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Chemical Modification of *Serratia marcescens* Acetolactate Synthase with Cys, Trp, and Arg Modifying Reagents

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Abstract: Acetolactate synthase purified from *Serratia marcescens* ATCC 25419 was rapidly inactivated by the thiol specific reagent p-chloromercuribenzoate (PCMB), the tryptophan specific reagent N-bromosuccinimide (NBS), and the arginine modifying reagent phenylglyoxal (PGO). Inactivation by PCMB was prevented by both α-ketobutyrate and pyruvate, and the second order rate constant for the inactivation was 2480 $M^{-1} \cdot min^{-1}$. The reaction order with respect to PCMB was 0.94. The inactivation of the enzyme by NBS was also substantially reduced by both α-ketobutyrate and pyruvate. The second order rate constant for inactivation by NBS was 15,000 $M^{-1} \cdot min^{-1}$, and the reaction order was 2.0. On the other hand, inactivation by PGO was partially prevented by α-ketobutyrate, but not by pyruvate. The second order rate constant for the inactivation was 1480 $M^{-1} \cdot min^{-1}$ and the order of reaction with respect to PGO was 0.75. These results suggest that essential cysteine, tryptophan and arginine are located at or near the substrate binding site.

Key words: Chemical modification, *Serratia*, Acetolactate Synthase.

Acetolactate synthase (ALS; EC 4.1.3.18), also known as acetohydroxyacid synthase, catalyzes the first common step in the biosynthesis of branched-chain amino acids. This enzyme catalyzes the condensation of two molecules of pyruvate, or one molecule of pyruvate and α-ketobutyrate to form acetolactate or acetohydroxybutyrate, respectively (Umbarger, 1978). Catalytic activity requires flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), and a divalent cation (Grimminger and Umbarger, 1979; Barak *et al.*, 1988a). Three major isozymes of ALS have been identified in enteric bacteria (Squires *et al.*, 1983; Wek *et al.*, 1985). Biochemical and genetic analysis indicates that ALS I and ALS II are composed of two dissimilar polypeptides of 60 kDa and 10 kDa (Eoyang and Silverman, 1984; Schloss *et al.*, 1985). ALS III is also composed of two subunits having molecular weights of approximately 60 kDa and 18 kDa (Lago *et al.*, 1985; Barak *et al.*, 1988a). A number of reports have suggested that ALS I has a relative preference for acetolactate formation, whereas ALS II and III are able to effectively catalyze acetohydroxybutyrate synthesis (Barak *et al.*, 1988b).

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An increased interest in the mechanism of action of ALS has ensued with the knowledge that this enzyme is the target of three chemical families of highly active herbicides, namely, sulfonyl urea, imidazolinone, and triazolo pyrimidine or sulfonanilide (LaRossa and Schloss, 1984; Muhitch *et al.*, 1984; Schloss *et al.*, 1988; Gerwick *et al.*, 1990). Mutants that are resistant to these herbicides have been described in bacteria, yeast, and higher plants (Kishore and Shah, 1988; Lee *et al.*, 1988) and resistance is a consequence of an altered ALS enzyme. Schloss *et al.* (1988) demonstrated that all three classes of herbicides are not only competitive with each other, but also with quinone, for binding to ALS. There are also indications that the quinone/herbicide binding site of ALS has some, as yet, undiscovered regulatory or mechanistic role (Schloss and Aulabangh, 1988). Furthermore, bromopyruvate affinity labeling of ALS II showed close proximity between the binding sites of FAD, TPP, and sulfometuron methyl (SM) herbicide (van Dyk and Schloss, 1987).

Despite detailed studies on the mechanism of action of herbicides, little is known about residues present in the enzymatic active site. This study investigates chemical modification of the purified valine sensitive ALS I isozyme from *Serratia marcescens* (Yang and Kim, 1992; 1993). Evidence is presented that cysteine, tryp-

tophan, and arginine residues are in or near the active site of the Serratia ALS isozyme.

Materials and Methods

Bacterial strain and culture conditions

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) broth (Difco Lab., Detroit) at 30℃ at 60 rpm on a reciprocal shaker. Cells were harvested by centrifugation (12,000 x g, 30 min) when the optical density of the culture at 660 nm was 1.0. The harvested cells were stored at −70℃ until use.

Chemicals

Hydroxyapatite was purchased from Bio-Rad; Sephacel S-200 was obtained from Pharmacia Fine Chemicals Inc.; α-naphtol was purchased from BHD Biochemical; and N-bromosuccinimide and trinitrobenzenesulfonic acid were obtained from Fluka Co. All other reagents and chromatographic resins used were of analytical grade purchased from Sigma Chemical Co.

Enzyme assay and protein measurements

The activity of ALS was assayed by the procedure of McEwen and Silverman (1980). One ml of reaction mixture contained 0.1 M potassium phosphate buffer (pH 8.0), 10 mM $MgCl_2$, 40 mM pyruvate, 0.1 mM TPP, 0.1 mM FAD, and the enzyme solution. The reaction mixture was incubated at 37℃ for 30 min, then the reaction was stopped by adding 1 ml 50% H_2SO_4. For the measurement of acetoin formation, the developing reagent, which consisted of 200 μl of 5% α-naphtol in 2.5 N NaOH and 200 μl of 0.5% creatine, was added to the reaction mixture. After incubation for 30 min the mixture was centrifuged at 12,000 x g for 20 min. The absorbance of supernatant was measured for acetoin formation at 540 nm using a Kontron 930 UV/VIS spectrophotometer (Model UVIKON 930). One unit of enzyme activity was defined as the amount of enzyme which was required for the production of 1 μmol acetolactate per minute, and the specific activity was U/mg protein. The amount of protein was determined by the method of Lowry *et al.* (1951).

Enzyme purification

The valine sensitive acetolactate synthase isozyme from *Serratia marcescens* ATCC 25419 was purified to homogeneity by ammonium sulfate fractionation, DEAE-Sephacel chromatography, and hydroxyapatite chromatography followed by Sephadex G-200 gel filtration chromatography as described by Yang and Kim (1993).

Chemical modification of the Cys, Arg and Trp residues of Acetolactate Synthase

Modifications of the cysteine, arginine, and tryptophan residues of acetolactate synthase were carried out as described by Lundblad and Noyes (1984). For modification of the Cys residues of the enzyme, the enzyme solution (70 μl, 86 μg/ml) was reacted with different concentrations of p-chloromercuribenzoate (PCMB) in 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$ and 1% glycerol at 25℃. Aliquots were removed after various incubation times and the remaining enzyme activity was measured. The effect of another sulfhydryl reagent, N-ethylmaleimide (NEM), was also tested by incubating the enzyme with this modifier in the same buffer at 25℃ for 10 min. The concentrations of NEM were fixed at 0.5 mM and 1.0 mM.

Modification of the Trp residues of the enzyme with N-bromosuccinimide (NBS) was performed in 50 mM sodium acetate buffer (pH 4.5) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 1% glycerol. The enzyme solution (70 μl, 86 μg/ml) was reacted with different concentrations of NBS at 25℃ and aliquots of the reaction mixture were removed for the measurement of residual enzyme activity.

Modification of the Arg residues of the enzyme was carried out by reacting 70 μl of enzyme solution (86 μg/ml) with different concentrations of phenylglyoxal (PGO) in 50 mM sodium bicarbonate buffer (pH 8.4) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 1% glycerol. At regular intervals, aliquots of the reaction mixture were removed for the measurement of residual enzyme activity.

Reaction of acetolactate synthase with other chemical modifiers

The purified enzyme solution (70 μl, 86 μg/ml) was incubated with 0.5 mM and 1 mM concentrations of the modifying reagents phenylmethylsulfonylfluoride (PMSF), pyridoxal-5′-phosphate (PLP) and trinitrobenzenesulfonic acid (TNBS) in 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM FAD, 10 μM TPP, 1 mM $MgCl_2$, and 1% glycerol for 10 min at 25℃. Stock solutions of PMSF were prepared by dissolution in 99% ethanol just before use. The ethanol concentration of the reaction mixture was kept at less than 5% so that it did not affect the enzyme activity.

Protection of enzyme activity by pyruvate and α-ketobutyrate

Protection against inactivation of the enzyme by PCMB, PGO and NBS was performed by preincubation of the enzyme with either 10 mM pyruvate or α-ketobutyrate for 10 min at 25℃ before the addition of each modifying reagent. Aliquots of the reaction mixture were removed for the measurement of enzyme activity.

Results and Discussion

Inactivation of acetolactate synthase by PCMB

The purified enzyme (0.1 μM) was inactivated by incubation with various concentrations of PCMB in 50 mM potassium phosphate buffer, pH 7.0. The time course for inactivation of the enzyme was dependent on the concenteration of the reagent used. A semi-log plot of residual activity versus incubation time yielded a biphasic inactivation pattern: initially rapid, then slowing, indicating that there may exist two types of modifiable sulfhydryl residues (Fig. 1A). From the slopes of the faster inhibition phase obtained at different concentrations of PCMB, the second order rate constant for inactivation was 2480 $M^{-1}\cdot min^{-1}$ (Fig. 1B). A double logarithmic plot of the reciprocal of the half-time of inactivation against PCMB concentrations yielded a reaction order of 0.94 with respect to the inhibitor (Fig. 1C), suggesting that there is one essential sulfhydryl residue per active molecule.

Inactivation of acetolactate synthase by NBS

The effect of NBS, which is an tryptophan modification reagent, on the activity of acetolactate synthase was examined in a 50 mM sodium acetate buffer, pH 4.5. The reaction of acetolactate synthase (0.1 μM) with 90 μM NBS caused a complete loss of enzyme activity within 6 min (Fig. 2A). A semi-log plot of activity against time shows a biphasic mode; initially rapid, then slowing, indicating the existence of two types of Trp residues whose modification affects enzyme activity (Fig. 2A). From the initial, fast inactivation reaction represented in Fig. 2A, the second order rate constant of 15,000 $M^{-1}\cdot min^{-1}$ was determined from the slope of the linear relationships between the k_{obs} values and NBS concentrations (Fig. 2B). A double logarithmic plot of the reciprocal of the half-time of inactivation against reagent concentration yielded a reaction order of 2.0 with respect to the inhibitor (Fig. 2C), suggesting that there may be two essential tryptophan residues per active molecule.

Inactivation of acetolactate synthase by PGO

Incubation of acetolactate synthase with various concentrations of PGO resulted in a time-dependent loss of enzyme activity. A semi-log plot of the residual activity versus time yielded a linear inactivation pattern (Fig. 3A). From this plot, the second order rate constant for inactivation was 1480 $M^{-1}\cdot min^{-1}$ (Fig. 3B). The reaction order with respect to PGO was 0.75 from a double logarithmic plot of the reciprocal of the half-

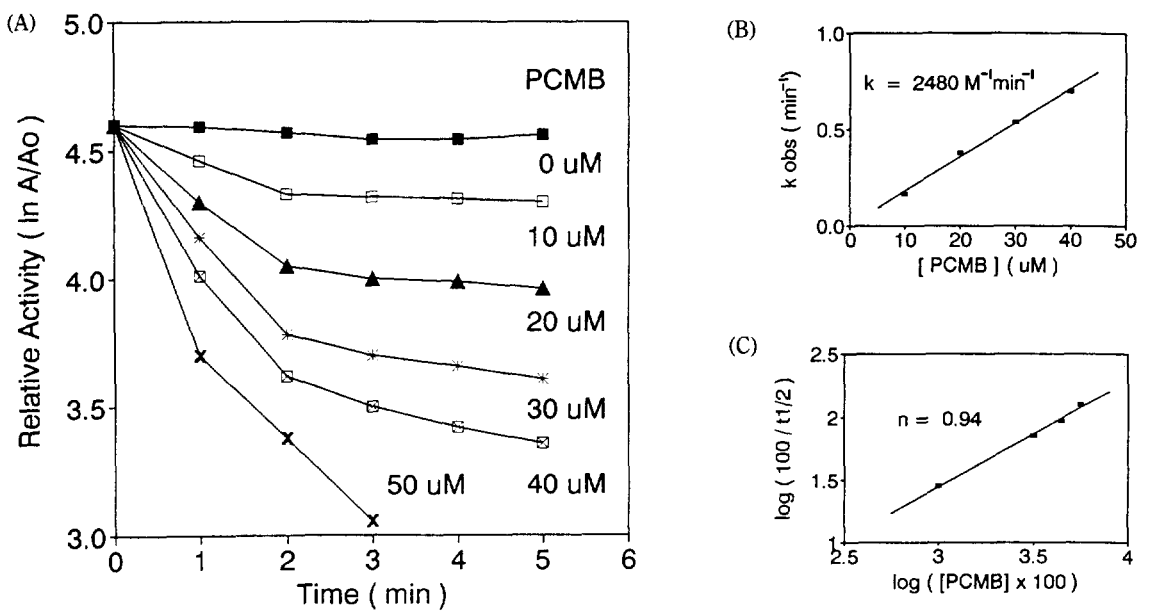

Fig. 1. (A) Inactivation of acetolactate synthase by p-chloromercuribenzoate (PCMB). The enzyme (0.1 μM) was incubated with various concentrations of PCMB in 50 mM potassium phosphate buffer (pH 7.0) at 25℃. (B) Plot of pseudo first-order rate constant for the inactivation of acetolactate synthase (k_{obs}) obtained at various concentrations (μM) of PCMB. (C) Double-logarithmic plot of the half-times of inactivation of acetolactate synthase at various concentrations of PCMB.

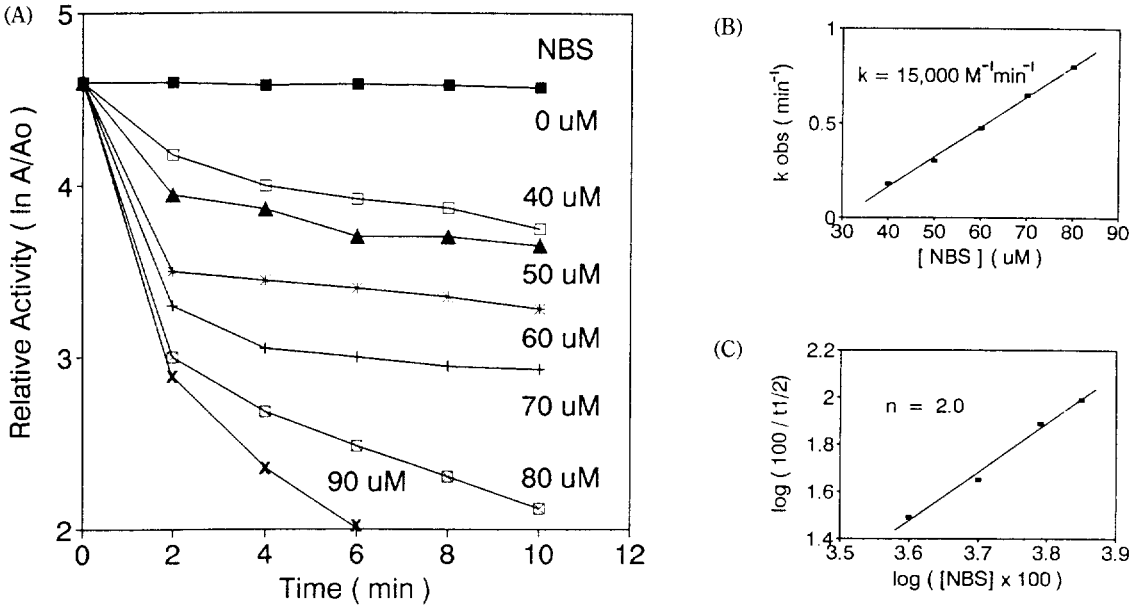

Fig. 2. (A) Inactivation of acetolactate synthase by N-bromosuccinimide (NBS). The enzyme (0.1 μM) was incubated with various concentrations of NBS in 50 mM sodium acetate buffer (pH 4.5) at 25℃. (B) Plot of pseudo first-order rate constant for the inactivation of acetolactate synthase (k_{obs}) obtained at various concentration (μM) of NBS. (C) Double-logarithmic plot of the half-times of inactivation of acetolactate synthase at various concentration of NBS.

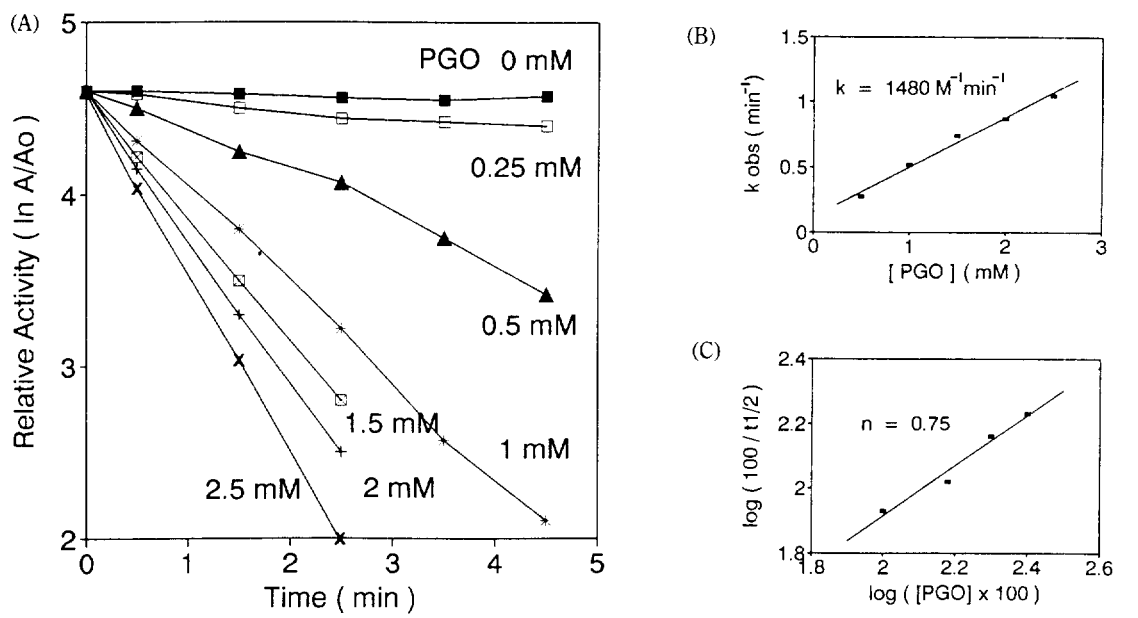

Fig. 3. (A) Inactivation of acetolactate synthase by phenylglyoxal (PGO). The enzyme (0.1 μM) was incubated with various concentrations of PGO in 50 mM sodium bicarbonate buffer (pH 8.4) at 25℃. (B) Plot of pseudo first-order rate constant for the inactivation of acetolactate synthase (k_{obs}) obtained at various concentration (mM) of PGO. (C) Double-logarithmic plot of the half-times of inactivation of acetolactate synthase at various concentration of PGO.

time of inactivation against reagent concentration (Fig. 3C). The result suggests that inactivation may be due to the reaction of one arginine residue per active acetolactate synthase molecule.

Protection against enzyme inactivation

The inactivation of enzyme activity by the above chemical modification reagents does not always directly imply that these chemically modifiable residues are present at the active site (Nakanishi *et al.*, 1989). However, protection of an enzyme by a substrate against inactivation does suggest that the amino acid residues that are protected from modification are located at or near the active site. As shown in Fig. 4, 10 mM α-keto-

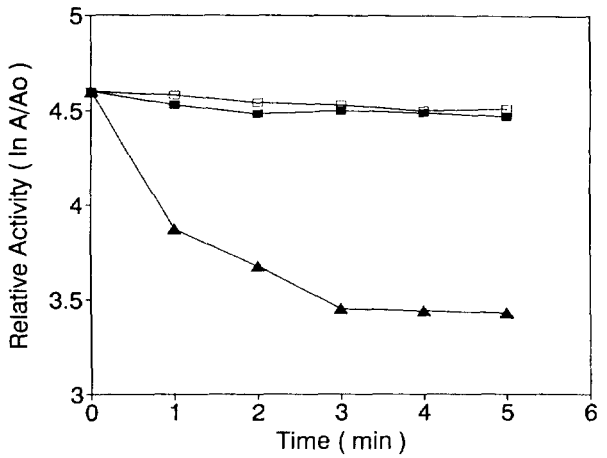

Fig. 4. Effect of substrates on the rate of inactivation of acetolactate synthase by PCMB modification. The enzyme solution (0.1 μM) was preincubated with 10 mM α-ketobutyrate or pyruvate prior to the addition of PCMB. (■–■); 10 mM α-ketobutyrate, ((□–□); 10 mM pyruvate, (▲–▲); 40 mM PCMB only.

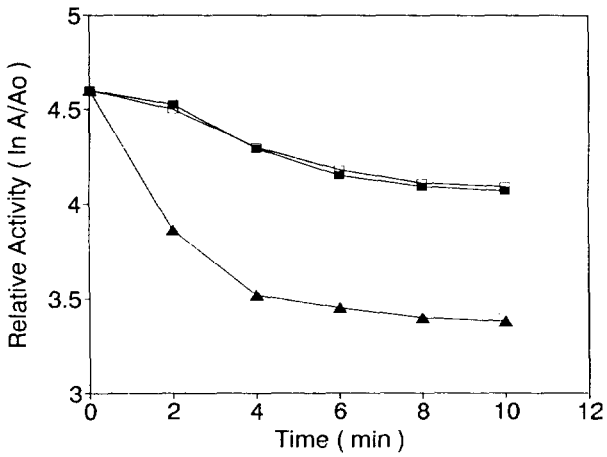

Fig. 5. Effect of substrates on the rate of inactivation of acetolactate synthase by NBS modification. The enzyme solution (0.1 μM) was preincubated with 10 mM α-ketobutyrate or pyruvate prior to the addition of NBS. (■–■); 10 mM α-ketobutyrate, (□–□); 10 mM pyruvate, (▲–▲); 70 mM NBS only.

butyrate or pyruvate almost completely protected the enzyme from inactivation by PCMB, indicating that a single cysteine residue is located in or near the active site. Inactivation of the enzyme by NBS was also partially protected by preincubation of the enzyme with either α-ketobutyrate or pyruvate (Fig. 5). However, only α-ketobutyrate partially protected the enzyme from inactivation by PGO (Fig. 6). These results suggest that the tryptophan residues and the arginine residue are also located in or near the active site of acetolactate synthase.

Effect of other chemical modifiers on acetolactate synthase activity

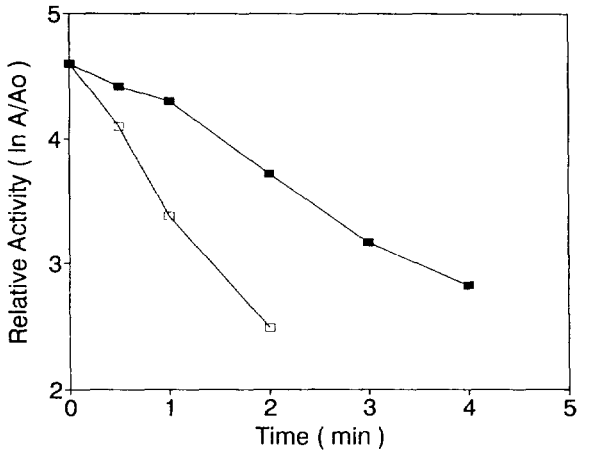

Fig. 6. Effect of α-ketobutyrate on the rate of inactivation of acetolactate synthase by PGO modification. The enzyme solution (0.1 μM) was preincubated with 10 mM α-ketobutyrate prior to the addition of PGO. (■–■); 10 mM α-ketobutyrate, (□–□); 2 mM PGO only.

Table 1. Inhibitory effect of modification reagent on Acetolactate Synthase

Conc. of reagent	Cys		Hydroxyl	Lys		Arg	Trp
	NEM	PCMB	PMSF	PLP	TNBS	PGO	NBS
0.5 mM	50	10	100	90	90	40	10
1.0 mM	50	10	100	80	90	10	10

Inhibitory effect was presented as % activity remaining compared to the control activity. Control enzyme activity which was not treated with modification reagent is considered as 100%.

The sulfhydryl specific reagent N-ethylmaleimide (NEM) inhibited enzyme activity approximately 50% at 1.0 mM concentration, whereas PCMB decreased the activity of acetolactate synthase approximately 90% at the same modifier concentration. However, use of the serine specific reagent phenylmethylsulfonyl fluoride (PMSF) and lysine reacting reagents, such as pyridoxal-5'-phosphate (PLP) and trinitrobenzenesulfonic acid (TNBS), had no effect on acetolactate synthase activity at 1.0 mM concentration (Table 1).

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