

Characterization of Cysteine Synthase in *Echinochloa crus-galli* L. and Its Inhibition by Substrate Analogues

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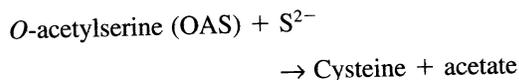
Cysteine synthase [CS; EC 4.2.99.8] catalyzes the terminal step of cysteine biosynthesis whereby cysteine is formed from *O*-acetylserine (OAS) and sulfide. CS was extracted from the leaves of *Echinochloa crus-galli* L., fractionated with ammonium sulfate, and then characterized. The optimum assay temperature of CS was 50°C, suggesting thermal stability. The activity was relatively low at pH 6.0, but increased sharply when the pH increased from 6.4 to 7.4. Maximal activity seemed between pH 7.4 and 9.0. The apparent K_m value was 2.53 mM for OAS and 0.88 mM for sulfide, which indicates that CS has a low affinity for OAS. Two inhibitors of pyridoxal-5'-phosphate (PLP)-dependent enzymes, amino-oxyacetate (AOA) and cycloserine, inhibited this enzyme by 76 and 42%, respectively, at 10 mM. An analogue of OAS, *O*-phosphoserine, inhibited CS slightly, but other analogues (e.g., *O*-phosphothreonine) were not significantly inhibitory, even at 30 mM. The I_{50} value of AOA was about 3 mM. When CS and AOA were preincubated together at 30°C, AOA inhibited CS in a time-dependent manner, by 51% with 0.1 mM AOA after a 4-h incubation. CS was stable during this incubation period. AOA and cycloserine inhibited the shoot elongation of *E. crus-galli* by 72 and 31%, respectively, at 5 kg a.i./ha postemergence. Growth inhibition by other compounds was less than 10%. These data suggest a correlation between CS inhibition and growth reduction; however, the supplementation of exogenous cysteine failed to reverse this inhibition in a test tube bioassay. This indicates that the growth inhibition could not be entirely attributed to the starvation of cysteine which might be caused by the inhibitor. © 2001 Academic Press

INTRODUCTION

Biosynthetic pathways of amino acids are excellent herbicide targets. Indeed, acetolactate synthase, the first enzyme in the biosynthesis pathways of the branched-chain amino acids, valine, leucine, and isoleucine, is inhibited by several classes of compounds such as sulfonylureas, e.g., chlorsulfuron (1), and imidazolinones, e.g., imazapyr (2, 3). EPSP synthase, which is involved in aromatic amino acids, is the site of action of glyphosate (4–6), and glutamine synthetase is inhibited by methionine sulfoximine and phosphinothricin (7, 8). Furthermore, intensive efforts have been made to discover new classes of herbicides that inhibit other enzymes involved in amino acid biosynthesis. For example, an inhibitor of isopropylmalate dehydrogenase, the third enzyme specific to leucine

biosynthesis, caused plant injury (9). Three triazole phosphonates also inhibited imidazole glycerol phosphate dehydratase (an enzyme of histidine biosynthesis) and these inhibitors showed wide-spectrum, postemergence herbicidal activity (10).

To our knowledge, cysteine biosynthesis has not been evaluated as a potential target site for herbicidal compounds and herbicide discovery efforts. In plants, sulfur is assimilated into cysteine through the cysteine biosynthesis pathway (11). Inorganic sulfate (SO_4^{2-}) is reduced to sulfite (SO_3^{2-}) and then to sulfide (S^{2-}) through the sulfate reduction pathway. Cysteine synthase [CS,² EC 4.2.99.8] catalyzes the assimilation of inorganic sulfide according to the reaction



² Abbreviations used: CS(s), cysteine synthase(s); OAS, *O*-acetylserine; PLP, pyridoxal-5'-phosphate; AOA, amino-oxyacetate.

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Cysteine plays important roles in most organisms as a precursor of protein cysteine, glutathione and the sulfur moiety of methionine. Inhibition of this enzyme might cause injury in higher plants because of cysteine starvation or accumulation of toxic sulfide or sulfite. Indeed, a transgenic tobacco overexpressing CS was resistant to acute toxicity of sulfite (12). This resistance was presumably due to the metabolic detoxification of sulfite by sequential reactions involving photoreduction and fixation into cysteine by overexpressed CS. In addition, transgenic tobacco seedlings expressing a wheat cytoplasmic CS cDNA were resistant to toxic levels of hydrogen sulfide (13), which was ascribed to metabolic detoxification of hydrogen sulfide by fixation into cysteine.

Cysteine is not an essential amino acid, and CS does not exist in animals. Animals can biosynthesize this amino acid from methionine. From the point of selectivity between plants and animals, this enzyme is attractive as a site for herbicide. CS has been purified and characterized from several plants (14, 15), but no studies on this enzyme derived from weeds have been reported. In this study, to obtain basic information on CS in weeds, this enzyme was extracted from the leaves of *Echinochloa crus-galli* L. (barnyardgrass), partially purified, and then characterized. In addition, several chemicals, including substrate analogues, were tested for inhibitory activity against this enzyme and for their *in vivo* phytotoxicity.

MATERIALS AND METHODS

Chemicals

All chemicals used in the cysteine synthase assay [OAS, Na₂S, dithiothreitol, EDTA, and pyridoxal-5'-phosphate (PLP)] and those used in the enzyme inhibition tests [*O*-phosphoserine, *O*-phosphothreonine, *O*-methylserine, azaserine, *N*-acetylserine, serine-*O*-sulfate, aminoxyacetate (AOA), and cycloserine] were purchased from Sigma Chemical Co. Ammonium sulfate was purchased from Mallinckrodt, Inc. All chemicals were reagent grade or higher in purity.

Plant Material

Seeds of *E. crus-galli* L. were germinated and seedlings were grown on wet paper towels placed in a plastic container. The containers were maintained under 12-h photoperiod (72 $\mu\text{E}/\text{s}\cdot\text{m}^2$) at 25°C for 14 days, at which time shoots were collected for enzyme preparation.

Enzyme Preparation

Shoots of *E. crus-galli* L. were homogenized at 4°C in 0.12 M phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, and 0.2% insoluble polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth and the filtrate was clarified by centrifugation at 15,000g at 4°C for 30 min. The resulting supernatant was then subjected to a 30–70% ammonium sulfate fractionation. The final pellet was gently resuspended in 0.12 M phosphate buffer, pH 7.5, and the suspension was desalted on a Sephadex-G25 column equilibrated with the same buffer. The desalted enzyme was stored at –45°C until use.

Assay of CS Activity

The routine CS assay (16) was performed in a final volume of 1 ml containing 50 mM phosphate buffer, pH 7.5, less than 0.3 mg protein, 5 mM OAS, 1 mM Na₂S, 1 mM dithiothreitol, and 0.025 mM PLP. The enzyme reaction was initiated by adding protein, and assay test tubes were sealed with rubber caps. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid (w/v), and precipitated protein was removed by centrifugation at 2000g for 10 min. An aliquot (1 ml) of the supernatant was collected and added to 1.5 ml of ninhydrin reagent (250 mg ninhydrin dissolved in 20 ml glacial acetic acid:concentrated HCl, 4:1 v/v). The mixture was boiled for 6 min in a hood and then cooled. Cysteine was determined by measuring the absorbance of the reaction mixture at 560 nm. The protein concentration was determined using the Bradford method (17) with bovine serum albumin as protein standard.

Effects of Temperature, pH, and Substrate Concentration on Enzyme Activity

The reaction mixtures for temperature and pH experiments were similar to those described above. The effects of different incubation temperatures on enzyme activity were examined at pH 7.5, and for pH curves, the activity measurement was performed at 37°C. In this case, 50 mM phosphate buffer (pH 6.0–7.8), 50 mM Tris-HCl (pH 7.8–8.6), and 50 mM glycine-NaOH (pH 8.6–10.0) were used. Tests for effects of substrate concentration on the enzyme activity were also conducted under similar conditions. The concentration of OAS was varied between 0 and 10 mM at constant 1 mM Na₂S, and in other tests, Na₂S concentration was varied between 0 and 1 mM at constant 5 mM OAS.

Enzyme Inhibition Test

Because some compounds tested were sufficiently acidic to decrease the reaction pH even in this buffer, they were dissolved in 200 mM phosphate buffer, pH 7.8, and pH was again adjusted to 7.8 with 5 N NaOH. Other conditions were as described above (e.g., concentrations of OAS and Na₂S were 5 and 1 mM, respectively), except that the reaction pH was 7.8 and the phosphate buffer concentration was 100 mM. In this experiment, the inhibitors and substrates were added to the enzyme simultaneously. In the preincubation test, the enzyme and AOA were preincubated at 30°C for 0–4 h; then OAS, Na₂S, dithiothreitol, and PLP were added to start the reaction at 37°C.

All enzyme experiments were repeated at least twice and each experiment was conducted with at least two replicates.

Postemergence Herbicidal Efficacy

Seeds of *E. crus-galli* L. were planted in a mixture of Jiffy-Mix Plus (Jiffy Products of America, Inc.) and field soil (silty clay loam, 1/1, v/v) in plastic pots (10 cm in diameter) in a greenhouse. At the one-leaf stage, plants were sprayed with compounds at 5 kg a.i./ha to an equivalent of 500 L/ha of water containing 0.1% Triton X-100. The plants were then grown in a

greenhouse and natural light was supplemented to provide 14-h photoperiod. The greenhouse temperature varied between 25 and 33°C. Four days after application, shoot length of the plants were measured. The original shoot length at the application was subtracted from the measurements to obtain shoot elongation. This experiment was conducted with four replicates and was repeated.

Cysteine Supplementation Test

Seeds of *E. crus-galli* L. were sterilized in a 0.5% bleach solution for 5 min and washed three times with sterile water. These were then germinated at 30°C for 48 h. Five seeds were placed in a test tube (2.2 cm in diameter, 11.6 cm in height) containing 5 ml of AOA water solution, with or without cysteine. Plants were grown under 12-h photoperiod (72 $\mu\text{E}/\text{s}\cdot\text{m}^2$) at 25°C for 10 days; then shoot length was measured. This test was conducted with three replicates and was repeated.

RESULTS AND DISCUSSION

Properties of CS from E. crus-galli L.

CS was extracted from the leaves of *E. crus-galli* L. and fractionated by ammonium sulfate. The strongest activity was found in the 50–60% ammonium sulfate fraction but that in the 30–70% fraction was used for routine assays. Enzyme activity increased with assay temperature between 15 and 50°C, and it was relatively high up to 60°C (Fig. 1). The activity decreased sharply above 60°C, but about 20% of that at optimum temperature (50°C) still remained at 70°C. This result implies that crude CS from *E. crus-galli* L. is relatively resistant to heat. In fact, heat treatment was utilized during the purification of CS from other plant species to concentrate protein (14, 15). The optimum temperature of CS of *Spinacia oleracea* L. (spinach) was also 50°C (14), and that of *Datura innoxia* ranged between 42 and 58°C (18). Glutathione concentrations increase in plants exposed to heat shock, suggesting an increase in biosynthesis of this tripeptide (19). Kuske *et al.* (18)

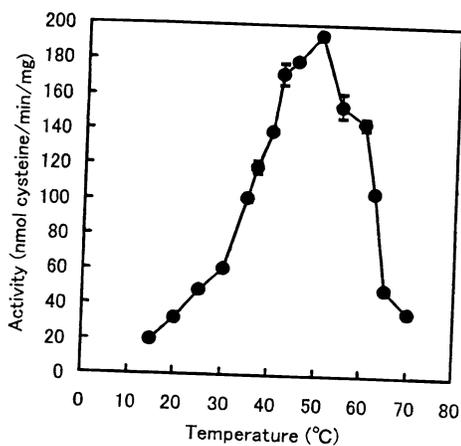


FIG. 1. Effect of reaction temperature on cysteine synthase activity. The vertical bars represent \pm SE.

indicated that the residual activity of CS at high temperatures suggests a mechanism for increased cysteine synthesis in response to elevated temperatures.

The activity of this enzyme sharply increased with increasing pH between 6.4 and 7.4 (Fig. 2). The optimum pH range of CS measured at 37°C was quite broad, from 7.4 to 9.0. A similar result was reported using chloroplasts from *S. oleracea* L. (14), indicating that the optimum pH range was pH 7.5–8.5. However, the enzymes from *D. innoxia* were highly dependent on reaction pH: they were active only in the range of 7 to 8, with optimum activity at 7.6. Even slight changes in pH drastically decreased the activity (18). However, it was impossible to obtain the precise optimum pH because, above pH 8.0, the OAS substrate rapidly undergoes an *O* to *N* shift and is thus no longer the substrate for the enzyme (20).

The effects of OAS and sulfide concentrations on CS activity were examined (Fig. 3). The K_m values for OAS and sulfide, estimated from double-reciprocal plots, were 2.53 and 0.88 mM, respectively. These values are similar to those found in other species. For example, the K_m s for OAS and sulfide (21) were 3.5 and 0.51 mM, respectively, from *Trifolium repens* (white clover) leaves and the K_m s for OAS and sulfide were 3.1 and 0.24 mM, respectively, from *Pisum*

sativum (pea shoots). In the case of *S. oleracea* L., the K_m s for OAS and sulfide were 1.3 and 0.25 mM, respectively (14). The K_m values of two CS isoenzymes from *Citrullus vulgaris* were 2.6 and 1.5 mM for OAS and 0.036 and 0.033 mM for sulfide (15). It is also reported that the K_m s of two isoenzymes from *Allium tuberosum* (chinese chive) for OAS were 6.7 and 9.0 mM (20). As shown in these examples, CSs exhibit a relatively low affinity for the substrate OAS. Droux *et al.* (14) suggested that this enzyme forms a supramolecular complex with serine acetyltransferase, the enzyme that produces OAS from serine. A high concentration of OAS can be maintained at the active site of CSs by this organization.

Effect of Several Compounds on the Activity of CS

Several compounds (Fig. 4) were selected for the inhibition test of CS from *E. crus-galli* L., including substrate analogues (*O*-phosphoserine, *O*-phosphothreonine, *O*-methylserine, azaserine, *N*-acetylserine, serine-*O*-sulfate). Inhibitors of PLP-dependent enzymes (serine-*O*-sulfate, AOA, and cycloserine) were also included because absorbance spectra studies

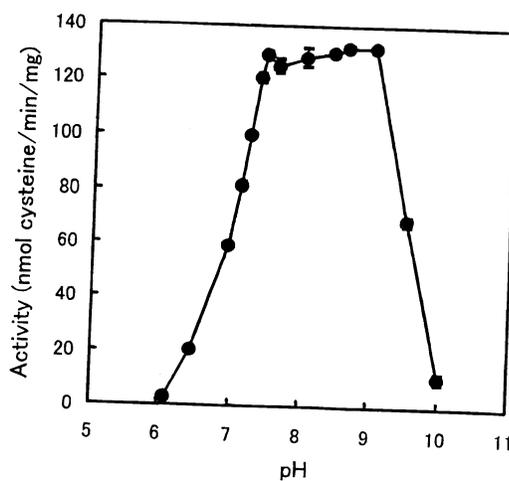


FIG. 2. Effect of reaction pH on cysteine synthase activity. The values between pH 8.0 and 10.0 are not valid because, above pH 8.0, the OAS substrate undergoes an *O* to *N* shift (18). The vertical bars represent \pm SE.

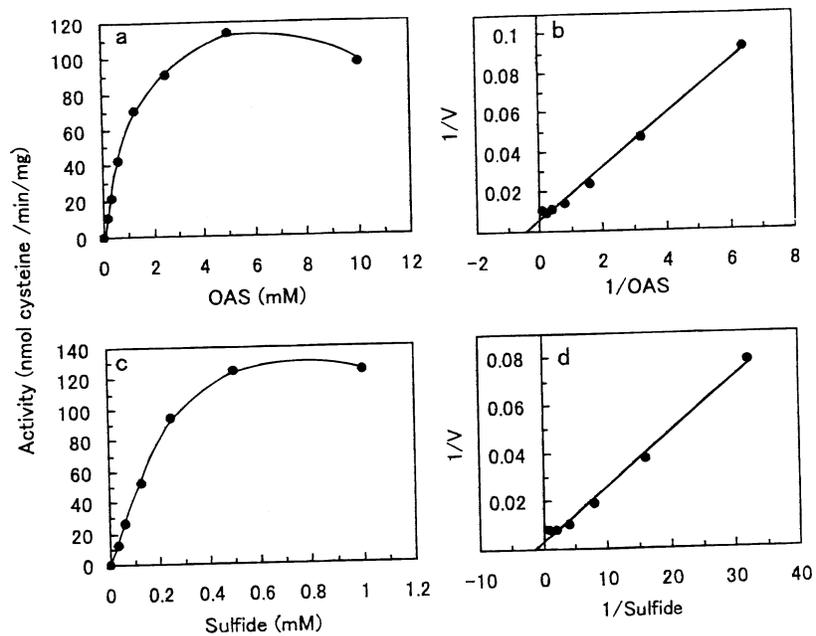


FIG. 3. Effect of (a) OAS and (c) sulfide concentration on cysteine synthase activity. Double-reciprocal plots of the data in (a) and (c) are shown in (b) and (d), respectively. The concentrations of sulfide in (a) and OAS in (c) were 1 and 5 mM, respectively.

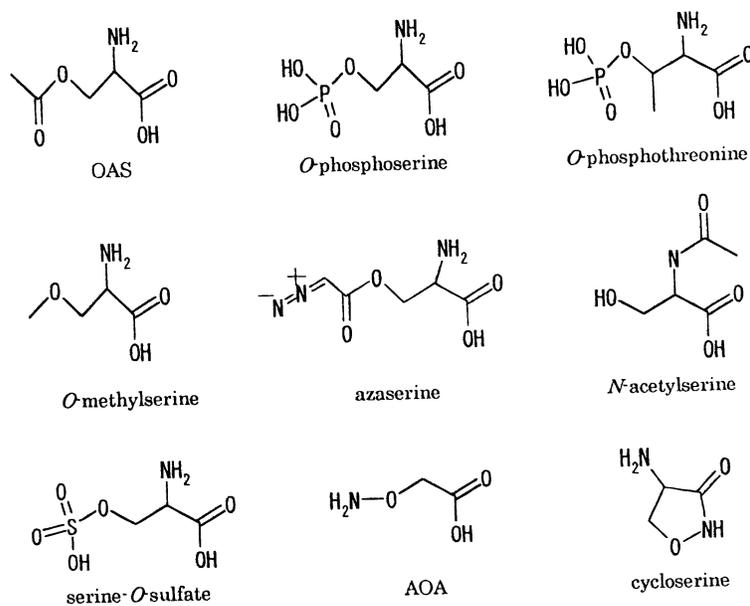


FIG. 4. Chemical structure of OAS (substrate) and compounds used as possible inhibitors of cysteine synthase.

with purified CS from other plants indicated the presence of PLP in the enzyme molecule (18, 22). AOA reportedly shows reactivity toward PLP-dependent enzymes (23). In addition, serine-*O*-sulfate and cycloserine are thought to be PLP-dependent enzyme inhibitors (24). AOA and cycloserine inhibited this enzyme by 76 and 42%, respectively, at 10 mM, and *O*-phosphoserine inhibited it by 13% at 30 mM (Table 1). Other compounds such as *O*-phosphothreonine, *O*-methylserine, and *N*-acetylserine had no significant inhibition, even at 30 mM. Neither azaserine nor serine-*O*-sulfate inhibited the enzyme at 10 mM. The substrate analogues tested here did not show significant inhibition, whereas inhibitors of PLP-dependent enzymes, except for serine-*O*-sulfate, had a little effect on this enzyme. As described above, it is suggested that CS forms a complex with serine acetyltransferase to maintain a high concentration of OAS at the active site. This aggregation might reduce the competence of substrate analogues to approach the active site. Some metabolites in cysteine metabolism, such as cystathionine and cysteine itself, were inhibitory at 10 mM (21). In preliminary tests, acidic compounds such as *O*-phosphoserine and *O*-phosphothreonine seemed to inhibit the enzyme at 10 mM. However, it was later revealed that these compounds slightly decreased the reaction pH at this concentration. As shown in Fig. 2, CS activity sharply decreased when pH dropped from 7.4 to 6.4.

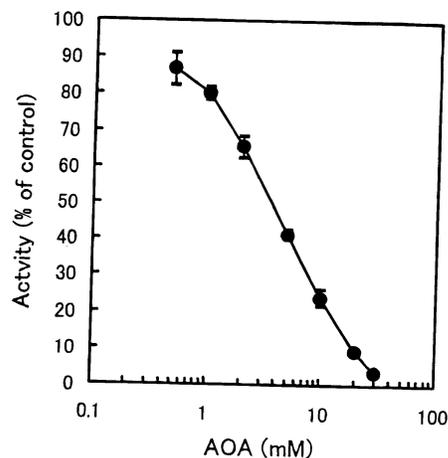


FIG. 5. Effect of AOA on cysteine synthase activity. The vertical bars represent \pm SE.

The pH change caused a decrease in the enzyme activity. Therefore, pH should be carefully monitored in the inhibition test of CS, in particular, when examining at high inhibitor concentration.

AOA inhibited this enzyme almost completely at 30 mM, and the I_{50} was about 3 mM (Fig. 5). AOA is not a potent inhibitor of this enzyme when added to the reaction mixture simultaneously with substrates. However, when CS was preincubated with AOA, the inhibition ratio increased in a time-dependent manner (Fig. 6), and AOA inhibited the enzyme more strongly than in the experiment described above. During 4-h incubation at 30°C, AOA inhibited the CS

TABLE 1
Effect of Several Compounds on Cysteine Synthase Activity and Postemergence Herbicidal Activity on *E. crus-galli* L.^a

| Compound | Cysteine synthase activity (% of control) \pm SE | | Herbicidal activity (shoot elongation, % of control) \pm SE (5kg a.i/ha) |
|----------------------------|---|-------------------|--|
| | 10 mM | 30mM | |
| <i>O</i> -Phosphoserine | 97.6 \pm 2.5 | 87.2 \pm 5.5 | 92.0 \pm 2.8 |
| <i>O</i> -Phosphothreonine | 103.3 \pm 5.0 | 97.4 \pm 2.4 | 96.2 \pm 2.9 |
| <i>O</i> -Methylserine | 112.9 \pm 1.6 | 103.1 \pm 2.4 | 93.8 \pm 2.9 |
| Azaserine | 96.4 \pm 0.4 | N.T. ^b | N.T. ^b |
| <i>N</i> -Acetylserine | 107.4 \pm 7.8 | 99.4 \pm 3.9 | 93.0 \pm 2.8 |
| Serine- <i>O</i> -sulfate | 100.6 \pm 3.2 | N.T. ^b | N.T. ^b |
| AOA | 23.9 \pm 2.4 | 3.7 \pm 0.1 | 27.6 \pm 1.7 |
| Cycloserine | 57.9 \pm 7.0 | 33.7 \pm 1.5 | 69.0 \pm 2.8 |

^a Postemergence testing conducted under greenhouse conditions.

^b N.T., not tested.

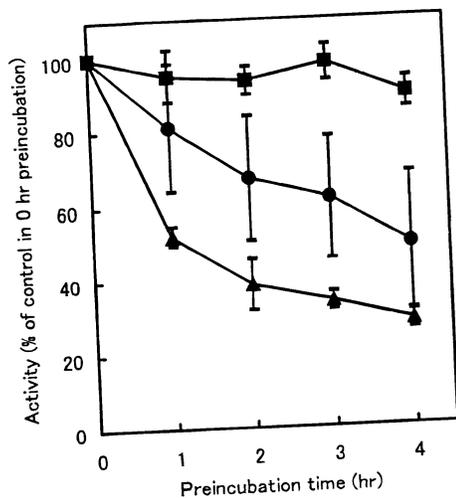


FIG. 6. Effect of preincubation of AOA on cysteine synthase activity. Incubation conditions: preincubation with AOA, 0 mM (■), 0.1 mM (●), or 1 mM (▲) at 30°C. The vertical bars represent \pm SE.

by about 50 and 72% at 0.1 and 1.0 mM, respectively. This suggests that CS from *E. crus-galli* L. requires PLP as a cofactor. Incubation of purified CS from roots of *Raphanus sativas* L. (radish) with hydroxylamine, which is another PLP-dependent enzyme inhibitor, resulted in a marked decrease of enzymatic activity (22). Time dependence of the inhibitory process may be indicative of irreversible inhibition but it cannot be regarded as providing conclusive proof of irreversibility (25). It is reported that purified CSs had an absorbance at 412 nm, which is characteristic of the aldoxime form of PLP and is due to a Schiff base formed between the PLP cofactor and the lysine side chain of the enzyme (18). In this case, the enzyme appeared to be saturated with tightly bound PLP, since the activity of the enzyme was not increased by addition of cofactor. Jenkins *et al.* (23) suggested that AOA were strongly complexed to the PLP-dependent enzymes via their bound cofactor. These suggestions considered, AOA probably reacted to CS on its PLP cofactor, resulting in inactivation during the preincubation period. The enzyme was stable during the 4-h incubation at 30°C.

Phytotoxicity Tests of CS Inhibitors

Postemergence herbicidal activity of several compounds against *E. crus-galli* L. is also shown in Table 1. AOA and cycloserine inhibited the shoot elongation of this weed by 72 and 31%, respectively. Inhibition by other compounds was less than 10%. For example, *O*-phosphoserine inhibited the growth by 8%. Although the number of compounds tested was small, there was a positive correlation between CS inhibition and growth inhibition. The correlation coefficient between the percentage of CS activity at 30 mM inhibitor concentration and the percentage of shoot elongation was 0.956 for the data in Table 1.

Since our preliminary experiment examined the relationship between CS inhibition and herbicidal activity, the effect of cysteine supplementation on the action of AOA was tested. AOA (100 μ M) inhibited shoot elongation of *E. crus-galli* L. seedlings by 80% after a 10-day incubation using a test tube (Fig. 7). Exogenous cysteine did not nullify this inhibition at 30–300 μ M (Table 2), which indicates that the growth inhibition caused by AOA was not solely attributed to cysteine starvation. It is reasonable to assume that AOA has multiple sites of action, including PLP-dependent enzymes (23, 26) as described above. For example, AOA reportedly

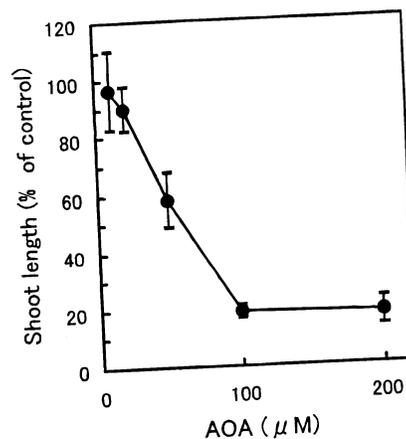


FIG. 7. Effect of AOA on shoot elongation of *E. crus-galli* L. in a test tube bioassay. The vertical bars represent \pm SE.

TABLE 2
Effect of Supplemental Cysteine on AOA-Caused Growth Inhibition of *Echinochloa crus-galli* L.

| Cysteine (μ M) | IC ₅₀ of AOA (μ M) |
|---------------------|------------------------------------|
| 0 | 74.0 |
| 30 | 75.4 |
| 300 | 74.7 |

Note. Growth of *E. crus-galli* was measured as shoot elongation in a test tube bioassay under conditions as described for tests presented in Fig. 7.

inhibited both chlorophyll and carotenoid formation in greening leaf segments and affected serine:glyoxylate aminotransferase (23). AOA inhibits aspartate aminotransferase (24) and phenylalanine ammonia-lyase (27). However, as an interesting possibility, toxic sulfide or sulfite might accumulate to injurious levels in tissue treated with a CS inhibitor just as plants treated with an inhibitor of glutamine synthetase accumulate toxic levels of ammonia (28).

Because it was found that AOA inhibited CS from *E. crus-galli* L. in this study, the effect of other PLP-dependent enzyme inhibitors on CS and their phytotoxicity will be examined as our next subject.

In conclusion, CS from *E. crus-galli* L. has enzymatic properties similar to CSs from other plant species. This enzyme is inhibited by AOA, which indicates that it is PLP dependent. Although AOA and cycloserine inhibited CS and were phytotoxic, the relationship between the CS inhibition and the growth inhibition of higher plant is unclear. Further investigation is necessary to determine whether CS is a sensitive target of herbicidal compounds.

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