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Expression in *Escherichia coli*, Purification, and Characterization of the Tobacco Sulfonylurea Herbicide-Resistant Recombinant Acetolactate Synthase and Its Interaction with the Triazolopyrimidine Herbicides

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Acetolactate synthase (ALS) is the first common enzyme in the biosynthesis of L-leucine, L-isoleucine, and L-valine. The sulfonylurea-resistant ALS gene from *Nicotiana tabacum* was cloned into the bacterial expression vector pGEX-2T. The resulting recombinant plasmid pGEX-ALS3 was used to transform *Escherichia coli* strain XL1-Blue, and the mutant tobacco ALS (mALS) was expressed in the bacteria as a protein fused with glutathione *S*-transferase (GST). The fusion product GST–mALS was purified in a single step on a glutathione-Sepharose column. ALS activities of 0.9–2.5 μmol/min/mg protein were observed in the GST–mALS, and the K_m values for pyruvate, FAD, and TPP were 10.8–24.1, (1.9–8.9) × 10^{-3}, and 0.14–0.38 mM, respectively. The purified GST–mALS was resistant to both the sulfonylurea and the triazolopyrimidine herbicides, and lost its sensitivity to end products, L-valine and L-leucine. For comparision, the tobacco wild-type recombinant ALS fused with GST, GST–wALS, was also characterized with respect to its pyruvate and cofactor bindings. These results suggest that the purified mutant recombinant tobacco ALS was functionally active, that the mutations resulting in herbicide resistance has affected pyruvate and cofactor bindings, and that the two classes of herbicides interact at a common site on the plant ALS.

Keywords: Acetolactate synthase, Herbicide resistant, Kinetics, Tobacco.

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Introduction

Acetolactate synthase (ALS, EC 4.13.18) is an enzyme catalyzing the first common step in the biosynthesis of branched chain amino acids in bacteria (Schloss *et al.*, 1985), yeast (Poulsen and Stougaard, 1989), and higher plants (Southan and Copeland, 1996; Chong *et al.*, 1997). It is feedback-inhibited by the end products L-valine, L-leucine, and L-isoleucine in both microorganisms and in plants (Miflin and Cave, 1972; Gollop *et al.*, 1983; Rathinasabapathi *et al.*, 1990; Mourad *et al.*, 1995). In addition, it is inhibited by several classes of structurally unrelated herbicides, including sulfonylureas (Chaleff and Mauvaris, 1984; LaRossa and Schloss, 1984; Ray, 1984), imidazolinones (Shaner *et al.*, 1984), triazolopyrimidines (Gerwick *et al.*, 1990), pyrimidyl-oxy-benzoates (Babczinski, 1991; Choi *et al.*, 1993), pyrimidyl-thio-benzenes (Choi *et al.*, 1993), and 4,6-dimethoxypyrimidines (Shim *et al.*, 1995). In plants, several structurally-conserved genomic sequences encoding the enzyme ALS have been isolated from *Arabidopsis thaliana*, *Nicotiana tabacum* or tobacco (Mazur *et al.*, 1987), *Brassica napus* (Wiersma *et al.*, 1989), cocklebur (*Xanthium* sp.) (Bernasconi *et al.*, 1995), *Zea mays* (Fang *et al.*, 1992), and *Pisum sativum* (Sin *et al.*, 1994). Mutants that are resistant to branched chain amino acids have been reported (Relton *et al.*, 1986; Wu *et al.*, 1994). Tobacco and *Arabidopsis* mutants that are resistant to the sulfonylurea herbicides have been isolated (Chaleff and Ray, 1984; Haughn and Somerville, 1986), as well as Cocklebur mutants that are resistant to the imidazolinone herbicides (Bernasconi *et al.*, 1995). In particular, the ALS genes from two herbicide-resistant mutants, C3 and S4-Hra, of *N. tabacum* have been sequenced and characterized (Lee *et al.*, 1988). The C3 mutant has a single amino acid change at Pro-196 in the

tobacco ALS enzyme specified by the *SuR*A locus. The S4-Hra mutant has two amino acid changes, one at Pro-196 and the other at Trp-573 in the *SuR*B locus. Introduction of these mutant genes into sensitive tobacco cells produced transgenic tobacco plants which were highly resistant to herbicides (Lee *et al.*, 1988). Despite these studies, the mutated ALS enzyme which produces a resistant phenotype has not been purified to homogeneity from the transgenic plants. Accordingly, knowledge about the kinetic properties of ALS resistant to herbicides is not well characterized. As a useful alternative, expression of the mutated ALS gene in bacteria could be used to purify the plant ALS which are in low amounts and are unstable when isolated from plants.

The purpose of this study was to prepare the plant ALS mutant that is resistant to herbicides. For this purpose, the ALS gene from the sulfonylurea-resistant mutant S4-Hra of *N. tabacum* was cloned, expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST), a protein partner that makes possible one-step purification using glutathione (GSH) agarose affinity column chromatography. Previously, the tobacco wild-type ALS gene was cloned in the bacterial expression vector pGEX-2T in a similar manner (Chang *et al.*, 1997).

In this study, we report the first purification of the sulfonylurea-resistant tobacco ALS enzyme in a highly pure and active form, and its interaction with the triazolopyrimidine and the sulfonylurea herbicides. The results suggest that the mutations resulting in herbicide-resistance has affected pyruvate and cofactor bindings, and that the two classes of herbicides interacts at a common site on the plant ALS.

Materials and Methods

Materials Bacto-tryptone, yeast extract, and bacto-agar were purchased from Difco Laboratories (Detroit, USA). Restriction and modifying enzymes were from Boehringer Mannheim (Mannheim, Germany) and Amersham Corporation (Arlington Heights, USA). Oligonucleotides were obtained from BIONEER (Chungbuk, Korea). The plasmid pAGS148 containing the sulfonylurea-resistant tobacco ALS gene (*Sur*B S4-Hra) was obtained from Dr. B. J. Mazur (E. I. Du Pont de Nemours & Co., Wilmington, USA). The expression vector pGEX-2T was obtained from Dr. Sang-Ki Paik (Chungnam National University, Taejon, Korea). Metsulfuron methyl, a sulfonylurea herbicide, was obtained from Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology, Taejon, Korea). Flumetsulam, a triazolopyrimidine herbicide, was obtained from Dr. Sung Keon Namgoong (Seoul Women's University, Seoul, Korea).

Construction of the expression vectors for GST–mALS, pGEX-ALS3 The sulfonylurea-resistant *Nicotiana tabacum* ALS gene (*Sur*B S4-Hra) was cloned into expression vector pGEX-2T, a *tac* promoter-based vector designed to express cloned inserts as a fusion with glutathione *S*-transferase (Smith and Johnson, 1988). This vector incorporates a linker and thrombin cleavage site between these two domains to allow cleavage and removal of the fusion partner after purification of the expression using glutathione (GSH) Sepharose column chromatography. First, the vector pMals was constructed by transfering the ALS gene into pBluescript SK(−) by simultaneously amplifying the gene from the plasmid pAGS148 and introducing both *Bam*HI and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, of the coding strand by polymerase chain reaµction (PCR) with the oligonucleotide primers 1 and 2:

1: 5′-CATCTCCGGATCCATGTCCACTACCCAAA-3′
*BamH*I
2: 5′-ATGCGGATCCTCAAAGTCAATAGG-3′
*BamH*I

The PCR was carried out as previously described (Saiki *et al.*, 1988). Each reaction contained 50 ng of template DNA, 100 pmol of the primers 1 and 2, 200 μM dNTPs in 50 mM KCl, 10 mM Tris (pH 8.3), and 1.5 mM $MgCl_2$ in 100 μl. The reactions were overlayed with 100 μl of mineral oil and subjected to 30 cycles of the following program: 94°C, 1 min 30 sec; 55°C, 2 min; 72°C, 3 min. PCR products were subcloned by ethanol precipitation, restriction digestion with *Bam*HI, and ligation with *Bam*HI-treated pBluescript SK(−). Finally, the ALS gene was excised from the pMals with *Bam*HI, isolated from 1% agarose gel, and cloned into the *Bam*HI site of pGEX-2T. The resulting expression vector pGEX-ALS3 was used to transform the *E. coli* strain XL1-Blue cells. Plasmid DNA was isolated according to the alkaline lysis method, and transformation of XL1-Blue cells was achieved using a standard $CaCl_2$ transformation protocol (Sambrook *et al.*, 1989). DNA sequence analysis of the PCR product was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Expression and purification of GST–mALS XL1-Blue cells containing the pGEX-ALS3 plasmid were grown at 30°C in Luria-Bertani (LB) medium in a 2-L round-bottomed flask to an A_{600} of 0.5–0.8. The tobacco mutant ALS fused with GST, GST–mALS, was induced with 0.1–1.0 mM isopropyl-β-D-thiogalactoside (IPTG). Cells were grown for an additional 4 h at 30°C and harvested by centrifugation at 5000 × *g* for 10 min. The cell paste was stored at −70°C.

For the purification of GST–mALS, the cell paste was thawed and suspended in resuspension buffer (50 mM N-[2-hydroxyethyl]piperazine-N′-[3-propanesulfonic acid] (HEPPS), 10% ethylene glycol, 10 mM $MgCl_2$, 1 mM pyruvate, 4 μg/ml leupeptin, 3 μg/ml aprotinin, 2 μg/ml pepstatin A, pH 7.5). The suspension was lysed by sonication at 4°C and centrifuged at 20,000 × *g* for 15 min. The supernatant was applied to a 5-ml GSH-Sepharose 6B column (Pharmacia-LKB, USA) pre-equilibrated with the resuspension buffer and then washed with 10 vol resuspension buffer to remove unbound proteins. The GST–mALS fusion was recovered from the column with a buffer containing 50 mM Tris-HCl, pH 9.6 and 15 mM GSH. The enzyme was stored in aliquots at −70°C. In order to obtain the recombinant mALS separated from GST, the purified GST–mALS (1 mg) was subjected to overnight digestion at 4°C in the presence of 10 units of thrombin. An aliquot was analyzed by SDS-PAGE (Laemmli, 1970). Protein concentrations were measured by the method of Bradford (1975).

Assays of ALS Enzyme activities of the purified GST–mALS or mALS were measured according to the colorimetric assay of Westerfeld (1945) with a modification. The reaction mixture (200 μl) contained 20 mM potassium phosphate, pH 7.0, 100 mM pyruvate, 0.5 mM thiamine-pyrophosphate (TPP), 0.5 mM $MgCl_2$, and 10 μM flavine adenine dinucleotide (FAD) in the presence or absence of various concentrations of herbicides or end products (L-valine and L-leucine). Assays were initiated by adding the purified GST–mALS or mALS enzymes and terminated by adding 20 μl of 6 N H_2SO_4 after 1 h at 37°C. The acidified reaction mixtures were heated for 15 min at 60°C after which 0.2 ml of 0.5% (w/v) creatine was added. Then, 0.2 ml of 5%-naphthol was added and the solutions were heated for an additional 15 min at 60°C. The absorbances of the solutions were determined at 525 nm. Specific activities of ALS were expressed as μmol acetoin/min/mg protein. Enzymatic activities of the purified GST–mALS were also measured using a continuous assay which monitors the consumption of pyruvate directly at 340 nm (ε_M = 22.13 $M^{-1}cm^{-1}$) on a microplate reader (THERMO max, Molecular Devices). The reaction mixture (200 μl) contained 50 mM potassium phosphate, pH 7.0, 100 mM pyruvate, 1 mM TPP, 10 mM $MgCl_2$, and 20 μM FAD. Assays were carried out in a microtitre plate at 30°C.

The IC_{50} value for inhibition by inhibitors is defined as the concentration of metsulfuron methyl or flumetsulam which inhibits ALS activity by 50% in a 60-min fixed time assay carried out as described above. The data were fitted to the equation

$$\% \text{ Activity} = \frac{100}{\left(1 + \frac{[I]}{IC_{50}}\right)} \quad (1)$$

where % activity equals the amount of activity in the presence of various inhibitor concentrations as a percent of an untreated control, and [I] equals the inhibitor concentration. IC_{50} was calculated by nonlinear least-squares and the Simplex method for error minimization (Nelder and Mead, 1965).

Results

Expression and purification of the sulfonylurea-resistant recombinant tobacco ALS We cloned the tobacco sulfonylurea-resistant ALS gene (Lee *et al.*, 1988) including part of the chloroplast transit peptide into the bacterial expression plasmid pGEX-2T, as described in Materials and Methods. Induction of the tobacco mutant ALS fused with GST, GST-mALS, by IPTG was established by analyzing crude bacterial extracts on SDS-PAGE as shown in Fig. 1, lane 3. A 91 kDa band corresponding to the fusion protein composed of GST (26 kDa) and the mutant tobacco ALS (65 kDa), mALS, was detected in pGEX-ALS3 after IPTG induction. In order to find the optimum conditions for the expression of GST–mALS, several parameters were adjusted. We found that the optimum growth temperature for the recombinant *E. coli* was 30°C, and that the optimum level of IPTG for GST–mALS induction was 0.3 mM, which was added when the culture reached an A_{600} of 0.7 (data not shown). Western blot analysis of the crude extract indicated that the fusion protein reacted with antibodies against GST (Chang *et al.*, 1996) or the amino-terminal fragment of sulfonylurea-resistant tobacco ALS (data not shown). The fusion protein was soluble and was found in the supernatant of the extract after centrifugation (data not shown). In contrast, the fusion protein was insoluble when the culture was grown at 37°C (data not shown). Purification of the fusion protein was carried out to near homogeneity in a single step by GSH-Sepharose affinity chromatography as shown in Fig. 1, lane 4, and resulted in the recovery of 2.5–5.0 mg GST–mALS per liter of bacterial culture with specific activity of 0.9–2.5 μmol/min/mg.

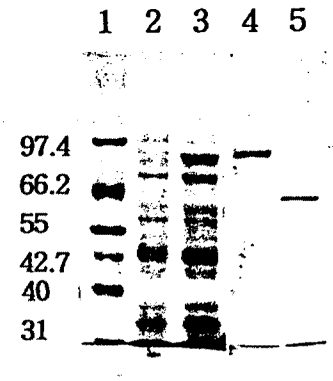

Fig. 1. Purification of GST–mALS fusion protein obtained after transformation of *E. coli* cell transformed with plasmid pGEX-ALS3. After 4 h induction of the *tac* promoter by 0.3 mM IPTG, *E. coli* (strain XL1-Blue) harbouring the plasmid pGEX-ALS3 were harvested and lysed, and the GST–mALS fusion protein and mALS were purified as described in Materials and Methods. Samples were electrophoresed on 10% polyacrylamide gels containing SDS. Lane 1: molecular weight markers; lane 2: total cell lysis before GST–mALS induction; lane 3: total cell lysis after GST–mALS induction; lane 4: purified GST–mALS; lane 5: purified mALS.

Purified GST–mALS was subjected to 16 h-digestion at 4°C in the presence of increasing amounts of thrombin. An aliquot was analyzed by SDS-PAGE, and another was assayed for ALS enzyme activity (Fig. 2). When aliquots of the reaction mixture were analyzed by SDS-PAGE, a band at 26 kDa, corresponding to GST, and a band at 65 kDa, corresponding to mALS, were obtained (Fig. 2A, lanes 5 and 6). Partially and completely cleaved enzyme retained activity of >80 and 80 (% of control), respectively (Fig. 2B). Enzymatic activity of GST–mALS, which was incubated without thrombin at 4°C for 16 h, was used as a control. Since the completely cleaved mALS enzyme was active, the cleaved ALS enzyme was purified to homogeneity by GSH-Sepharose affinity chromatography as shown in Fig. 1, lane 5.

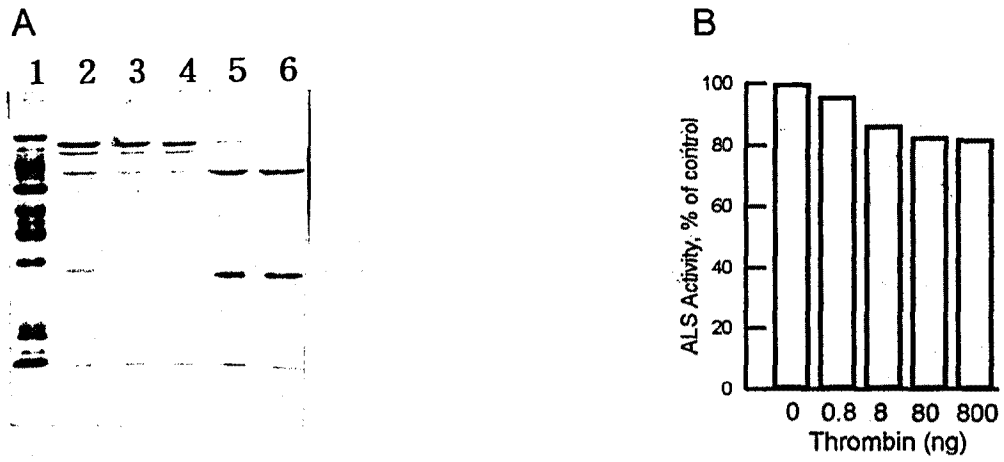

Fig. 2. Thrombin cleavage of the purified GST–mALS fusion protein. A. GST–mALS fusion protein, which was purified by the method described in Materials and Methods, was incubated with thrombin at 4°C for 16 h. Lane 1: molecular weight markers 97.4, 66.2, 55, 42.7, 40, 31, and 21.5 kDa; lane 2: 15 μg of GST–mALS; lanes 3–6: 20 μg of GST–mALS, which was incubated with 0.8, 8, 80, 800 ng of thrombin, respectively. The molecular weight of GST–mALS fusion protein is 91 kDa. After thrombin cleavage, GST and mALS are 26 and 65 kDa, respectively. B. Enzymatic activity of each reaction fraction, which was assayed as described in Materials and Methods. Enzymatic activity of GST–mALS, which was incubated without thrombin at 4°C for 16 h, was used as a control.

Characterization of the sulfonylurea-resistant recombinant tobacco ALS

(1) Kinetics The GST fusion system used in this study, for the first time, allowed us to measure the enzymatic activity of the tobacco mutant recombinant ALS enzyme in a highly pure form. Figure 3 shows the pyruvate saturation kinetics of GST–mALS. The kinetic data were fitted to the equations

$$v = \frac{V_{max}}{\left(1 + \frac{K_m}{[S]}\right)} \tag{2}$$

or

$$v = \frac{V_{max}[S](RK_{m2} + [S])}{(K_{m1}K_{m2} + 2K_{m2}[S] + [S]^2)} \tag{3}$$

by nonlinear least-squares and the Simplex method for error minimization (Nelder and Mead, 1965). In these equations, $[S]$ represents the total concentration of substrate, V_{max} is the maximum velocity, K_m is the Michaelis constant for pyruvate, R is the ratio of catalytic rate constants k_{c1}/k_{c2} (Chang and Duggleby, 1997), K_{m1} is the Michaelis constant for pyruvate at the first active site, and K_{m2} is that at the second active site. The rate equation (3) was derived from the mechanism for the negatively cooperative substrate kinetics of *Arabidopsis* ALS (Chang and Duggleby, 1977), and this mechanism assumes: (i) ALS is a dimer, and (ii) there are interactions between the subunits where substrate binding to one subunit impairs binding to the second.

Figure 3A shows the pyruvate saturation kinetics of GST–mALS by using the colorimetric assay method. The broken line in Fig. 3A has been calculated with Eq. (2) and the best-fit parameters $V_{max} = 0.93 \pm 0.03$ μmol/min/mg protein and $K_m = 24.1 \pm 1.8$ mM. The solid line in Fig. 3A has been calculated with Eq. (3), with K_{m2} set equal to 100 mM, and the best-fit parameters $V_{max} = 1.3 \pm 0.2$ μmol/min/mg protein, $R = 1.0 \pm 0.3$, and $K_{m1} = 30.2 \pm 7.3$ mM. For comparision, pyruvate saturation kinetics of the wild-type tobacco recombinant ALS (Chang *et al.*, 1997) was also carried out. The K_m values for pyruvate in the wild-type ALS were determined to be 8.2 ± 0.1 mM with Eq. (2), and K_{m1} values for that were determined to be 12.8 ± 1.9 mM with Eq. (3), with K_{m2} set equal to 100 mM and $R = 1.4 \pm 0.2$ (Table 1).

Figure 3B shows the pyruvate saturation kinetics of GST–mALS by using the continuous assay method. The broken line in Fig. 3B has been calculated with Eq. (2) and the best-fit parameters $V_{max} = (1.25 \pm 0.04) \times 10^{-3}$ A_{340}/min and $K_m = 10.8 \pm 1.3$ mM. The solid line in Fig. 3B has been calculated with Eq. (3), with K_{m2} set equal to 100 mM, and the best-fit parameters $V_{max} = (1.5 \pm 0.4) \times 10^{-3}$ A_{340}/min, $R = 1.4 \pm 0.5$, and $K_{m1} = 17.0 \pm 4.8$ mM. For comparision, pyruvate saturation kinetics of the wild-type tobacco recombinant ALS (Chang *et al.*, 1997) was also carried out. The K_m values for pyruvate in the wild-type ALS were determined to be 5.3 ± 0.5 mM with Eq. (2), and K_{m1} values for that were determined to be 8.4 ± 1.1 mM with Eq. (3), with the K_{m2} set equal to 100 mM and $R = 1.5 \pm 0.2$ (Table 1).

The effects of cofactors on the enzymatic activity of the tobacco mutant recombinant ALS enzyme were examined. Figures 4A and 4B show FAD and TPP saturation kinetics

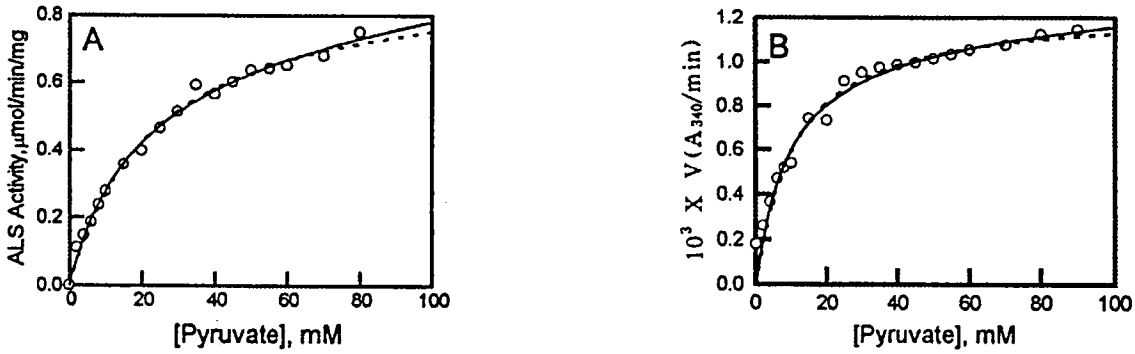

Fig. 3. A. Pyruvate saturation kinetics of the purified GST–mALS fusion proteins by using the colorimetric assay method. The substrate pyruvate concentration was varied from 1 to 100 mM, and other assay conditions were as described in Materials and Methods. The broken line is the best fit to Eq. (2), with the best-fit parameters V_{max} = 0.93 ± 0.03 μmol/min/mg protein and K_m = 24.1 ± 1.8 mM. The solid line is the best fit to Eq. (3), with the K_{m2} set to 100 mM, and the best-fit parameters V_{max} = 1.3 ± 0.2 μmol/min/mg protein, K_{m1} = 30.2 ± 7.3 mM, and R = 1.0 ± 0.3. B. Pyruvate saturation kinetics of the purified GST–mALS fusion proteins by using the continuous assay method. The substrate pyruvate concentration was varied from 1 to 100 mM, and other assay conditions were as described in Materials and Methods. The broken line is the best fit to Eq. (2), and the best-fit parameters V_{max} = (1.25 ± 0.04) × 10^{-3} A_{340}/min and K_m = 10.8 ± 1.3 mM. The solid line is the best fit to Eq. (3), with K_{m2} set to 100 mM, and the best-fit parameters V_{max} = (1.5 ± 0.4) × 10^{-3} A_{340}/min, K_{m1} = 17.0 ± 4.8 mM, and R = 1.4 ± 0.5.

Table 1. Kinetic constants of pyruvate from the sulfonylurea herbicide-resistant mutant versus wild-type recombinant acetolactate synthase of *N. tobacum*.

	GST–mALS	GST–wALS
	Pyruvate	
Colorimetric assay[a]		
Eq. (2): K_m (mM)	24.1	8.1
V_{max} (μmol/min/mg protein)	0.93	2.8
Eq. (3): K_{m1} (mM)	30.2	12.8
V_{max} (μmol/min/mg protein)	1.2	3.4
Continuous assay[b]		
Eq. (2): K_m (mM)	10.8	5.3
V_{max} (10^3 × A_{340}/min)	1.3	2.0
Eq. (3): K_{m1} (mM)	17.0	8.4
V_{max} (10^3 × A_{340}/min)	1.5	2.4

[a] The reaction mixture (200 μl) contained 20 mM potassium phosphate, pH 7.0, 1–100 mM pyruvate, 0.5 mM thiamine-pyrophosphate (TPP), 0.5 mM $MgCl_2$, and 10 μM flavine adenine dinucleotide (FAD).

[b] The reaction mixture (200 μl) contained 50 mM potassium phosphate, pH 7.0, 1–100 mM pyruvate, 1 mM TPP, 10 mM $MgCl_2$, and 20 μM FAD.

of GST–mALS by using the colorimetric assay method, respectively. Saturation of GST–mALS by the cofactors appeared hyperbolic.The curve in Fig. 4A has been calculated with Eq. (2) and the best-fit parameters V_{max} = 2.19 ± 0.04 μmol/min/mg protein and K_m = 8.9 ± 0.6 μM. The curve in Fig. 4B has been calculated with Eq. (2) and the best-fit parameters V_{max} = 2.5 ± 0.1 μmol/min/mg protein and K_m = 0.38 ± 0.07 mM. The K_m values for FAD and TPP in the wild-type ALS were determined to be 2.6 ± 0.4 μM and 0.21 ± 0.04 mM, respectively (Table 2).

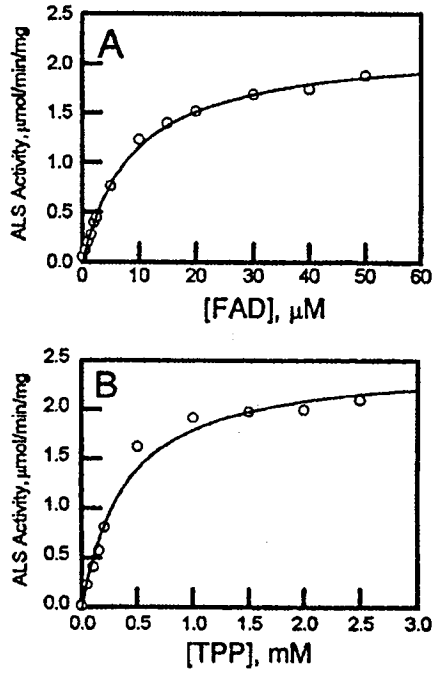

Fig. 4. A. FAD saturation kinetics of the purified GST–mALS fusion proteins by using the colorimetric assay method. The cofactor FAD concentration was varied from 1 to 50 μM, and other assay conditions were as described in Materials and Methods. The curve is the best fit to Eq. (2), and the best-fit parameters V_{max} = 2.19 ± 0.04 μmol/min/mg protein and K_m = 8.9 ± 0.6 μM. B. TPP saturation kinetics of the purified GST–mALS fusion proteins by using the colorimetric assay method. The cofactor TPP was varied from 1 to 2.5 mM, and other assay conditions were as described in Materials and Methods. The curve is the best fit to Eq. (2), and the best-fit parameters V_{max} = 2.5 ± 0.1 μmol/min/mg protein and K_m = 0.38 ± 0.07 mM.

Table 2. Kinetic constants of FAD and TPP from the sulfonylurea herbicide-resistant mutant versus wild-type recombinant acetolactate synthase of *N. tobacum*.

	GST-mALS		GST-wALS	
	FAD[c]	TPP[d]	FAD[c]	TPP[d]
Colorimetric assay				
K_m (mM)[a]	8.9×10^{-3}	0.38	2.6×10^{-3}	0.21
V_{max} (μmol/min/mg protein)[a]	2.2	2.5	0.74	0.87
K_m (mM)[b]	7.2×10^{-3}	0.17	1.4×10^{-3}	0.08
V_{max} (μmol/min/mg protein)[b]	0.7	0.61	0.34	0.33
Continuous assay				
K_m (mM)[b]	1.9×10^{-3}	0.14	0.50×10^{-3}	0.10
$V_{max}(10^3 \times A_{340}/\text{min})$[b]	3.0	2.6	1.8	1.6

[a] The reaction mixture (200 μl) contained 20 mM potassium phosphate, pH 7.0, 100 mM pyruvate, and 0.5 mM $MgCl_2$.
[b] The reaction mixture (200 μl) contained 50 mM potassium phosphate, pH 7.0, 100 mM pyruvate, and 10 mM $MgCl_2$.
[c] The cofactor flavine adenine dinucleotide (FAD) concentration was varied from 1 to 50 μM but the cofactor thiamine-pyrophosphate (TPP) concentration was 0.5 mM (colorimetric assay) or 1 mM (continuous assay).
[d] The cofactor TPP concentration was varied from 1 to 2.5 mM but the cofactor FAD concentration was 10 μM (colorimetric assay) or 20 μM (continuous assay).

FAD and TPP saturation kinetics of GST–mALS were also carried out using the continuous assay method. The K_m values for FAD and TPP in the mutant ALS were determined to be 1.9 ± 0.2 μM and 0.14 ± 0.02 mM, respectively (Table 2). For comparision, FAD and TPP saturation kinetics of the tobacco wild-type recombinant ALS (Chang *et al.*, 1997) was carried out using the continuous assay method. The K_m values for FAD and TPP in the wild-type ALS were determined to be 0.50 ± 0.08 μM and 0.10 ± 0.03 mM, respectively (Table 2).

(2) Interaction of ALS with herbicides ALS activity of GST–mALS was assayed in the presence of metsulfuron methyl, a sulfonylurea herbicide, or flumetsulam, a triazolopyrimidine herbicide. IC_{50} values were determined for GST–mALS. GST–mALS was resistant to inhibition by both metsulfuron methyl (Fig. 5A, closed circle) and flumetsulam (Fig. 5B, closed circle). The curves in Figs. 5A and 5B have been calculated with Eq. (1) and the best-fit parameter IC_{50} = 465.9 ± 39.7 nM and 938.5 ± 132.0 nM, respectively. For comparision, the tobacco wild-type recombinant ALS (Chang *et al.*, 1997) was assayed in the presence of flumetsulam. The IC_{50} value for flumetsulam was 31.6 ± 1.2 nM (Fig. 5B, open circle). Previously, the IC_{50} value for metsulfuron methyl in the tobacco wild-type recombinant ALS were determined to be 10.3 ± 0.01 nM (Fig. 5A, open circle).

(3) Interaction of ALS with end products ALS activity of GST–mALS was also assayed in the presence of 0.2–1.4 mM end products, L-valine and L-leucine. The purified GST–mALS lost its sensitivity to the end products, as shown in Fig. 5C.

Discussion

In this study, we cloned the tobacco sulfonylurea-resistant acetolactate synthase (ALS; also known as acetohydroxyacid synthase) coding region with part of a chloroplast transit peptide (Mazur *et al.*, 1987; Lee *et al.*, 1988) into pGEX-2T as described in Materials and Methods, and active protein was expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST) from the expression vector, pGEX-ALS3. The oligonucleotide primer 1 indicates that the coding sequence starts at Ser-72 in the *SurB* S4-Hra mutant ALS gene (Lee *et al.*, 1988). When we, however, cloned a full coding region of the sulfonylurea-resistant ALS gene (*SurB* S4-Hra), a 2000 bp *Bam*HI/*Bam*HI fragment of pBals (Kim and Chang, 1995), into pGEX-2T, no correct product was detected upon induction by IPTG (data not shown). Recently, Chang and Duggleby (1997) reported the functional expression of wild-type *Arabidopsis* ALS in *E. coli* from the construct which starts at Thr-86 in *Arabidopsis* ALS, which is a few residues beyond the Ser-72 in tobacco ALS (Lee *et al.*, 1988). These results are consistent with that of Wiersma *et al.* (1990) who reported the functional expression of *Brassica napus* ALS in *Salmonella typhimurium*, and demonstrated that efficient expression of *B. napus* ALS in bacteria requires the removal of at least part of the chloroplast transit peptide sequences. In contrast, Bernasconi *et al.* (1995) reported the functional expression

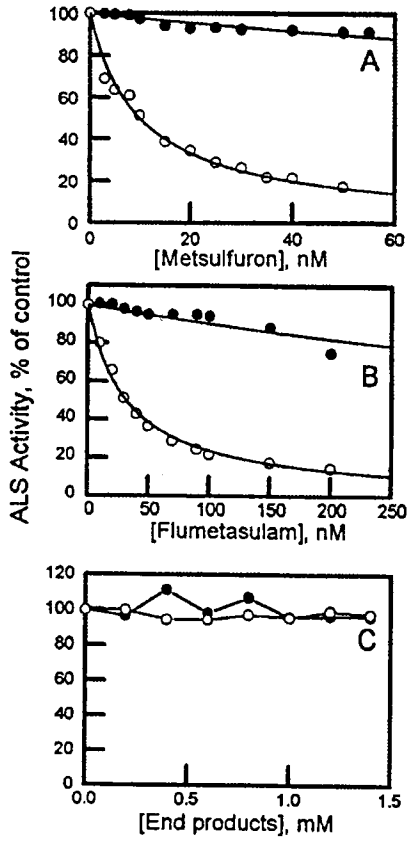

Fig. 5. A. Inhibition of the purified GST–mALS versus GST–wALS fusion proteins by a sulfonylurea herbicide, metsulfuron methyl. The inhibitor metsulfuron methyl concentrations were varied from 2 to 50 nM, and other assay conditions were as described in Materials and Methods. The values are given as the percent of an untreated control. The upper curve (GST–mALS) is the best fit to Eq. (1), and the best-fit parameter IC_{50} is 465.9 ± 39.7 nM. The lower curve (GST–wALS) is the best fit to Eq. (1), and the best-fit parameter IC_{50} is 10.3 ± 5.6 nM. B. Inhibition of the purified GST–mALS versus GST–wALS fusion proteins by flumetsulam, a triazolopyrimidine herbicide. The inhibitor flumetsulam concentrations were varied from 2 to 200 nM, and other assay conditions were as described in Materials and Methods. The values are given as the percent of an untreated control. The upper curve (GST–mALS) is the best fit to Eq. (1), and the best-fit parameter IC_{50} is 938.5 ± 132.0 nM. The lower curve (GST–wALS) is the best fit to Eq. (1), and the best-fit parameter IC_{50} is 31.6 ± 1.2 nM. C. Effect of end products on the purified GST–mALS fusion proteins activity. The end products, L-valine (•) and L-leucine (○), concentrations were varied from 0.2–1.4 mM, respectively, and other assay conditions were as described in Materials and Methods.

of wild-type cocklebur (*Xanthium* sp.) ALS in *E. coli* as a fusion protein with GST, and demonstrated that the complete proform of the ALS was needed to obtain an active enzyme.

The use of a GST fusion allowed us to study mutant tobacco ALS that is present in only minute quantities in herbicide-resistant plants. The mutant recombinant glutathione *S*-transferase-acetolactate synthase (GST–mALS) was purified in a highly pure and active form using one simple chromatography step on GSH-Sepharose (Fig. 1, lane 4). The design of the expression vector pGEX-2T allowed cleavage of the fusion protein and removal of the fusion partner, GST, by thrombin (Smith and Johnson, 1988). The recombinant mALS separated from GST was active (Fig. 2B), and the cleaved mALS was also purified in a highly pure form (Fig. 1, lane 5).

Knowledge about the kinetic properties of ALS resistant to herbicide is limited. The K_m values for the substrate pyruvate and the cofactors FAD and TPP in the purified mutant tobacco ALS were higher than that for the wild-type tobacco recombinant ALS (Tables 1 and 2). These results suggest that the mutations resulting in sulfonylurea resistance have affected pyruvate and cofactor bindings. Saari *et al.* (1990) have reported that the K_m values for pyruvate in sulfonylurea-resistant *Koichia* ALS were similar to that for the sensitive *Koichia* ALS. In contrast, Rathinasabapathi and King (1990) have reported that the K_m values of the ALS for pyruvate from three sulfonylurea-resistant variants were many folds greater than that of the wild-type. It is worth noting that none of these sulfonylurea-resistant plants ALS enzymes have not been purified to homogeneity, and thus partially purified fractions of these mutants were used for the determination of the kinetic properties of the sulfonylurea-resistant *Koichia* ALS (Saari *et al.*, 1990) and variants of *Datura innoxia* ALS (Rathinasabapathi and King, 1990).

Previously, the kinetics of the native plant ALS enzyme in crude extract from plants (Singh *et al.*, 1992; Mourad *et al.*, 1995) and of the purified recombinant tobacco ALS (Chang *et al.*, 1997) have been analyzed as hyperbolic saturation curves. Recently, Chang and Duggleby (1997) suggested the negatively cooperative substrate kinetics for the plant ALS enzyme since close examination of the substrate saturation kinetic data of the enzyme revealed deviations from Michaelis-Menten kinetics. In this study, the kinetic data of GST–mALS were also fitted to Eq. (3), which was derived from the mechanism for the negative cooperativity. The K_{m1} values for pyruvate in sulfonylurea-resistant tobacco ALS were higher than that for the wild-type tobacco recombinant ALS (Table 1).

We showed that the purified mutant recombinant tobacco ALS was resistant to inhibition by the two classes of herbicides: metsulfuron methyl, a sulfonylurea herbicide, having IC_{50} values of 465.9 ± 39.7 nM, and flumetsulam, a triazolopyrimidine herbicide, having IC_{50} values of 938.5 ± 132.0 nM. Previously, Lee *et al.* (1988) reported that introduction of the S4-Hra mutant gene into sensitive tobacco cells produced the sulfonylurea-resistant tobacco plants. The results from the current studies suggest that the transgenic tobacco plants are also resistant to the triazolopyrimidine herbicides. In contrast, the wild-type

enzyme is extremly sensitive to inhibition by metsulfuron methyl having IC_{50} values of 10.30 ± 0.01 nM (Chang *et al.*, 1997) and flumetsulam having IC_{50} values of 31.6 ± 1.2 nM. These results suggest that the two classes of herbicides interact at a common site on ALS.

The amino acid sequences specifically interacting with different herbicides are not known since the three-dimensional structure of the plant ALS has not been determined. Recently, Ott *et al.* (1996) proposed a structural model which was generated based on homology to pyruvate oxidase, and the imidazolinone herbicide was positioned in the proposed binding site using structure-activity data. The herbicide-binding pocket contains the mutation site for sulfonylurea resistance, Trp-574 in *Arapidopsis* ALS. The results from the current studies and from Ott *et al.* (1996) suggest that the imidazolinone and the sulfonylurea herbicides interact at a common site, Trp-573, on the tobacco ALS enzyme. Bernasconi *et al.* (1995) reported that the mutation that changed Trp-552 in the cocklebur (*Xanthium* sp.) ALS to Leu conferred a dramatically reduced sensitivity toward the sulfonyureas (6250-fold resistence), the triazolopyrimidines (10,000-fold resistance), and the pyrimidinyl oxybenzoates (2200-fold resistence) but at a lesser extent to the imidazolinones (>7.2-fold resistance).

The purified recombinant tobacco ALS was insensitive to the end products, L-valine and L-leucine (Fig. 5C). A similar observation was noted before in the purified wild-type recombinant tobacco ALS (Chang *et al.*, 1997). The reasons for the insensitivity of both the mutant and the wild-type recombinant plant ALS to the end products, the feedback inhibitors, are not known. Recently, a second subunit has been identified for a red alga ALS as well as yeast ALS (Duggleby, 1997), and thus a second subunit which is responsible for the feedback sensitivity of ALS may not exist in the purified mutant and wild-type recombinant tobacco ALS.

Analysis of a wide range of mutations in a plant ALS gene for a herbicide-resistant phenotype would normally involve: (1) screening of plant tissue for random mutational events, and (2) the introduction of specifically mutated genes into plants by transformation (Wiersma *et al.*, 1990). However, in both cases, the use of plant tissues makes the process very labor-intensive. In this study, we demonstrated that this problem can be circumvented by expression of the mutated ALS gene in bacteria. The purified mutant ALS enzyme was used for determining the level of catalytic efficiency and resistance to the herbicides. As demonstrated by Ott *et al.* (1996), by employing a rational structure-based approach, novel ALS mutations that exhibit selective herbicide resistance can be identified and then the mutant genes can be used to generate selective herbicide-resistant plants.

In conclusion, we demonstrated that the expression of the plant ALS gene in *E. coli* as a fusion protein with GST can provide a useful alternative to purification of the herbicide-resistant plant ALS enzyme, which is a valuable material for the development and rational design for new herbicides and their resistant plants. The purified plant ALS resistant to herbicides was used to study their kinetic properties and the mode of action of herbicide inhibition.

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