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Chemical Modification and Feedback Inhibition of *Arabidopsis thaliana* Acetolactate Synthase

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Abstract : Acetolactate synthase (ALS) was partially purified from *Escherichia coli* MF2000/pTATX containing *Arabidopsis thaliana* ALS gene. The partially purified ALS was examined for its sensitivity toward various modifying reagents such as iodoacetic acid, iodoacetamide, N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), p-chloromercuribenzoic acid (PCMB), and phenylglyoxal. It was found that PCMB inhibited the enzyme activity most strongly followed by DTNB and NEM. Since iodoacetic acid did not compete with substrate pyruvate, it appeared that cysteine is not involved in the substrate binding site. On the other hand, the substrate protected the enzyme partly from inactivation by phenylglyoxal, which might indicate interaction of arginine residue with the substrate. The partially purified enzyme was inhibited by end products, valine and isoleucine, but not by leucine. However, the ALS modified with PCMB led to potentiate the feedback inhibition of all end products. Additionally, derivatives of pyrimidyl sulfur benzoate, a candidate for a new herbicide for ALS, were examined for their inhibitory effects.(Received March 18, 1997; accepted June 19, 1997)

Introduction

Acetolactate synthase (ALS) condenses an acetaldehyde moiety derived from pyruvate either with another molecule of pyruvate to form 2-acetolactate or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate. These compound lead to the branched amino acids, valine, leucine, and isoleucine.[1] ALS is highly conserved protein with substantial sequence similarities among the enzymes of bacteria, yeast, and higher plants.[2] Animals do not have the branched-chain amino acid biosynthesis pathways, and therefore, must ingest these amino acids in the diet. In *Escherichia coli* and *Salmonella typhimurium*, three ALS isozymes have been characterized.[3] Further, ALS have been cloned and sequenced from yeast *Saccharomyces cerevisiae*[4,5] and from the higher plants *Arabidopsis thaliana* and *Nicotiana tabacum*.[2] The nature of ALS subunit from plant is unclear. In addition, regulation of the biosynthesis of valine, leucine, and isoleucine in plants is still not fully understood.

ALS is the target of six classes of structurally unrelated herbicides, sulfonylureas,[6,7] imidazolinones,[8] triazolopyrimidines,[9] N-phthalyl valine anilide, sulfonyl carboxamide, pyrimidyl oxy benzoate. ALS enzymes from a wide range of organisms are sensitive to these compounds. Mutants that are resistant to the herbicides have been described in bacteria,[7,10] yeast,[5,10] and higher plants,[6,11] and resistance has been shown to be the consequence of an altered ALS enzyme.[12,13]

Analysis of mutant with herbicide-resistant ALS has provided valuable information about the action of these herbicides. Schloss *et al.*[14] proposed that sulfonylureas, imidazolinones, and triazolopyrimidines compete for a common binding site on the ALS II from *Salmonella typhimurium* based on herbicide enzyme binding studies. Overlap among the binding sites of different herbicides, substrate, and branched chain amino acids can also be inferred from studies with herbicide resistant mutant.[15-18] Altered feedback sensitivity and kinetic constants of ALS from some herbicide resistant mutants compared with the wild type have been found.[16-21] Studies on herbicide-resistant ALS mutants were also carried out in *Lolium rigidum*,[22-24] *Chlorella emersonii*,[25] *Xanthium strumarium*.[26,27] These observation led to a hypothesis that the herbicide binding on the ALS molecules could directly or indirectly affect the sites involved in substrate and/or branched chain amino acids binding. No previous work has been done in identifying amino acid residues of catalytic or regulatory sites on ALS.

In order to identify specific amino acids at the catalytic or regulatory sites of the enzyme which are in-

Key words : acetolactate synthase, *Arabidopsis thaliana*, feedback inhibition, chemical modification, herbicide
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volved in catalysis or binding, ALS enzyme cloned from *Arabidopsis thaliana* into *E. coli* was partially purified and chemical modification of ALS by various chemical reagents was undertaken. Additionally, effect of pyrimidyl sulfur benzoate derivatives on catalytic activity was examined as a potential candidate of herbicide for ALS.

Materials and Methods

Materials

DEAE-cellulose, flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), 3-β-indoleacrylic acid (IAA), ampicillin, sodium pyruvate, iodoacetic acid, iodoacetamide, N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and amino acids were obtained from Sigma Chemical Co. Tryptone, yeast extract were obtained from Difco Laboratories. *p*-Chloromercuribenzoic acid (PCMB) and phenylglyoxal were obtained from Tokyo Chemical Industry Co.,Ltd. All other chemicals were reagent grade commercially available.

Bacteria and bacterial growth condition

E. coli strain MF2000 containing the *ilv*B800 plasmid pTATX was originally donated by Dr. B. J. Mazur of E. I. du Pont de Nemours & Co. Bacteria were grown in LB medium base, and ampicillin (100 μg/m*l*) was added to medium used for propagating bacteria containing pTATX derived from pBR322.[28] The inducing agent, 3-β-indoleacrylic acid (IAA), (100 μg/m*l*) was added at optical density (600 nm) of 0.22. The cells were harvested by centrifugation at optical density of 1.64 and stored at -70℃.

Acetolactate synthase assay

The ALS activity was measured by estimation of the product, acetolactate, after conversion by decarboxylation in the presence of acid to acetoin. Standard reaction mixtures contained the enzyme in 50 mM potassium phosphate buffer (pH 7.0) containing 200 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM thiamine pyrophosphate (TPP), and 10 mM flavin adenine dinucleotide (FAD). The total volume of reaction mixture was 1 mL. This mixture was incubated at 37℃ for 20 min after which time the reaction was stopped with addition of H_2SO_4 to make a final concentration of 0.85% H_2SO_4 in a microcentrifuge tube. The reaction product was decarboxylated at 60 oC for 20 min. The acetoin formed was determined by incubation with creatine (0.17 %) and 1-naphthol (1.7% in 4 N NaOH) by the method of Westerfeld.[29] Maximum color was observed by incubation at 60℃ for 15 min and then incubated further at room temperature for 15 min. The absorption of colored complex was measured at 525 nm. One unit is defined as 1 μmol acetoin derived from acetolactate produced per minute in the enzyme reaction. All experiments had taken an average of duplication and average values usually were within ±5% error range, otherwise we had repeated it again.

Partial purification of acetolactate synthase

All operation were carried out at 4℃. Frozen cells of 15 g were thawed and suspended uniformly in 30 mL of standard buffer (20 mM potassium phosphate buffer pH 7.0 containing 1 μM FAD, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM thiamine pyrophosphate, and 20% glycerol (v/v)). The cells were disrupted by sonication. Sonication power was applied in 1 min bursts with intervals for cooling of 1 min until the suspension turned dark, usually 10 times. The crude extract was then subjected to centrifugation at 100,000×g for 1 hour and the supernatant was directly loaded to 3.9×25 cm column of DEAE-cellulose equilibrated with the standard buffer without FAD. The column was developed with 1.2 L linear NaCl gradient from 0 M to 1 M. The active fraction was emerged from about 300 mM NaCl and this step enriched the enzyme activity almost ten folds (usually about 0.1 unit/mg).

Chemical modification

Enzyme solution used for this study was pooled from active fractions on DEAE-cellulose chromatography and dialyzed against the standard buffer solution without β-mercaptoethanol. The incubation for modification was carried out in the same buffer solution with the modifying reagents at the concentration indicated in the figures. Modification reactions were initiated by adding enzyme solution of 20 μL to the incubation mixtures of 180 μL at 37℃. After 20 minutes, aliquots of 0.1 mL of the incubation mixtures were diluted into ice cold standard reaction mixture of 0.9 mL and assayed for their activities. Therefore, final concentrations of the modifying reagents in the assay mixtures were diluted to 10 folds. A control was treated in a manner identical with that of the diluted sample except that no modifying reagents were added.

For protection experiments, enzyme solutions of 60 μL were preincubated for 5 minute in standard buffer of 120 μL containing 0.1 M pyruvate. For feedback inhibition experiments, unmodified and modified enzymes with 1 μM PCMB or 1 mM phenylglyoxal were assayed for activity in assay mixtures containing each amino acid of indicated concentration. In the case of experiments with modified enzymes, amino acids were not added to the control sample, and modifying reagents were present in all the assay mixtures at the control concentration of 0.1 μM PCMB or 0.1 mM phenyl-

glyoxal. Since the enzyme activity was considerably affected by the presence of DMSO, which was used to dissolve herbicide, DMSO was added to the control to compensate the effect.

Synthesis of ALS inhibitors

In an attempt to develope new types of herbicide for ALS, following compounds were synthesized:[30,31] Pyrimidyl thio benzene derivatives, 4,6-dimethyl-2-(2-chlorophenylthio) pyrimidine (Comp. A) and 4,6-dimethyl-2-(4-chlorophenylthio) pyrimidine (Comp. B); pyrimidine thio pyridine derivative, 4,6-dimethyl-2-(3-nitropyridine-2-thio) pyrimidine (Comp. C); pyrimidine thio benzoate derivative, 4,6-dimethyl-2-(2-leucine methyl ester benzoamide-2-thio) pyrimidine (Comp. D).

Results and Discussion

Chemical modification

In view of genetic sequences, deduced number of cysteines and arginines in *Arabidopsis* ALS genes are 6 and 30, respectively. We tested inactivation of ALS with iodoacetic acid, iodoacetamide, N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), *p*-chloromercuribenzoic acid (PCMB) as sulfhydryl modifying reagents, and phenylglyoxal, and 2,3-butanedione as arginine modifying reagents. Because all reagents, except liquid 2,3-butanedione, are barely soluble in water or ethanol, stock solutions were made in dimethyl sulfoxide. Fig. 1 shows the concentration dependence of inactivation of ALS by various compounds. *Arabidopsis thaliana* ALS was most sensitive to PCMB among the reagents tested. At 0.1 mM concentration of PCMB, the activity was inhibited more than 90%. This observation strongly implies that the sulfhydryl group(s) could be involved in catalytic or regulatory activity of ALS. DTNB and NEM caused the enzyme to lose its activity more sensitively than iodoacetic acid, iodoacetamide, and phenylglyoxal. The effect of 2,3-butanedione on ALS was not included owing to uncertainty in the assay, since it interfered to the colorimetric measurement at 525 nm.

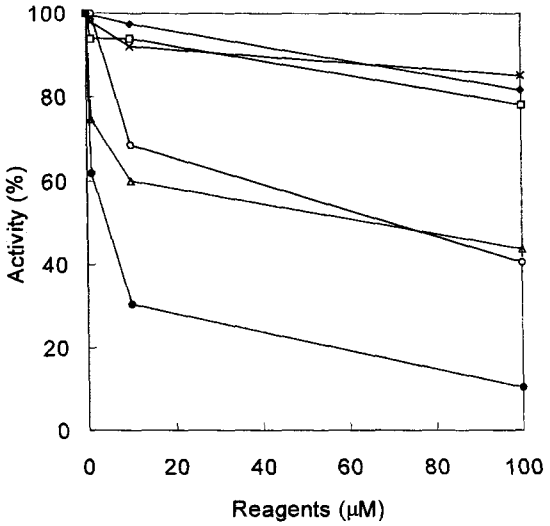

Fig. 1. Inactivation of ALS by various reagents. ◆—◆, iodoacetic acid; □—□, iodoacetamide; ○—○, N-ethylmaleimide; ×—×, phenylglyoxal; △—△, 5,5'-ditiobis(2-nitrobenzoic acid); ●—●, *p*-chloromercuribenzoic acid. The enzyme was incubated with the various reagents in standard buffer at 37°C for 20 minutes. Aliquots of 0.1 mL were removed and diluted into ice cold standard reaction mixture of 0.9 mL and assayed for activity as described in "Materials and Methods".

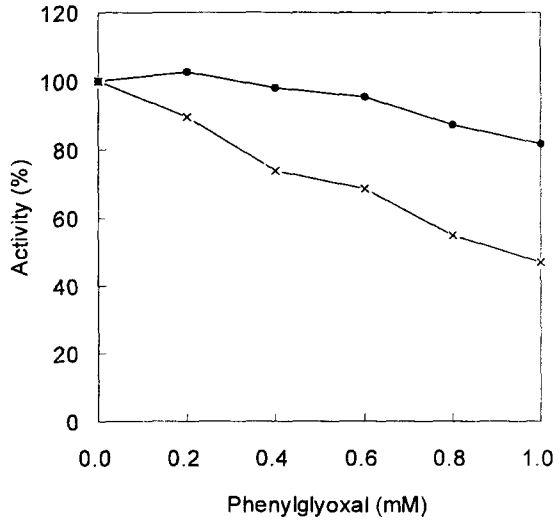

Fig. 2. Effect of substrate on the inactivation of ALS by phenylglyoxal. ×—×, control enzyme without pyruvte; ●—●, enzyme preincubated with pyruvate. After preincubation of the enzyme with or without pyruvate, modifying reagents of 20 μL were added to the incubation mixtures. Incubation with the reagent was then carried out for 20 minutes at 37°C in the same medium. Aliquots were removed and assayed for activity as described in Fig. 1.

Protection test by pyruvate

In order to identify specific amino acids at the active site of the enzyme which might be involved in catalysis, we examined protection capability of substrate by adding pyruvate to the enzyme solution in preincubation period in the modification studies. After preincubation in the presence of an excess amount of the pyruvate, the enzyme assay was performed with PCMB, iodoacetic acid or phenylglyoxal. As shown in Fig. 2, the substrate protected somehow the enzyme against the inhibition by phenylglyoxal. However, pyruvate did not protect the enzyme from inactivation by PCMB or iodoacetic acid (data not shown). This observation suggests a possibility that an arginine residue(s) might be at or near the pyruvate binding site, but the sulfhydryl group(s) are not involved directly in the pyruvate binding site.

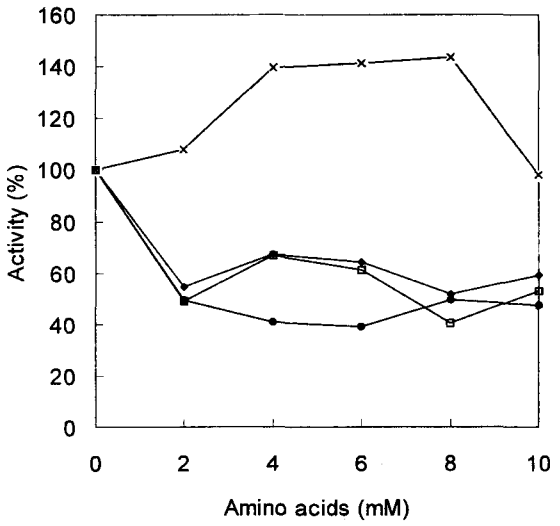

Fig. 3. Feedback inhibition of *Arabidopsis* ALS by end products. ◆—◆, valine; ●—●, isoleucine; ×—×, leucine; □—□, valine+leucine. Enzymatic activities in the presence of each amino acid were measured as described in "Materials and Methods".

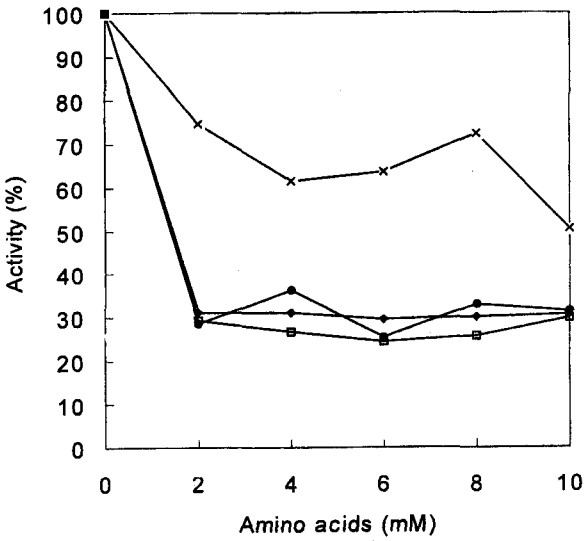

Fig. 4. Effect of PCMB on the feedback inhibition of *Arabidopsis* ALS. ◆—◆, valine; ●—●, isoleucine; ×—×, leucine; □—□, valine+leucine. After preincubation of the enzyme with 1 mM PCMB, the enzymatic activities were assayed in the presence of each amino acid as described in Fig. 3.

Effects of chemical modification on feedback inhibition

Previously, it was reported that ALS from *Arabidopsis thaliana* seedling was sensitive to inhibition by the end products, valine and leucine.[32] At the highest concentration of 1 mM for each amino acid, ALS activity of *Arabidopsis* seedling was inhibited up to 60%. However, *Arabidopsis* ALS expressed in *E. coli* is less sensitive to the feedback inhibitors, valine and leucine.[32] When cloned enzyme was purified extensively, the insensitivity of feedback inhibition prevailed further.[33] In our enzyme source with partially purified *Arabidopsis* ALS from *E. coli* MF2000/pTATX, it was observed that the enzyme was less but still sensitive to inhibition by the end products, valine and isoleucine. Unexpectedly leucine exerted activational effect on the cloned ALS in the range of 4~8 mM. Similar activational effect of end product was observed in yeast ALS by combination of leucine and isoleucine.[34] At the concentration of 2 mM of valine and isoleucine, the ALS activity was inhibited up to 50% (Fig. 3).

We have investigated to test a possible correlation of feedback regulatory site by chemical modification of ALS. Fig. 4 shows feedback inhibition of sulfhydryl modified *Arabidopsis* ALS expressed in *E. coli* by PCMB. PCMB, at the concentration of 1 μM in the modification mixture and then 0.1 μM in the assay mixture containing amino acids, led to potentiation of the inhibition by valine and isoleucine by about 70%. The control sample had identical concentration of PCMB, but no amino acids. Leucine now inhibits the enzyme by about 40% at the concentration of 4 mM. It has been suggested that the allosteric binding sites of these amino acids on the *Arabidopsis thaliana* ALS molecule probably overlap.[20] Hence, the simplest interpretation of our results is that the modifications of the cysteine residue(s) had led to structural or functional changes in the binding site for the amino acids. The observation of sulfhydryl modified ALS with alteration in feedback sensitivity and unprotected ALS in the assay mixture containing excess pyruvate that had unchanged sensitivity to sulfhydryl modifying reagents suggests that ALS binding sites for the end products and pyruvate are not identical. In contrast, effect of feedback inhibition by the arginyl modified enzyme with phenylglyoxal up to 1 mM showed no potentiation of inhibition (data not shown). Furthermore, we could speculate that the cysteine residue(s) seemed to influence the feedback inhibition. However, we cannot rule out the possibility that the chemically modified residue(s) of ALS could have some yet undiscovered regulatory or mechanistic role and existence of 0.1 μM PCMB molecules in the assay mixtures containing the amino acids could directly or indirectly affect the end product binding involved in feedback inhibition.

Testing of a new ALS herbicide compounds

Pyrimidyl oxy compound is one of the six classes of known ALS herbicide. We converted the pyrimidyl oxy compounds to pyrimidyl sulfur derivatives and tested a potential herbicide effect for ALS. Three types of pyrimidyl sulfur compounds were synthesized.[30,31] The pyrimidyl thio phenyl derivatives, compounds A and B did not show any inhibition, and pyrimidine thio pyridine, compound C showed a little inhibition in the range of

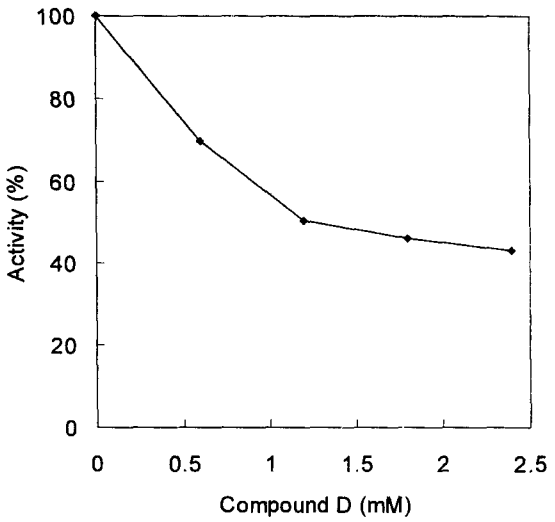

Fig. 5. Effect of compound D on ALS activity. Compound D is a pyrimidyl sulfur benzoate containing leucine as the benzoamide, 4,6-dimethyl-2-(2-leucine methyl ester benoamide-2-thio) pyrimidine.

mM concentration. Therefore, the pyrimidyl sulfur compounds series as a whole seems not to be a good candidate for the herbicide. However, compound D, which is pyrimidyl thio benzoate combined with leucine, showed an inhibitory activity at 1 mM by 50% (Fig. 5). This is very interesting because leucine containing substance like compound D potentiate inhibitory effect significantly although the leucine itself was not a typical feedback inhibitor for the cloned *Arabidopsis thaliana* ALS. Besides, chemically modified ALS at cysteine residues apparently affects the leucine effect. Another word, modified leucine presumably can be a lead compound that alters the conformation of ALS or influence the regulatory site of ALS. Further studies on binding site of inhibitor will enable us to design a new series of herbicides.

Acknowledgments

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아라비돕시스 탈리아나 Acetolactate Synthase의 화학적 변형과 되먹임 방해

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초록 : 아라비돕시스 탈리아나의 아세토락테이트 합성 효소 (ALS)를 그 유전자를 포함하고 있는 대장균 MF 2000/pTATX로부터 부분 정제하였다. 부분 정제된 이 효소를 가지고 여러 가지 변형 화학물질들 즉, 요오드아세트산, 요오드아세타마이드, N-에틸말레이미드 (NEM), 5,5'-디티오비스(2-니트로벤조산) (DTNB), 파라염화수은벤조산 (PCMB), 그리고 페닐글리옥살 등에 대한 민감성을 조사하였다. PCMB가 가장 민감하게 저해를 했으며, DTNB와 NEM이 그 뒤를 따랐다. 이 효소의 기질인 피루브산이 요오드아세트산에 의한 활성 저해를 보호하지 못하였으므로 기질의 결합에 시스테인의 관련이 없는 것 같이 보인다. 한편, 기질이 페닐글리옥살에 의한 효소의 활성 저해를 부분적으로 보호하는 것으로 보아 기질이 아르기닌기와 상호 작용함을 암시하고 있다. 부분 정제된 효소는 발린과 이소루신에 민감하게 방해를 받았으나 루신은 그렇지 않았다. 그러나, PCMB로 변형시킨 효소는 되먹임 방해를 더 강하게 받았다. 그 외 ALS에 대한 새로운 제초제 후보인 피리미딜설퍼 벤조산 유도체의 저해 효과를 검토하였다.

찾는말 : acetolactate synthase, *Arabidopsis thaliana*, feedback inhibition, chemical modification, herbicide
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