was 3 to 10. The gel contained 2% ampholytes without denaturing agents, and focusing conditions were adapted from Robertson *et al.* (1987).

Activity staining The method of activity staining was adapted from Grimminger and Umbarger (1979). After nondenaturing gel electrophoresis or native IEF, gels were sliced into 1.5 mm pieces and incubated in the assay solution for 1 to 3 h at 37°C. Gels were then stained with creatine and α-naphthol as described in the enzyme assay.

Results

Separation of two forms of acetolactate synthase Acetolactate synthase was partially purified from etiolated pea seedlings by a procedure using polyethylene glycol 2000 fractionation, DEAE-Sephacel, hydroxylapatite, and Sephadex G-200 filtration chromatography. Approximately 340-fold purification was achieved, and the overall yield was 7% of the initial activity in the crude extract. After hydroxylapatite chromatography, the partially purified sample yielded two active peaks on gel filtration chromatography (Fig. 1A). However, the two peaks of ALS activity did not show significant differences in enzymatic properties, in sensitivity to feedback inhibition by branched-chain amino acids, or inhibition by a herbicide, Cadre (data not shown).

Heparin-affinity chromatography after gel filtration resolved partially purified ALS into two peaks of activity (Fig. 1B). Both peaks of ALS activity from the gel filtration showed the same elution pattern on Heparin chromatography. The minor peak (designated ALS I) did not bind to Heparin. On the other hand, the major peak (designated ALS II) was eluted with 0.5 M NaCl. The two peaks were significantly different with respect to enzymatic properties and sensitivity to feedback inhibition by leucine and valine and inhibition by Cadre.

Characterization of ALS I and ALS II The physical and kinetic properties of ALS I and ALS II are summarized in Table 1. The substrate saturation curves for both ALS I and ALS II showed a hyperbolic pattern in contrast to a

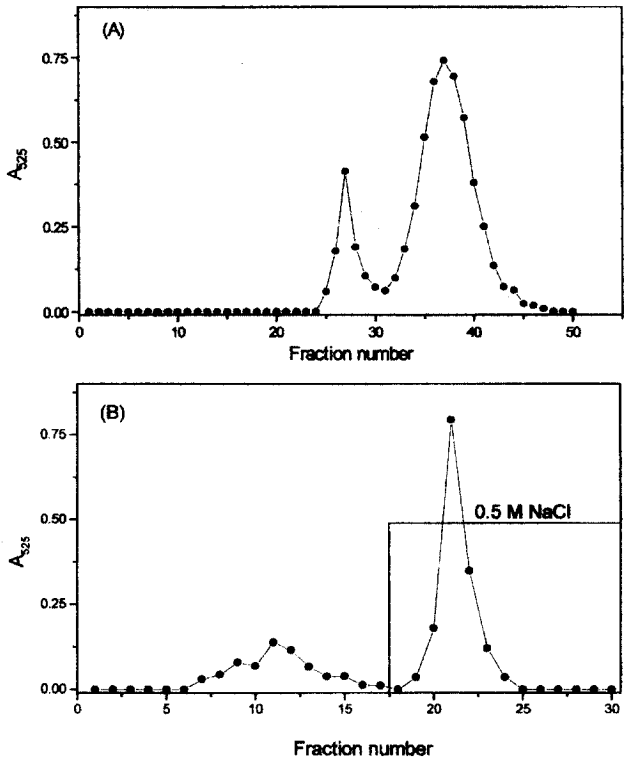

Fig. 1.A. Chromatography of acetolactate synthase on a Sephadex G-200 gel filtration column. **B.** Chromatography of acetolactate synthase on a Heparin affinity column.

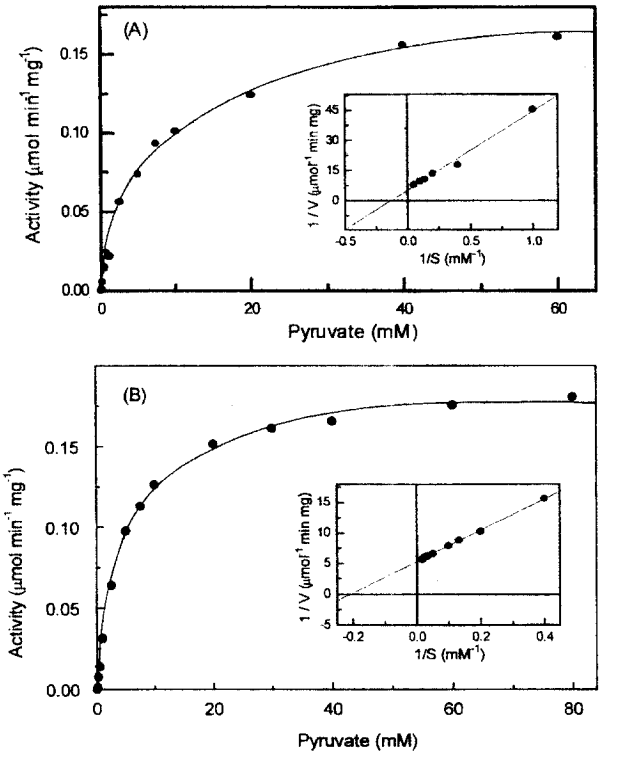

Fig. 2.A. Substrate saturation curve for pyruvate with ALS I. **B.** Substrate saturation curve for pyruvate with ALS II. The insert is a Lineweaver-Burk plot of each saturation curve.

previous report (Lee *et al.*, 1991) (Fig. 2). Lineweaver-Burk plots of the data for ALS I and ALS II showed no significant deviation from straight lines and gave the K_m values of 9.0 mM and 4.8 mM, respectively (Figs. 2A and 2B, Insert, Table 1). The native molecular weights of ALS I and ALS II were estimated to be 124 kDa and 244 kDa, respectively, by nondenaturing gel electrophoresis after activity staining (Fig. 3). ALS I and ALS II in their native forms appeared to be a dimer and tetramer, respectively, based on the ALS subunit molecular weight of about 60 kDa in *arabidopsis* as reported by Singh *et al.* (1991). Both ALS I and ALS II were assayed over a pH range from 5.0 to 9.0 using three different buffer systems, citrate, phosphate, and Tris buffer. There was a broad pH optimum for ALS I between pH 6.5–7.0, while ALS II had a distinct pH optimum at pH 6.5 (Figs. 4A and 4B). The pI values of ALS I and ALS II were determined to be 5.30 and 5.75, respectively, by isoelectric focusing after activity staining (Fig. 5). This indicates that ALS I is somewhat more acidic than ALS II.

To determine the effect of branched-chain amino acids on ALS activity, the two forms of ALS were assayed in the presence of various concentrations of leucine and valine (Fig. 6A). The results showed that there was little inhibition of ALS I by these amino acids. On the other hand, ALS II was considerably inhibited by each of these

Table 1. Summary of the physical and kinetic properties of ALS I and ALS II.

	Molecular weight (kDa)	K_m for pyruvate (mM)	pH optima	pI	Inhibition (%)		
					8 mM Leucine	8 mM Valine	40 μM Cadre
ALS I	124	9.0	6.5 – 7.0	5.30	9	3	34
ALS II	244	4.8	6.5	5.75	37	20	50

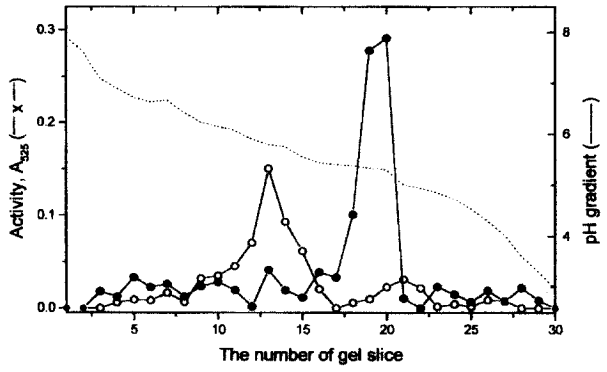

Fig. 3. Activity staining profiles of ALS I (○) and ALS II (●) after IEF-gel electrophoresis. The dot line indicates the pH gradient on the gel.

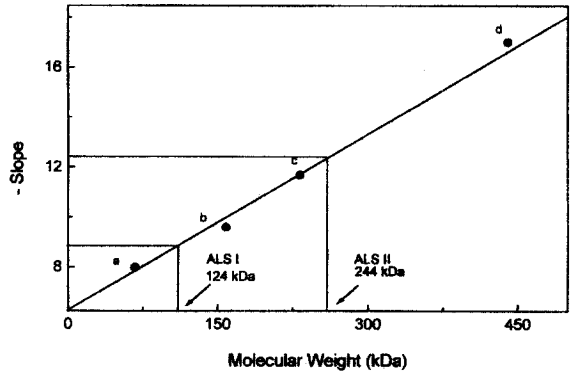

Fig. 4. Molecular weight determination of the active forms of ALS I and ALS II by nondenaturing gel electrophoresis and activity staining. Graph of -slope of 100 × log (R_f × 100) versus gel concentration (%) as a function of molecular weight. The slopes were obtained for the four proteins, which were a, BSA; b, lactate dehydrogenase; c, catalase; d, ferritin.

amino acids. Leucine was more effective in the inhibition of both forms of ALS than valine. Both forms of ALS were almost insensitive to feedback inhibition by isoleucine at millimolar levels (data not shown). Inhibition of the two forms of ALS by Cadre was also examined. ALS II was significantly more sensitive to the herbicide than ALS I (Fig. 6B, Table 1).

Discussion

In bacteria, three ALS isozymes have been purified and characterized with respect to substrate specificity, subunit organization, and feedback regulation (Grimminger and Umbarger, 1979; Schloss *et al.*, 1985). Each of the isozymes exists as a tetramer composed of two catalytic subunits of about 60 kDa and two smaller, regulatory subunits (Grimminger and Umbarger, 1979; Eoyang and Silverman, 1984; Schloss *et al.*, 1985). The quaternary

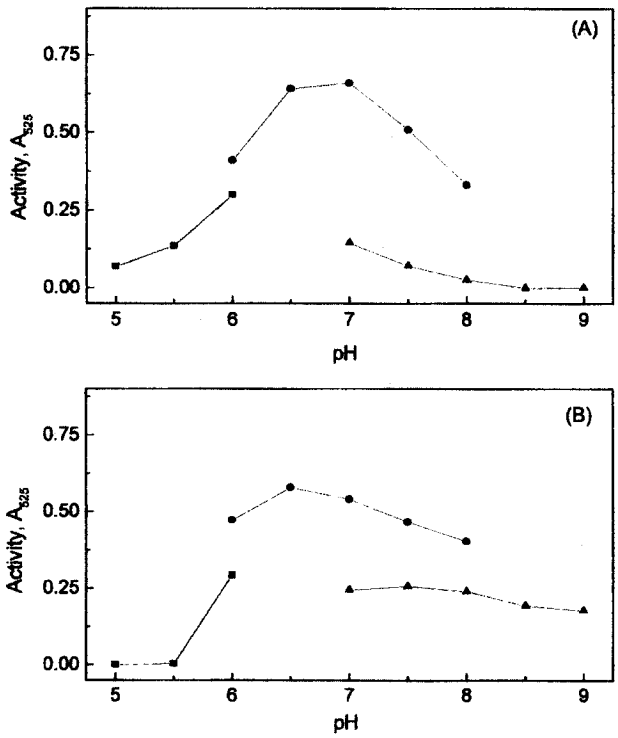

Fig. 5. Optimum pH of ALS I (**A**) and ALS II (**B**) (■, citrate; ●, phosphate; ▲, Tris)

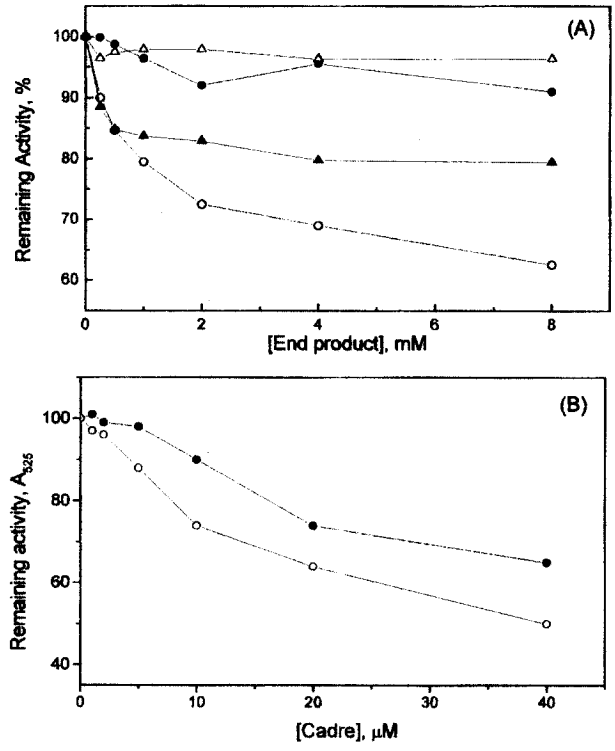

Fig. 6. A. Inhibition of ALS I and ALS II by leucine (●, ALS I; ○, ALS II) and valine (▲, ALS I; △, ALS II). **B.** Inhibition of ALS I (●) and ALS II (○) by Cadre.

structure of plant ALS is far less clear than that of the bacterial enzymes. Plant enzymes have frequently been assumed to be composed of a single kind of polypeptide (Simth *et al.*, 1989; Durner and Böger, 1990), but the evidence for this is not clear. One piece of evidence for this assumption is the apparent absence of small subunits in enzymes from plant sources. Although the presence of isozymes of ALS in plants had long been speculated, evidences for this had never been reported. In this report, we present biochemical evidence for the existence of two forms of ALS in etiolated pea shoots. The two forms of ALS were well-separated by Heparin-affinity chromatography (Fig. 1B). Re-chromatography of the two separated peaks back on a Heparin column gave a single peak of ALS activity, suggesting that the resolution of two peaks is not an artifact of chromatography. The two forms of ALS showed significant differences in their molecular weights, K_m, pI, pH optima, and inhibition by Cadre and branched-chain amino acids (Table 1). Based on the ALS subunit molecular weight reported by Singh *et al.* (1991), ALS I unbound to Heparin is a dimer (MW = 124 kDa) and ALS II bound to Heparin is a tetramer (MW = 244 kDa). ALS II was more sensitive to feedback inhibition by leucine and valine and more strongly inhibited by Cadre than ALS I. At this stage, we do not have sufficient data to define the origin of the two enzyme forms, but they are probably two distinct isozymes. On the other hand, a number of other possibilities also exist. It is possible that the two forms of ALS are different oligomeric forms of the same enzyme. However, this possibility could be excluded in the present case since the two different oligomeric forms of ALS, the dimer and the tetramer, resolved on gel filtration column were not significantly different in their biochemical properties (data not shown). Another possibility is that the one form represents the native enzyme form and the other form may be a degraded form. To solve these problems, purification and amino acid analysis of these polypeptides are required. Singh *et al.* (1988) reported firstly the isolation of two forms of ALS from a plant source, black mexican sweet corn cells. More recently, Bekkaoui *et al.* (1991) reported the separation of two forms of ALS from *Brassica napus*, which had similar molecular weights, but different biochemical properties.

Many organisms regulate the flow of carbon in branched biosynthetic pathways by having multiple isozymes of a regulatory enzyme. Typically, each isozyme is differently feedback-regulated by the end products of different pathways. However, the two forms of ALS in this case do not fit this model, since both ALS I and ALS II are regulated by end-products of only one pathway, valine and leucine. More experiments are needed to elucidate the nature and role of these forms of ALS in plants.

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