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Effect of Pyrimidylsalicylate on the Valine Sensitive Acetolactate Synthase Purified from *Serratia marcescens*

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Abstract : The inhibitory effect of herbicides such as sulfonylurea derivatives, imidazolinones and pyrimidylsalicylate has been examined on the purified valine sensitive acetolactate synthase (ALS) from Serratia marcescens. The concentration of sulfometuron methyl which inhibits 50% of the ALS activity was 2.5 mM. The required concentrations of triasulfuron, primisulfuron methyl and imazaquin for the 50% inhibition of the ALS activity were 1 mM. The resistance of *Serratia* ALS to sulfometuron methyl, imazapyr and imazaquin is similar to that of *E. coli* ALS I. However, pyrimidylsalicylate showed a potent inhibitory effect on the *Serratia* ALS almost 13 times more potent than on *E. coli* ALS II, which is known as herbicide-sensitive isozyme. The inhibitory mode was competitive against pyruvate. I_{50} value was determined to be 17 μM in an assay mixture containing 20 mM pyruvate, and the K_i value was calculated to be 0.4 μM from the modified double reciprocal plot of 1/V versus $1/S^2$.

Key words : enterobacterial acetolactate synthase isozymes, pyrimidylsalicylate, *Serratia* acetolactate synthase.

The widely distributed acetolactate synthase (EC 4.1.13.18) in bacteria, yeast and higher plants has attracted the attention of many researchers because it has been known as the target enzyme of structurally unrelated herbicides, sulfonylureas, imidazolinones, and triazolopyrimidines (Chaleff and Mauvais, 1980; La Rossa and Schloss, 1984; Ray, 1984; Mazur *et al.*, 1987; Kleschick *et al.*, 1990). Genetic analysis of the three types of acetolactate synthase (ALS) isozymes in bacteria has shown that ALS I is encoded by the *ilvBN* gene, ALS II by the *ilvGM*, and ALS III by the *ilvIH* gene, respectively (De Felice *et al.*, 1982; Squires *et al.*, 1983; Friden *et al.*, 1985). The nucleotide sequence of ALS isozymes showed that the homology between large subunits of these isozymes is above 40%, and small subunits are about 20% (De Felice *et al.*, 1982; Wek *et al.*, 1985). The purification of ALS has been mainly done in *E. coli* and *Salmonella typhimurium*, and their physical and chemical properties such as molecular size, substrate specificity, feedback regulation, and cofactor requirements have been well characterized (Grimminger and Umbarger, 1979; Eoyang and Silverman, 1984; Schloss *et al.*, 1985).

In *Salmonella typhymurium*, sulfometuron methyl binds reversibly to the active site of ALS II (La Rossa and Schloss, 1984). The evidence that imazapyr binds to bacterial ALS II and ALS III has been reported by Schloss *et al.* (1988). The herbicidal inhibitory effect of imidazolinones on the activity of plant ALS has also been shown (Stidham and Shaner *et al.*, 1990; Durner *et al.*, 1991). On the other hand, the resistance of bacterial ALS I to sulfometuron methyl has been reported (La Rossa and Sumulski, 1984). The sulfometuron methyl-resistant mutant forms of ALS have also been isolated from yeast, *Nicotiana tobacum* and *Arabidopsis thaliana* (Chaleff and Ray, 1984; Falco and Dumas, 1985). A biochemical and genetic study of these mutant forms of ALS have shown that their herbicidal resistance to sulfometuron methyl was derived from the production of altered forms of ALS by a single amino acid substitution (Yadav *et al.*, 1986).

The valine sensitive *Serratia* ALS has somewhat unusual enzymatic characteristics in terms of a substrate saturation curve against pyruvate and a different molecular size of a small subunit than any other bacterial ALS isozymes (Yang and Kim, 1992; Yang and Kim, 1993). Previous studies on the active site of the *Serratia* ALS using a chemical modification method utilizing group specific reagents suggested that Arg, Cys and Trp might be present on the active site of the *Serratia* ALS (Choi and Kim, 1995). In this study, we have

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investigated the inhibitory effect of various herbicides on the ALS purified from *Serratia marcescens*. These studies indicate that the valine-sensitive *Serratia* ALS shows sulfonylurea and imidazolinone resistance like *E. coli* ALS I. On the other hand, the enzyme was strongly inhibited by pyrimidylsalicylate, unlike *E. coli* ALS isozymes.

Materials and Methods

Reagents

The analytical grade of herbicides, sulfometuron methyl, triasulfuron, cinosulfuron, primisulfuron methyl, imazapyr, imazaquin, and pyrimidylsalicylate were kindly supplied by the LG Biotechnology Corporation (Taejon, Korea). Pyruvate, thiamine pyrophosphate (TPP), flavine adenine dinucleotide (FAD), $MgCl_2$, potassium phosphate (mono basic), α-naphtol, and glycerol were purchased from Sigma Chemical Co. BHI medium was the product of Gibco. All other reagents used were reagent grade.

Bacterial strain and culture condition

The bacterial strain used in this study was *Serratia marcescens* ATCC 25419, obtained from Professor H. D. Braymer of Louisiana State University. Cells (2 l/batch) were grown aerobically for 15 h in 5 l culture flasks containing brain heart infusion (BHI medium) at 30°C at 60 rpm on a reciprocal shaker. Cells were harvested by centrifugation (12,000×*g*, 30 min) when the optical density of the culture at 660 nm was 1.0. The harvested cells were stored at −70°C until use.

Purification of the *Serratia* ALS

The valine sensitive ALS isozyme from *Serratia marcescens* ATCC 25419 was purified to homogeneity by the procedures of ammonium sulfate fractionation, DEAE-Sephacel chromatography with potassium phosphate gradient elution, hydroxylapatite chromatography with potassium phosphate gradient elution, and Sephadex G-200 gel filtration chromatography as described by Yang and Kim (1993).

Enzyme assay

One ml of reaction mixture containing 10 mM $MgCl_2$, 40 mM pyruvic acid, 0.17 mM TPP, 24 μM FAD and enzyme solution in 0.1 M potassium phosphate buffer (pH 7.5) was incubated at 37°C for 30 min. The enzyme reaction was stopped by adding 5 μl of 50% (v/v) H_2SO_4. After the addition of 200 μl of 0.5% (w/v) creatine and 200 μl of 5% (w/v) α-naphtol in 2.5 N NaOH solution at 37°C for 30 min, the absorbance of supernatant of the reaction product was measured at 540 nm. One unit of ALS activity is defined as the amount of enzyme that is required for the production of 1 mol acetolactate per min. The quantity of protein was determined by the Lowry method.(Lowry *et al.*, 1951)

Effect of various herbicides on the activity of *Serratia* ALS

The enzyme (10 nM) was incubated with various concentrations of herbicides for 30 min at 37°C in a standard assay condition. The reaction solution was comprised of 10 mM $MgCl_2$, 20 mM pyruvic acid, 0.17 mM TPP, 24 μM FAD in 0.1 M potassium phosphate buffer(pH 7.5). The remaining activity was measured as described above. The herbicides used were sulfometuron methyl, cinosulfuron, triasulfuron, primisulfuron methyl, imazapyr, imazaquin, and pyrimidylsalicylate.

The inhibitory effect of pyrimidylsalicylate on *Serratia* ALS

The concentration of pyrimidylsalicylate which inhibits 50% of ALS activity in standard assay condition containing 20 mM pyruvic acid at 37°C is defined as I_{50} value. The I_{50} value was calculated from the following equation:

$$\% \text{ activity} = 100/(1+[I]/I_{50})$$

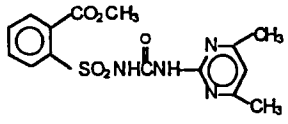
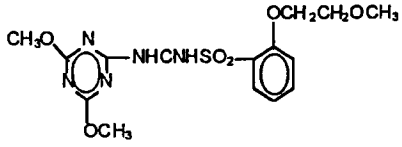
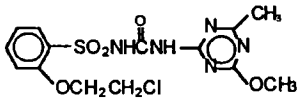
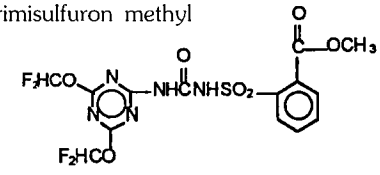
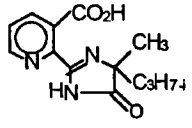
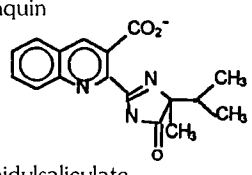
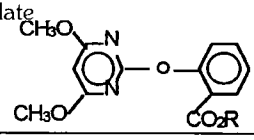
where % activity equals the amount of activity in the presence of pyrimidylsalicylate concentration as a percent of an untreated control, and [I] equals the inhibitor concentration. To determine the type of inhibition by pyrimidylsalicylate, ALS activities were measured with different concentrations of pyrimidylsalicylate in a standard assay condition containing 4, 6, 8 and 10 mM pyruvic acid, respectively. The concentrations of pyrimidylsalicylate used were 0, 0.05 and 0.1 μM. The apparent K_i value was calculated by using a Dixon plot (Dixon, 1953).

Results and Discussion

The effect of various herbicides on the activity of *Serratia* ALS

In this report we have examined the inhibitory effect of well known herbicides such as sulfometuron methyl, cinosulfuron, triasulfuron, primisulfuron methyl, imazapyr, imazaquin, cinosulfuron and pyrimidylsalicylate on valine-sensitive ALS purified from *Serratia marcescens* ATCC 25419. In weed species, *Stellaria media*, chlorsulfuron, metasulfuron, triasulfuron and sulfometuron have potent inhibitory effects with I_{50} values

Table 1. Effect of various herbicides on the activity of ALS purified from *Serratia marcescens* ATCC 25419

Herbicide	Structure	Concentration (mM)	% Activity[a]
Sulfometuron methyl		0.25	97
		0.5	91
		0.75	82
		1.0	80
		2.5	50
Cinosulfuron		0.25	94
		0.5	90
		0.75	85
		1.0	80
Triasulfuron		0.25	86
		0.5	66
		0.75	63
		1.0	49
Primisulfuron methyl		0.25	87
		0.5	72
		0.75	65
		1.0	50
Imazapyr		0.25	95
		0.5	90
		0.75	85
		1.0	80
Imazaquin		0.25	98
		0.5	97
		0.75	70
		1.0	50
Pyrimidylsalicylate (a) R=H		0.001	91
		0.0025	77
		0.005	72
		0.025	40

[a] The data are expressed as a percentage of the nonherbicide treated control.

ranging 10~100 μM (Devine *et al.*, 1991). It has also been reported that triasulfuron and primisulfuron methyl have strong inhibitory effects in cereal and corn with I_{50} values of 10~100 nM (Brown, 1990). As shown in Table 1, the inhibitory effect of pyrimidylsalicylate on *Serratia* ALS was very powerful requiring only 25 μM to inhibit about 60% enzyme activity. The inhibitory effect of other herbicides were not so potent, requiring mM herbicide concentration ranges to inhibit 50 to 80% activity. It is interesting to compare the structural features of pyrimidylsalicylate with that of other nonsensitive herbicides such as sulfonylureas. For example, sulfonylureas have two aromatic rings linked by a sulfonamide bond (-SO_2-NH-CO-NH-), whereas pyrimidylsalicylate has two aromatic rings linked by ether (-O-) bond. Therefore, the shorter ether bond between two aromatic rings in pyrimidylsalicylate might be the reason why it is easily accessible to the active site pocket of *Serratia* ALS.

Table 2. Comparison of the I_{50} value for sulfometuron methyl on ALS from various sources of bacteria and plants

Enzyme sources	I_{50} value Sulfometuron methyl (nM)	References
Serratia ALS	2.500.000	
Bacterial ALS I	>1.000.000	La Rossa and Sumulski (1984)
ALS II	65	La Rossa and Schloss (1984)
ALS III	<1.000.000	La Rossa and Sumulski (1984)
Yeast	20	Falco and Dumas (1985)
Pea	16	Ray (1984)
Tobacco	8	Chaleff and Mauvais (1980)

Comparision of sulfometuron and imidazolinone inhibition among ALSs from various sources

The inhibitory effect and its action mechanism of structurally unrelated well known herbicides, sulfometuron methyl and imidazolinone(imazapyr and imazaquin) on ALS have been extensively studied on bacterial and plant ALS previously. The herbicidal action of sulfometuron methyl on *Salmonella typhimurium* ALS II showed a reversible biphasic inhibition with an initial K_i of 660~60 nM and a final steady state K_i of 60 nM suggesting competition between the herbicide and pyruvate at the active site (La Rossa and Schloss, 1984). In plants, the herbicidal inhibitory effect of imidazolinone has been observed on corn, peas and Black Mexican sweet corn cells with a noncompetitive or uncompetitive pattern (Shaner *et al.*, 1984; Singh *et al.*, 1988; Stidham and Shaner *et al.*, 1990). Imidazolinone and imazaquin also compete with radiolabeled sulfometuron methyl for a common site on ALS (Schloss *et al.*, 1988). In this study, the resistance of the *Serratia* ALS to sulfometuron methyl is similar to that of *E. coli* ALS I. The concentration of sulfometuron methyl and imazaquin for 50% inhibition on *Serratia* ALS was 2.5 mM and 1 mM, respectively (Table 1 and 2). Especially, the I_{50} value for sulfometuron methyl inhibition of *Serratia* ALS was very high as compared with many sulfometurom methyl sensitive ALSs from other bacterial and plant sources (Table 2).

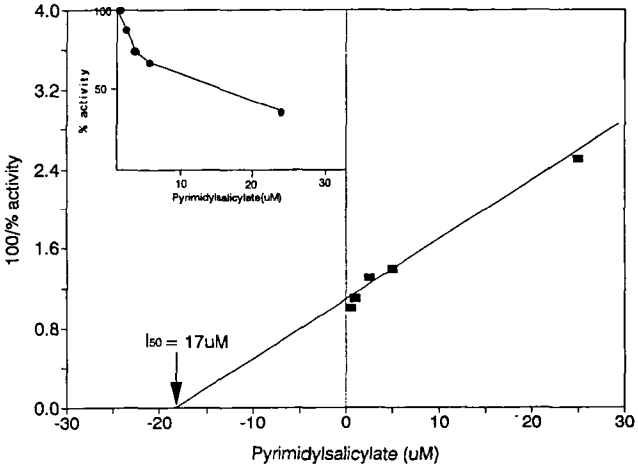


Fig. 1. Determination of I_{50} value of pyrimidylsalicylate.

Table 3. Comparison of I_{50} values of pyrimidylsalicylate on ALS from *Serratia*, yeast, and plant cell cultures of *Catharanthus roseus*

Enzyme sources	I_{50} (μM)	References
Serratia marcescens	17	
Yeast	21	Babczinski and Zelinski (1991)
C. roseus	0.042	Babczinski and Zelinski (1991)
E. coli ALS II	217	Babczinski and Zelinski (1991)

The inhibitory effect and mode of action of pyrimidylsalicylate on *Serratia* ALS

The inhibitory effect of pyrimidylsalicylate on *Serratia* ALS shows that the I_{50} value for pyrimidylsalicylate is 17 μM (Fig. 1 and Table 3). The inhibitory effect of pyrimidylsalicylate on *Serratia* ALS is comparable to that on yeast ALS but almost 400 times less sensitive when compared with *Catharanthus roseus* ALS (Table 3). The substrate saturation curve of *Serratia* ALS against pyruvate has shown sigmoidity, and the modified double reciprocal plot of 1/V versus $1/S^2$ shows that pyrimidylsalicylate competes with pyruvate on the binding site of *Serratia* ALS (Fig. 2). Analysis of the Dixon plot indicated that the K_i value is 0.4 μM (Fig. 3). In a time course assay, inhibition of *Serratia* ALS by pyrimidylsalicylate increases with incubation time and reversible slow binding kinetics was observed similar to sufometuron methyl inhibition on *S. typhimurium* ALS II (J. H. Yang and S. S. Kim, unpublished observation). The slow phase of inhibition was suggested by a slow change in the oligomeric state of ALS by FAD, or it could be due to the tightening of the interaction between the enzyme and inhibitor in a time-dependent manner as previously reported (Grimminger and Umbarger, 1979). A previous study indicates that *E. coli* ALS I has common physical and chemical properties with *Serratia* ALS as far as substrate specificity and valine sensitivity are concerned (Yang and Kim, 1993). Among the three isozymes of ALS from enteric bacteria, only ALS II is unique in terms of a high level of sensitivity to all three major classes of herbicides, such as sulfoylurea, imidazolinone, and triazolo pyrimidine (Table 2 and 3). *Serratia* ALS showed a very low sensitivity to sulfometuron methyl like ALS I and a high level of sensitivity to pyrimidylsalicylate. Especially, pyrimidylsalicylate has about a 13-fold less inhibitory effect on *E. coli* ALS II when compared with the *Serratia*

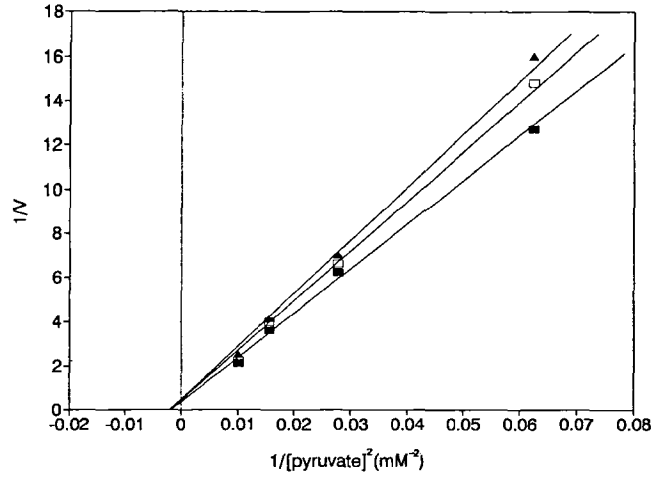


Fig. 2. Inhibition of the *Serratia* ALS by pyrimidylsalicylate. Activities were measured in 0.1 M potassium phosphate buffer (pH 7.5) at 37°C with different concentrations of pyruvate. The concentrations of pyrimidylsalicylate used were 0 μM (■), 0.05 μM (□), and 0.1 μM (▲).

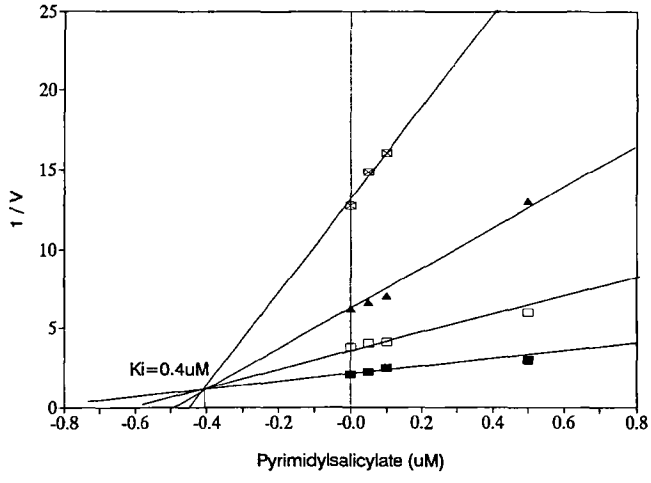


Fig. 3. Dixon plot for the inhibition of *Serratia* ALS by pyrimidylsalicylate. The concentrations of pyruvate used were 4 mM (○), 6 mM (●), 8 mM (□), and 10 mM (■).

ALS (Table 3). These results suggest that the *Serratia* isozyme has a somewhat different active site environment when compared with other enterobacterial ALS isozymes.

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