Functional Expression of *Nicotiana tabacum* Acetolactate Synthase Gene in *Escherichia coli*

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(Received January 9, 1995)

Abstract: Acetolactate synthase (ALS, EC 4.1.3.18) is the first common enzyme in the biosynthesis of leucine, isoleucine, and valine. It is the target enzyme for several classes of herbicides, including the sulfonylureas, the imidazolinones, the triazolopyrimidines, the pyrimidyl-oxy-benzoates and the pyrimidyl-thio-benzens. The sulfonylurea-resistant ALS gene (*SurB*) from *Nicotiana tabaccum* [Lee *et al.* (1988) *The EMBO J.* **7**, 1241-1248] was cloned into the bacterial expression plasmid pT7-7. The resulting recombinant plasmid pT7-ALS was used to transform an ALS-deficient *Escherichia coli* strain MF2000. MF2000 cells transformed with pT7-ALS grew in the absence of valine and isoleucine. ALS activities of 0.042 and 0.0002 μmol/min/mg protein were observed in the crude extracts prepared from MF2000 cells transformed with plasmids pT7-ALS and pT7-7, respectively. In addition, the former crude extract containing mutant ALS was insensitive to inhibition by K11570, a new chemical class of herbicides. IC_{50} values for K11570 were 0.13± 0.01 mM. For comparison, a plasmid pTATX containing the wild-type Arabidopsis thaliana ALS coding sequences was also expressed in MF2000. ALS activities of 0.037 μmol/min/mg protein were observed, and the wild type ALS was sensitive to two different classes of herbicides, K11570 and ALLY, a sulfonylurea. IC_{50} values for K11570 and ALLY were 0.63± 0.07 and 80± 5.6 nM, respectively. Thus, the results suggest that the sulfonylurea-resistant tobacco ALS was functionally expressed in the bacteria, and that K11570 herbicides bind to the regulatory site of ALS enzymes.

Key words: acetolactate synthase, expression, sulfonylurea, tobacco.

Acetolactate synthase (ALS, EC 4.13.18) is the first enzyme unique to the biosynthesis of branched chain amino acids leucine, isoleucine, and valine in bacteria, yeast, and higher plants. It is the target of several classes of structurally unrelated herbicides, including the sulfonylureas (Chaleff and Mauvis, 1984; LaRossa and Schloss, 1984; Ray, 1984), the imidazolinones (Shaner *et al.*, 1984), the triazolopyrimidines (Gerwick *et al.*, 1990), the pyrimidyl-oxy-benzoate (Babczinski and Zelinski, 1991; Choi *et al.*, 1993), and the pyrimidyl-thio-benzenes (Choi *et al.*, 1993). 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), and *Pisum sativum* (Sin *et al.*, 1994). Tobacco and *Arabidopsis* mutants that are resistant to sulfonylurea herbicides have been isolated using a seed mutagenesis protocol, and in particular two herbicide-resistant forms of ALS genes (*SurA* and *SurB*) were isolated from the *N. tabacum mutant* (Lee *et al.*, 1988). A single and two amino acid(s) change(s) in *SurA* and *SurB* ALS genes, respectively, resulted in the production of the enzyme ALS which was resistant to the herbicide. Since ALS is present in low amounts and is unstable when isolated from plants, none of the plant ALS enzymes have been purified yet. Accordingly, data on biochemical and structural characterization of ALS from wild type and in particular herbicide-resistant plants is very limited. The amino-terminal residue in the mature plant ALS is unknown. Both the wild-type and mutant Arabidopsis ALS genes have been functionally expresed in *Escherichia coli* (Smith *et al.*, 1989; cf. Singh *et al.*, 1992). In addition, ALS from *Brassica napus* has been expressed in *Salmonella typhimurium* (Wiersma *et al.*, 1990).

In this study, we report the functional expression of sulfonylurea-resisitant ALS gene (*SurB*) from the *Nicotiana tabacum* in *E. coli*. The mutant *SurB* gene was cloned in the bacterial expression plasmid pT7-7, in which expression of the tobacco ALS gene is under the control of the T7 RNA polymerase promoter (Studier *et al.*, 1990). Kinetic properties of the herbicide-

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resistant tobacco ALS were characterized, and its interaction with K11570, a new chemical class of herbicide, were examined. This study provides valuable information in assessing herbicide-resistant mutants for use in gene transfer, and will provide large amounts of tobacco ALS for its biochemical and structural characterization.

Materials and Methods

Enzymes, chemicals, and oligonucleotides

Bacto-tryptone, yeast extract, and bactor-agar were purchased from Difco. Restriction and modifying enzymes were from Boehringer Mannheim, Toyobo, and Amersham. ECL direct nucleic acid labeling and detection system and Hybond ECL membrane were from Amersham. DNA sequencing reagents including Sequenase version 2.0 were obtained from United States Biochemicals. K11570 and ALLY were a gift from Dr. Dae-Whang Kim of Korea Research Institute of Chemical Technology in Taejon, Korea. Oligonucleotides were obtained from Korea Biotechnology, Taejon, Korea.

Bacterial strains and plasmids

E. coli strain MF2000, and the plasmids pAGS148 and pTATX (Smith *et al.*, 1989) were obtained from Dr. B. J. Mazur (Du Pont). The expression vector pT7-7, M13 phage mGP1-2, and pGP1-2 were obtained from Drs. C. C. Richardson and S. Tabor (Harvard Medical School).

Construction of the expression vector pT7-ALS

The Nicotiana tabacum ALS gene (*SurB*) that is resistant to sulfonylurea herbicides was cloned into expression vector pT7-7 as follows: First, the shuttle vector pBals 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 *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, of the coding strand by polymerase chain reaction (PCR) with the oligonucleotide primers 1 and 2:

1: 5′-ATTCGGATCCAACACATATGGCGGCGGCTGCGG-3′
*Bam*HI *Nde*I

2: 5′-ATGCGGATCCTCAAAGTCAATAGG-3′
*Bam*HI

The PCR was carried out as described (Saiki *et al.*, 1988). Each reaction contained 10 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℃, 1 min; 37℃, 2 min; 72℃, 3 min. PCR products were subcloned by ethanol precipitation, restriction digestion with *Bam*HI, and ligation with *Bam*HI-treated pBluscript SK(−). Finally, the ALS gene was excised from the pBals with *Nde*I and *Bam*HI, isolated from 1% agarose gel, and cloned into the *Nde*I and *Bam*HI sites of pT7-7. The resulting expression vector pT7-ALS was used to transform the *E. coli* strain MF2000. Plasmid DNA was isolated according to the alkaline lysis method, and transformation of MF2000 cells was achieved using a standard $CaCl_2$ transformation protocol (Sambrook *et al.*, 1989).

PCR products were separated by electrophoresis on agarose gels. After transfer to Hybond ECL membrane, the PCR products were hybridized with labeled DNA probe generated from the Arabidopsis ALS gene (Southern, 1975). The membranes were washed and probed with the ECL substrate (Amersham, RPN 2106), according to the instruction of the supplier. ECL was detected with Kodak X-Omat film for variable exposure times. Following subcloning of the PCR product into XbaI treated pUC119, DNA sequence analysis of the PCR product was performed by dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Expression and extraction of a mutant and a wild-type plant ALS

The plasmid pT7-ALS was transformed into the strain MF2000, and the mutant tobacco ALS was expressed as described by Tabor (1992). The culture were then centrifuged at 5000 g for 10 min and the pellet was resuspended in 3 vol. of 100 mM Tris-HCl, pH 8.1. Lysozyme was added to 1 mg/mL, and the cells were 3~4 times frozen in liquid N_2 and thawed at 0℃. The lysate was centrifuged at 20,000 g for 15 min at 4℃, and the supernatant was collected. Extracts were stored at −70℃ with 10% (vol/vol) glycerol.

Expression of wild type *Arabidopsis* ALS were carried out as described by Smith *et al.* (1989), and extraction was carried out as above.

Assays of ALS

ALS activity in the cell extracts was measured according to the method of Westerfeld (1943) with a modification. The reaction mixture (200 μL) contained 50 mM potassium phosphate, pH 7.0, 20 mM pyruvate, 25 mM thiamine-pyrophosphate, 50 mM $MgCl_2$, 5 mM flavine adenine dinucleotide, and various concentrations of ALS inhibitors K11570 or ALLY. Assays were initiated by adding the cell extracts and terminated by adding 20 μL of 6 N H_2SO_4 after 1 h at 37℃. The acidified reaction mixtures were heated for 15 min at

60℃ 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℃. The absorbances of the solutions were determined at 525 nm. Specific activities of ALS were expressed as mol acetoin/min/mg protein. Protein was determined by the method of Lowry *et al.* (1951). The IC_{50} value for inhibition by inhibitors is defined as the concentration of K11570 or ALLY which inhibits ALS activity 50% in a 60-min fixed time assay carried out as described above. The data were fit to the equation

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

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

The cell extracts were electrophoresed on 7% polyacrylamide gels in the absence of SDS. The gel was sliced into 5 mm pieces, and incubated for 1 h at 37℃ in 50 mM potassium phosphate, pH 7.0, 100 mM pyruvate, 25 mM thiamine-pyrophosphate, 50 mM $MgCl_2$, and 5 mM flavine adenine dinucleotide. The eluted ALS enzyme activities were measured as described as above.

Results and Discussion

We wished to have the bacterial expression system for herbicide-resistant mutant tobacco ALS. In this study, we cloned surfonylurea resistant tobacco *SurB* gene into the bacterial expression plasmid pT7-7 as descried in Fig. 1. Southern blot analysis of the PCR product indicated that 2.0 kb PCR product hybridized with the labeled DNA probe generated from the *Arabidopsis* ALS gene (Mazur *et al.*, 1987), and the DNA sequences of the PCR product were consistent with those expected (data not shown). Plasmids pT7-ALS (with insert) and pT7-7 (without an insert) were separately transformed into the *E. coli* strain MF2000. The cells were selected on medium supplemented with valine, isoleucine, and ampicilline. Cells from individual colonies were then streaked on medium lacking valine and isoleucine. MF2000 cells transformed with pT7-7 did not grow in the absence of valine and isoleucine. On the other hand, cells transformed with pT7-ALS grew normally in the absence of valine and isoleucine. The result indicates that the herbicide resistant tobacco ALS gene genetically complements ALS-deficient *E. coli* strain MF2000.

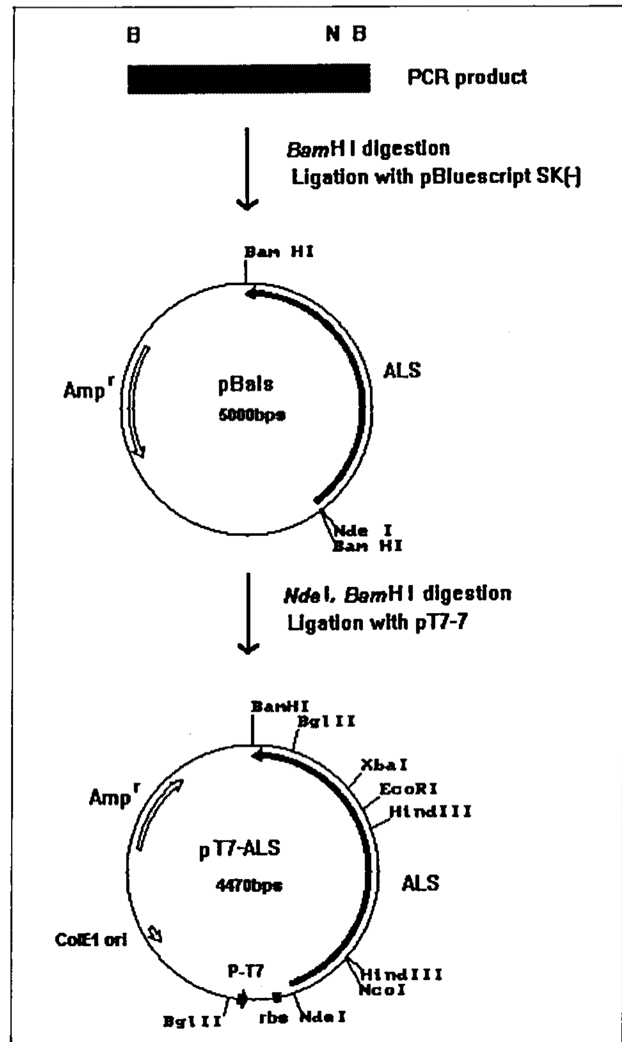

Fig. 1. Scheme of construction of the expression vector pT7-ALS. The shuttle vector pBals was constructed by transfering the PCR products of ALS gene into pBluescript SK (−). The PCR was carried out as described in "Material and Methods". The ALS gene was excised from the pBals with *Nde*I and *Bam*HI and cloned into the *Nde*I and *Bam*HI sites of pT7-7. ColE1 ori: origin of replication; rbs: ribosome binding site; Amp: ampicillin resistance; P-T7: T7 promoter; B: *Bam*HI; N: *Nde*I.

The subunit structure of ALS from higher plants is unknown. In *E. coli* and *S. typhimurim*, ALS occurs as a tetramer of two large 59~60 kD and two small 9~17 kDa subunits (Schloss *et al.*, 1985). In yeast, ALS occurs as a dimer of two large 75 kDa subunits (Poulsen *et al.*, 1989). Since the herbicide-resistant tobacco ALS gene can complement an ALS deficiency in *E. coli*, ALS from tobacco may not have a second subunit. Smith *et al.* (1989) suggested that ALS from *Arabidopsis* does not have a second subunit. Recently, Singh *et al.* (1992) suggested that plant ALS requires a second subunit for the feedback sensitivity since expression of the *Arabidopsis* ALS gene alone yields an enzyme that is insensitive to inhibition by valine+leu-

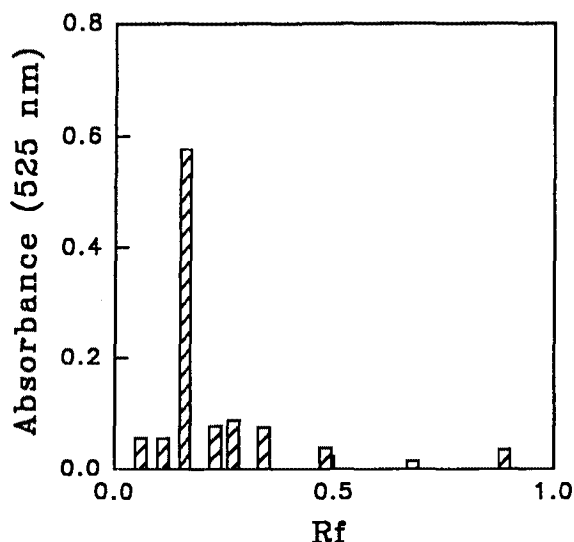

Fig. 2. Activity profile of tobacco ALS in native polyacrylamide gel. *E. coli* MF2000 cells carrying the pT7-ALS were grown, centrifuged, resuspended as described as in "Materials and Methods". The cell extracts were electrophoresed on 7% polyacrylamide gels in the absence of SDS. The gel was sliced into 5 mm pieces, and incubated for 1 h at 37℃ in 50 mM potassium phosphate, pH 7.0, 100 mM pyruvate, 25 mM thiamine-pyrophosphate, 50 mM $MgCl_2$, and 5 mM flavine adenine dinucleotide. The eluted ALS enzyme activities were measured as described as in "Materials and Methods".

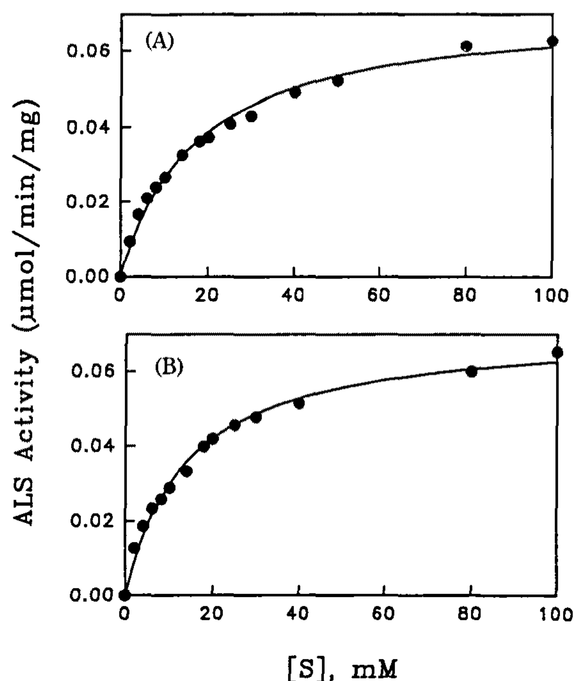

Fig. 3. Pyruvate saturation kinetics of ALS activity in crude extracts from *E. coli* MF2000 cells carrying the expression vectors pTATX (A) and pT7-ALS (B). The substrate pyruvate concentration was varied from 1 to 100 mM, and other assay conditions were as described in "Material and Methods". The curves are the best fit to equation 2, and the best fit parameters K_m are 16.8± 1.4 mM and 13.9± 1.1 mM for pTATX (A) and pT7-ALS (B), respectively.

cine. Further study of ALS, however, is needed to elucidate the subunit structure of the plant enzyme.

We demonstrated here that the herbicide-resistant tobacco ALS gene was functionally expressed in *E. coli*. ALS activites were 0.042 and 0.0002 μmol/min/mg protein in the extracts prepared from MF2000 cells transformed with pT7-ALS and pT7-7, respectively. For comparison, extracts prepared from MF2000 cells transformed with pTATX, which carried the wild type Arabidopsis ALS gene, were assayed and had a specific activity of 0.037. Smith *et al.* (1989) have reported that the wild type and mutant Arabidopsis ALS had specific activities of 0.065 and 0.043, respectively. Cell extracts prepared from MF2000 cells transformed with plasmid pT7-ALS were further examined by measuring ALS activity in the gel after electrophoresis of the extracts in the absence of SDS. A protein band with a relative mobility, R_f, of 0.16 was detected as shown in Fig. 2.

Fig. 3B shows pyruvate saturation kinetics of the herbicide-resistant tobacco ALS. Saturation of the ALS by pyruvate appreared hyperbolic. The kinetic data were fit to the equation

$$v=V_{max}/(1+K_m/[\text{pyruvate}]) \qquad (2)$$

by non-liner least-squares and the Simplex method for error minimization (Nelder and Mead, 1965). In this equation V_{max} is maximum velocity, and K_m is the Michaelis constant for pyruvate. The curve in Fig. 3B has been calculated with equation 2 and the best-fit parameters $V_{max}=5.30\pm 0.20\times 10^{-2}$ μmol/min/mg protein and $K_m=13.7\pm 1.1$ mM. For comparison, pyruvate saturation kinetics of the wild-type Arabidopsis ALS was also carried out. The curve in Fig. 3A has been calculated with equation 2 and the best-fit parameters $V_{max}=5.30\pm 0.45\times 10^{-2}$ μmol/min/mg protein and $K_m=16.8\pm 1.4$ mM. The K_m value for pyruvate in herbicide resistant tobacco ALS were similar to that for the wild-type *Arabidopsis* ALS. The results suggest that the mutations resulting in sulfonylurea resistance has not affected pyruvate binding and catalysis. Saari *et al.* (1990) have reported that the K_m values for pyruvate in sulfonylurea resistant kochia ALS were similar to that for the susceptible kochia ALS.

ALS activity of the extracts was also assayed in the presence of K11570, a new chemical class of herbicide. The chemical structure of K11570 is shown in Fig. 4A. IC_{50} values were determined for the resistant and sensitive plant enzymes extracted from the pT7-ALS and pTATX, respectively, as described in "Material and Methods". The mutant enzyme is insensitive to inhibition by K11570 having IC_{50} values of 0.13± 0.01 mM

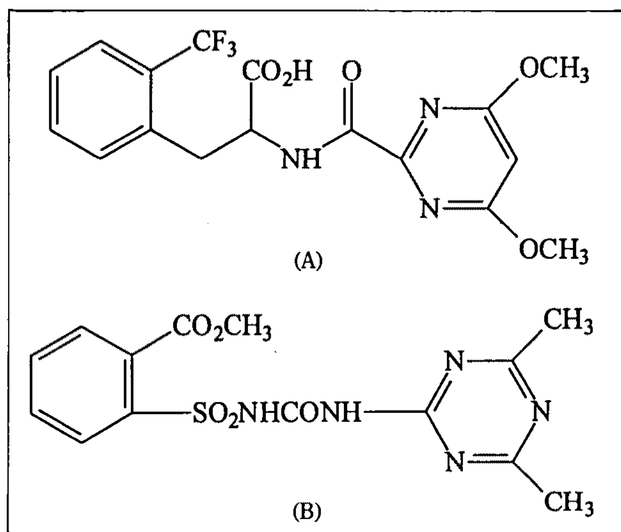

Fig. 4. The chemical structures of K11570 (A) and ALLY (B).

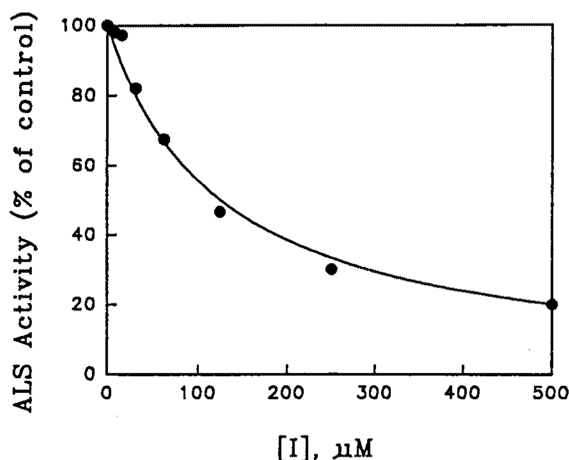

Fig. 5. Inhibition of tobacco ALS in crude extracts from *E. coli* MF2000 cells carrying the pT7-ALS by K11570. *E. coli* MF2000 cells carrying the pT7-ALS were grown, centrifuged, resuspended as drscribed as in "Material and Methods". The inhibitor K11570 concentration was varied, and other assay conditions were as described in "Material and Methods". The values are given as the percent of an untreated control. The curve is the best fit to equation 1, and the best fit parameter IC_{50} is 0.13± 0.01 mM.

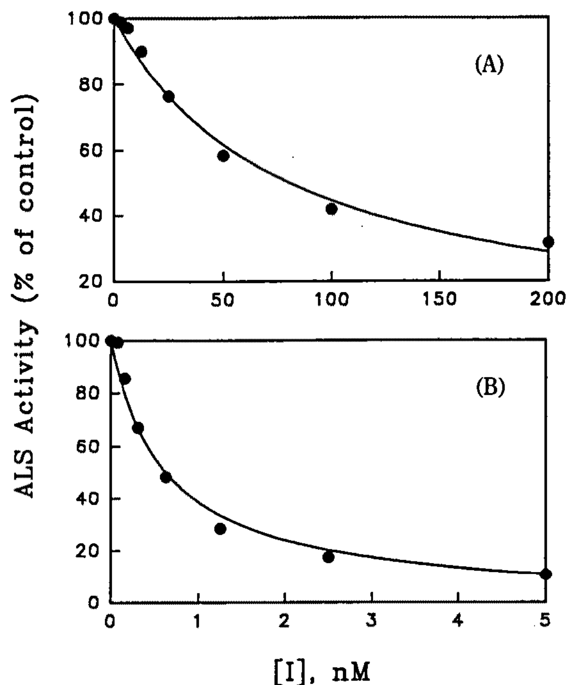

Fig. 6. Inhibition of Arbidopsis ALS in crude extracts from *E. coli* MF2000 cells carrying the pTATX by ALLY (A) and K11570 (B). *E. coli* MF2000 cells carrying the pTATX were grown, centrifuged, resuspended as drscribed as in "Material and Methods". The inhibitors ALLY and K11570 concentrations were varied, and other assay conditions were as described in "Material and Methods". The valuse are given as the percent of an untreated control. The curves are the best fit to equation 1, and the best fit parameters IC_{50} are 80± 5.6 nM and 0.63± 0.07 nM for ALLY (A) and K11570 (B), respectively.

(Fig. 5). In contrast, the wild-type enzyme is extremely sensitive to inhibition by K11570 having IC_{50} values of 0.63± 0.07 nM (Fig. 6B). For comparison, ALS activity of the extracts was assayed in the presence of ALLY, surfonylurea herbicides. The chemical structure of ALLY is shown in Fig. 4B. IC_{50} values for ALLY was 80± 5.6 nM (Fig. 6A). These results suggest that K11570 herbicides bind to the regulatory site of ALS enzyme, and that the two classes of herbicides interact at a commom site on ALS since the mutant is also insensitive to inhibition by sulfonylurea herbicides. Lee *et al.* (1988) have used cloned gene segments containing a sulfonylurea resistant form of *SurB* to transform sensitive lines of tobacco to a sulfonylurea resistance. It is likely that the transgenic plant is also resistant to K11570.

In summary, we demonstrated here that herbicide-resistant tobacco ALS gene can complement an ALS deficiency in *E. coli* and that the plasmid pT7-ALS can functionally express the mutant ALS which is insensitive to inhibition by K11570 herbicide. The next step in biochemical charaterization of this enzyme is to purify the mutant ALS and to carry out N-terminal amino acid sequencing.

Acknowledgement

This work was supported by a research grant for genetic engineering from the Ministry of Education (1993) and in part a research grant from the Korea Science and Engineering Foundation (92-46-00-01). We thank Dr. Babara J. Mazur and Tony Guida (Du Pont) for the plasmids pAGS148 and pTATX, and *E. coli* strain MF2000. We thank Drs. Charles C. Richardson and Stanley Tabor (Harvard Medical School) for the M13 phage mGP

1-2, and plasmids pT7-7 and pGP1-2. We thank Dr. Ook-Joon Yoo and Mr. S. B. Hong (KAIST) for their help in DNA sequencing. We thank Drs. Kyeong-A Sin, Jung-Do Choi, and Tae-Ju Cho (Chungbuk National University), and Dr. Young Tae Kim (National Fisheries University of Pusan) for the many fruitful discussions which we had during the course of this work. We thank Dr. Dae-Whang Kim (Korea Research Institute of Chemical Technology) for providing us with herbicides K11570 and ALLY.

References

Babczinski, P. and Zelinski, T. (1991) *Pest. Sci.* **31**, 305.

Chaleff, R. S. and Mauvaris, C. J. (1984) *Science* **224**, 1443.

Choi, J.-D., Moon, H., Chang, S.-I., Chae, J.-K. and Shin, J.-H. (1993) *Korean Biochem. J.* **26**, 638.

Gerwick, B. C., Subramanian, M. V., Loney-Gallant, V. and Chandler, D. P. (1990) *Pest. Sci.* **29**, 357.

LaRossa, R. A. and Schloss, J. V. (1984) *J. Biol. Chem.* **259**, 8753.

Lee, K. Y., Townsend, J., Tepperman, J., Black, M., Chui, C. F., Mazur, B., Dunsmuir, P. and Bedbrook, J. (1988) *EMBO J.* **7**, 1241.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265.

Mazur, B. J., Chui, C. F. and Smith, J. K. (1987) *Plant physiol.* **85**, 1110.

Nelder, J. A. and Mead, R. (1965) *Comput. J.* **7**, 308.

Poulsen, C. and Stougaard, P. (1989) *Eur. J. Biochem* **185**, 433.

Ray, T. B. (1984) Plant Physiol. **75**, 827.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. (1988) *Science* **239**, 487.

Saari, L. L., Cotterman, J. C. and Primiani, M. M. (1990) *Plant Physiol.* **93**, 55.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, A laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463.

Schloss, J. V., Dyk, D, E., Vasta, J. F. and Kutny, R. M. (1985) *Biochemistry* **24**, 4952.

Shaner, D. L., Anderson, P. C. and Stidham, M. A. (1984) *Plant Mol. Biol.* **76**, 545.

Sin, K.-A, Kim, H.-J., Choi, J.-D. and Chang, S.-I. (1994) *Korean Biochem. J.* **27**, 534.

Singh, B., Szamosi, I., Hand, J. M. and Misra, R. (1992) *Plant Physiol.* **99**, 812.

Smith, J. K., Schloss, J. V. and Mazur, B. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4179.

Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60.

Tabor, S. (1992) in *Current Protocals in Molecular Biology* (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Scidman, J. G., Smith, J. A., Struhl, K. eds.) 2nd ed., pp. 16-6, Wiley, New York.

Westerfeld, W. W. (1943) *J. Biol. Chem.* **161**, 495.

Wiersma, P. A., Hachey, J. E., Crosby, W. L. and Moloney M. M. (1990) *Mol. Gen. Genet.* **224**, 155.

Wiersma, P. A., Schmiemann, M. G., Condie, J. A., Crosby, W. L. and Moloney, M. M. (1989) *Mol. Gen. Genet.* **219**, 413.