

## Effect of Inhibitors of Pyridoxal-5'-Phosphate-Dependent Enzymes on Cysteine Synthase in *Echinochloa crus-galli* L.

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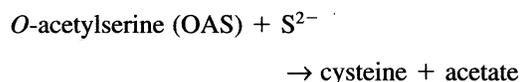
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The effect of inhibitors of pyridoxal-5'-phosphate-dependent enzymes (IPEs) on cysteine synthase (CS; EC 4.2.99.8), which synthesizes cysteine from *O*-acetylserine and sulfide, was examined. CS was extracted from the leaves of *Echinochloa crus-galli* L., fractionated with 30–70% ammonium sulfate, and then used for the enzyme assay with seven IPEs. When substrates of CS and 10 mM IPEs were added simultaneously, DL-allylglycine, DL-propargylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, amino-oxyacetate (AOA), and hydroxylamine inhibited CS by 64, 45, 62, 61, 89, and 33%, respectively. All the IPEs inhibited shoot elongation of *E. crus-galli* in a seedling growth bioassay; however, there was a very low correlation between the inhibition of CS and that of shoot elongation ( $R^2 = 0.173$ ). When CS was preincubated with 10 mM IPEs at 30°C for 1 h, DL-allylglycine, DL-propargylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, AOA, and hydroxylamine inhibited CS by 47, 27, 49, 40, 96, and 98%, respectively. CS inhibition by AOA and hydroxylamine increased during the preincubation period, suggesting that AOA and hydroxylamine might be irreversible inhibitors of CS. The correlation coefficient between CS inhibition by preincubation and inhibition of shoot elongation was  $R^2 = 0.630$ . These results suggest that CS inhibition in *E. crus-galli* might affect its growth. © 2001 Academic Press

### INTRODUCTION

In the sulfur assimilation process in plants, sulfur is reduced to sulfite by ATP-sulfurylase and adenosine 5'-phosphosulfate reductase and then to sulfide by sulfite reductase before the incorporation to cysteine (1). Cysteine synthase (CS;<sup>2</sup> EC 4.2.99.8) catalyzes the assimilation of inorganic sulfide according to the reaction



CSs have been purified from many plants and their enzymatic properties are well studied (2–8). Amino acid sequences of CSs from several plants have been compared, and CSs have

been classified into several families (9–11). Cysteine is a precursor to methionine, glutathione, and other sulfur-containing compounds. CS may have an important role in detoxification of excess sulfite and hydrogen sulfide in plants because a transgenic tobacco overexpressing CS was resistant to sulfite (12) or hydrogen sulfide (13). The resistance was presumably due to the metabolic detoxification of sulfite or hydrogen sulfide by fixation into cysteine.

CS in *Echinochloa crus-galli* was characterized and the analogues of the CS substrate, OAS, such as *O*-phosphothreonine, *O*-methylserine, and *N*-acetylserine, did not inhibit CS even at 30 mM, although cycloserine inhibited CS by 42% at 10 mM (14). On the other hand, amino-oxyacetate (AOA), an inhibitor of pyridoxal-5'-phosphate-dependent enzymes (IPE), had some effects on CS activity. For example, the  $I_{50}$  of AOA was about 3 mM, and when CS was preincubated with AOA at 30°C for 4 h, the  $I_{50}$  value was about 0.1 mM. CS from *E. crus-galli* may require pyridoxal-5'-phosphate (PLP) as a cofactor.

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<sup>2</sup> Abbreviations used: CS(s), cysteine synthase(s); OAS, *O*-acetylserine; IPE(s), inhibitor(s) of pyridoxal-5'-phosphate-dependent enzymes; AOA, amino-oxyacetate; PLP, pyridoxal-5'-phosphate; AEP, 1-aminoethylphosphonic acid.



PLP is a common cofactor for many other enzymes, such as transaminases. There are many studies on transaminases in animals and microorganisms and transaminase inhibitors including AOA (15). Plant transaminases were also inhibited by IPEs such as semicarbazide, cyanide, and isoniazid (16). To our knowledge, no studies have examined the effect of IPEs on CS from a view point of weed control except for the above-mentioned paper (14). CS is an interesting enzyme for herbicide target site studies because inhibition of CS would deprive plants of necessary amino acids and may limit the ability to detoxify sulfite or sulfide.

In the present study, several IPEs were tested for inhibitory activity against CS from *E. crus-galli* leaves. The *in vivo* phytotoxicity of the IPEs was determined and then the relationship between CS inhibition and phytotoxicity was assessed.

#### MATERIALS AND METHODS

##### Chemicals

All chemicals used in the CS assay [OAS, Na<sub>2</sub>S, dithiothreitol, EDTA, and PLP] and those used in the enzyme inhibition tests [1-aminooethylphosphonic acid (AEP), DL-allylglycine, DL-propargylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, 3-bromopyruvate, AOA, and hydroxylamine] 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 planted in a mixture (1/1, v/v) of Jiffy-Mix Plus (Jiffy Products of America, Inc.) and field soil (silty clay loam) in plastic pots (10 cm in diameter). The pots were placed in a greenhouse (maintained at 33  $\pm$  2°C in the daytime and 25  $\pm$  2°C at night) and natural light was supplemented to provide 14-h photoperiod. At the one leaf stage, shoots were collected for enzyme preparation.

##### Enzyme Preparation

Shoots of *E. crus-galli* 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 cheese cloth and the filtrate was clarified by centrifugation at 15,000g at 4°C for 30 min. An aliquot of the resulting supernatant (referred to as the crude enzyme) was used for preincubation, and the other portion of the supernatant was 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 (referred to as the ammonium sulfate fraction) was stored at –45°C until use.

##### Assay of CS Activity

The CS assay (14,17) was performed in a final volume of 1 ml containing 100 mM phosphate buffer, pH 7.8, less than 0.05 mg protein, 5 mM OAS, 1 mM Na<sub>2</sub>S, 1 mM dithiothreitol, 0.025 mM PLP, and 1 or 10 mM IPEs. Because some IPEs were sufficiently acidic to decrease the reaction pH, they were dissolved in 200 mM phosphate buffer, pH 7.8, and pH was again adjusted to 7.8 with 5 N NaOH before use. The substrates and IPEs were added to the enzyme simultaneously to initiate the reaction, 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 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 heated in a boiling water bath for 6 min and cooled. Cysteine was determined by measurement of the absorbance of the reaction mixture at 560 nm. The protein concentration was determined with the Bradford method (18) with bovine serum albumin as protein standard. All enzyme assays were conducted with two replications and repeated three times unless indicated.

##### Preincubation CS Assay

The preincubation CS assay was performed with the crude enzyme and the ammonium sulfate fraction. The preincubation was conducted

in a volume of 0.5 ml containing 200 mM phosphate buffer, pH 7.8, 1 or 10 mM IPEs, and the enzyme. The mixture was incubated at 30°C for 10, 30, 60, 120, and 180 min and then OAS, Na<sub>2</sub>S, dithiothreitol, and PLP were added to start the reaction at 37°C. The final volume of the assay mixture was 1 ml, containing 100 mM phosphate buffer, pH 7.8, less than 0.05 mg protein, 5 mM OAS, 1 mM Na<sub>2</sub>S, 1 mM dithiothreitol, 0.025 mM PLP, and 0.5 or 5 mM IPEs. The other procedures of the CS assay were the same as those described above. Only freshly prepared CS was used in the crude enzyme assay, and this assay was conducted with two replications and repeated twice.

#### *Reactivity of IPEs to Cysteine*

The reactivity of IPEs with cysteine was performed in a final volume of 1 ml containing 100 mM phosphate buffer, pH 7.8, less than 0.05 mg protein (ammonium sulfate fraction), 1 mM dithiothreitol, 0.025 mM PLP, 0.1–10 mM IPEs, and 0.125 mM cysteine. The mixture was incubated at 37°C for 10 min, and the reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid (w/v). The remaining cysteine was determined as described above. This test was conducted with two replications and repeated twice.

#### *Shoot Growth Bioassay*

Seeds of *E. crus-galli* were sterilized in a 0.5% bleach solution for 5 min and washed three times with deionized water. Ten seeds were placed in a test tube (4 cm in diameter, 6 cm in height) containing 5 ml of nutrient solution. One liter of the culture solution contained 10 mg (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.4 mg NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 4 mg KCl, 1 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mg MgCl<sub>2</sub>, and 0.3 mg FeCl<sub>3</sub>·6H<sub>2</sub>O; the pH of this solution was adjusted to 5.5. At 3 days after sowing, shoot length was measured, and IPE solutions that had been neutralized by NaOH were added to the culture solution. Plants were grown under 16-h photoperiod (150 μE/s·m<sup>2</sup>) at 25°C for another 3 days at which time shoot length was measured again.

The percentage difference in growth was calculated. This experiment was conducted with three replications and repeated twice.

#### *Data Transformation*

Data were transformed by the following equation (19):

$$\begin{aligned} \text{\% of control (transformed)} = \\ \arcsin \sqrt{\text{remaining CS activity (\% of control)}} \\ \text{or arcsine } \sqrt{\text{shoot elongation (\% of control)}}. \end{aligned}$$

## RESULTS

#### *Reactivity of IPEs to Cysteine*

Cysteine is reactive with many molecules, such as 5,5'-dithiobis(2-nitrobenzoic acid) and methylglyoxal (20). Because CS activity was determined by the amount of cysteine produced, compounds that are reactive to cysteine may interfere with the CS assay. A reactivity test was conducted under the same conditions as those of the CS assay, which contain protein, PLP, and dithiothreitol to determine whether there was interaction between IPEs and cysteine. Most compounds did not reduce cysteine in this test, which indicates that these compounds had no interference with CS assay (data not shown). However, 3-bromopyruvate (1 to 10 mM) significantly reduced cysteine, suggesting that this compound interferes with the CS assay (data not shown). The correct inhibitory activity of this compound against CS would not be determined because of its reactivity with cysteine; therefore 3-bromopyruvate was omitted in further experiments.

#### *Simultaneous Treatment*

When IPEs and substrates were added to CS simultaneously, DL-allylglycine, DL-propargylglycine, β-chloro-L-alanine, 3-bromopropionate, AOA, and hydroxyamine inhibited CS by 64, 45, 62, 61, 89, and 33%, respectively, at 10

mM (Table 1). However, AEP did not inhibit the enzyme at 10 mM. At 1 mM, DL-allylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, and AOA inhibited CS by 17, 16, 16, and 32%, respectively. The other compounds did not inhibit CS at 1 mM.

#### *In Vivo* Phytotoxicity of IPEs

The effect of IPEs on shoot elongation of *E. crus-galli* was examined in a shoot growth bioassay. IPEs were applied to the *E. crus-galli* seedlings, when the shoot length was  $1.12 \pm 0.38$  cm. The untreated control obtained a length of  $2.90 \pm 0.46$  cm at 3 days after treatment. AOA and hydroxylamine were the most phytotoxic compounds, inhibiting the elongation by 94 and 89%, respectively, at 10 mM (Table 2). AEP, DL-allylglycine, DL-propargylglycine,  $\beta$ -chloro-L-alanine, and 3-bromopropionate inhibited the elongation by 46, 63, 79, 63, and 40%, respectively, at 10 mM. AOA and hydroxylamine were also effective at 1 mM, producing about 90% growth inhibition, and DL-propargylglycine inhibited the elongation by 64% at 1 mM. Other IPEs inhibited shoot growth by less than 50% at 1 mM.

The correlation between the remaining CS activity [percentage of control (transformed), calculated from Table 1] and the shoot elongation of *E. crus-galli* [percentage of control

TABLE 1  
Effect of Inhibitors of PLP-Dependent Enzymes on Cysteine Synthase in *E. crus-galli*

Compound	Remaining CS activity (% of control) <sup>a</sup>	
	1 mM	10 mM
AEP	104.9 $\pm$ 1.8	105.1 $\pm$ 4.0
DL-Allylglycine	83.3 $\pm$ 5.2	35.9 $\pm$ 0.9
DL-Propargylglycine	100.1 $\pm$ 3.5	55.3 $\pm$ 3.2
$\beta$ -Chloro-L-alanine	83.6 $\pm$ 3.3	37.7 $\pm$ 3.0
3-Bromopropionate	83.7 $\pm$ 6.0	38.5 $\pm$ 6.0
AOA	67.5 $\pm$ 4.8	11.2 $\pm$ 7.3
Hydroxylamine	92.5 $\pm$ 7.2	66.6 $\pm$ 4.5

Note. IPEs and substrates were added to the enzyme simultaneously to initiate enzyme reaction.

<sup>a</sup> Values are means  $\pm$  standard error.

TABLE 2  
Effect of Inhibitors of PLP-Dependent Enzymes on the Growth of *E. crus-galli*

Compound	Shoot elongation (% of control) <sup>a</sup>	
	1 mM	10 mM
AEP	72.9 $\pm$ 3.9	53.8 $\pm$ 4.0
DL-Allylglycine	56.8 $\pm$ 0.1	37.2 $\pm$ 3.7
DL-Propargylglycine	36.0 $\pm$ 7.0	21.2 $\pm$ 4.6
$\beta$ -Chloro-L-alanine	52.6 $\pm$ 9.3	37.4 $\pm$ 4.9
3-Bromopropionate	82.1 $\pm$ 6.2	60.3 $\pm$ 2.6
AOA	10.9 $\pm$ 0.7	6.4 $\pm$ 0.3
Hydroxylamine	6.7 $\pm$ 3.0	10.5 $\pm$ 6.5

Note. The shoot elongation of *E. crus-galli* was measured in the test tube bioassay.

<sup>a</sup> Values are means  $\pm$  standard error.

(transformed), calculated from Table 2] at 10 mM was  $R^2 = 0.173$  when all IPEs were considered together. However, when just DL-allylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, and AOA were included in the calculation, the correlation was improved ( $R^2 = 0.863$ ).

#### Preincubation Test

CS was preincubated with IPEs at 30°C for 1 h prior to the CS assay with both the ammonium sulfate fraction and the crude enzyme (Table 3). When the ammonium sulfate fraction was used, DL-allylglycine, DL-propargylglycine,  $\beta$ -chloro-L-alanine, 3-bromopropionate, AOA, and hydroxylamine inhibited CS by 47, 27, 49, 40, 96, and 98%, respectively, at 10 mM. AEP did not inhibit CS at 10 mM. AOA and hydroxylamine inhibited CS by 65 and 47%, respectively, at 1 mM, and other IPEs inhibited the enzyme by 30% or less at this concentration. The inhibition of CS with the crude enzyme was similar to that with the ammonium sulfate fraction. AOA and hydroxylamine reduced the CS activity by 98 and 92%, respectively, at 10 mM. AEP had no efficacy on CS at this concentration, and the reduction in CS activity by other IPEs ranged between 30 and 44%.

The inhibition of CS by AOA and hydroxylamine was greater in the preincubation test than in the simultaneous treatment, suggesting that

TABLE 3  
Effect of Preincubation with Inhibitors of PLP-Dependent Enzymes on Cysteine synthase in *E. crus-galli*

Compound	Remaining CS activity (% of control) <sup>a</sup>		
	Ammonium sulfate fraction <sup>b</sup>		Crude enzyme <sup>c</sup>
	1 mM <sup>d</sup>	10 mM <sup>e</sup>	10 mM <sup>e</sup>
AEP	98.4 ± 18.8	97.7 ± 16.6	93.7 ± 3.8
DL-Allylglycine	73.1 ± 5.3	52.6 ± 4.8	55.6 ± 6.5
DL-Propargylglycine	93.9 ± 24.9	72.9 ± 8.1	70.3 ± 6.8
$\beta$ -Chloro-L-alanine	87.0 ± 8.6	50.6 ± 0.3	62.6 ± 6.1
3-Bromopropionate	69.7 ± 7.8	59.5 ± 2.7	60.4 ± 6.7
AOA	34.6 ± 2.5	3.9 ± 0.3	2.3 ± 0.3
Hydroxylamine	53.2 ± 6.4	1.6 ± 1.3	8.0 ± 3.9

Note. IPEs and the enzyme were preincubated at 30°C for 1 h before the enzyme reaction was initiated.

<sup>a</sup> Values are means ± standard error.

<sup>b</sup> Ammonium sulfate fraction was used as the enzyme solution. See Materials and Methods.

<sup>c</sup> Enzyme solution before the ammonium sulfate fractionation was used. See Materials and Methods.

<sup>d</sup> The enzyme was preincubated with the IPEs at 1 mM; then enzyme activity was measured with 0.5 mM IPEs.

<sup>e</sup> The enzyme was preincubated with the IPEs at 10 mM; then enzyme activity was measured with 5 mM IPEs.

inhibitory activity of AOA and hydroxylamine increased during the preincubation period. On the other hand, the inhibition of CS by other IPEs was not increased by the preincubation, suggesting that these compounds have no significant influence on CS during the preincubation period. It appears that AOA and hydroxylamine have a mode of inhibition different from that of the other compounds.

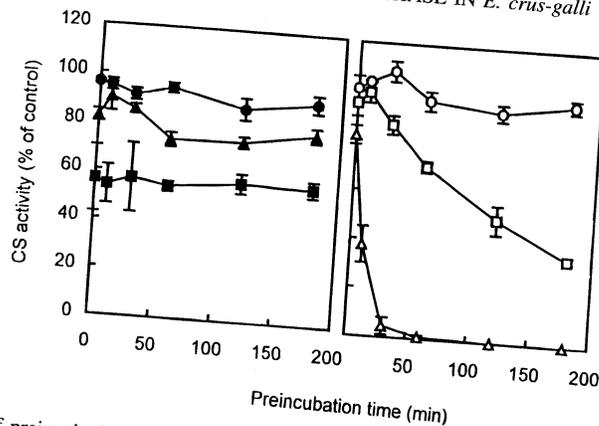
The effect of the preincubation time on the inhibitory activity of AEP, DL-allylglycine, DL-propargylglycine, and hydroxylamine was examined with the ammonium sulfate fraction. AEP showed no inhibitory activity after a 3-h preincubation at 10 mM (Fig. 1). The inhibition of CS by DL-allylglycine and DL-propargylglycine was about 45 and 25%, respectively, at 10 mM, and these values did not change after a 3-h preincubation. It has been previously reported that CS was stable at 30°C during a 4-hr incubation period (14).

CS activity decreased as the incubation time with hydroxylamine increased (Fig. 1). CS activity was decreased by 62% in 10 min and 95% in 30 min at 10 mM and by 30% in 1 h and 65% in 3 h at 1 mM. No significant CS inhibition by hydroxylamine was observed during a 3-h preincubation at 0.1 mM. These results suggest that CS inhibition by hydroxylamine is concentration and time dependent.

The relationship between the remaining CS activity [percentage of control (transformed), calculated from Table 3, 10 mM] with preincubation and the shoot elongation [percentage of control (transformed), calculated from Table 2, 10 mM] of *E. crus-galli* was examined. A correlation of  $R^2 = 0.630$  was observed when the ammonium sulfate fraction was used for the preincubation test (Fig. 2). The correlation was similar when the crude enzyme was used in the CS assay instead of the ammonium sulfate fraction ( $R^2 = 0.680$ ). The phytotoxicity of weak inhibitors of CS (e.g., AEP) was relatively low, and compounds that inhibited CS, such as AOA and hydroxylamine, showed relatively higher phytotoxicity. These results suggest that the reduction in CS activity may be correlated to the inhibition of shoot elongation in *E. crus-galli*. In addition, the crude enzyme and ammonium sulfate fraction gave equivalent results, indicating that there was no effect of the enzyme preparation method on the assay and that these preparations did not alter the activity of the inhibitors.

#### DISCUSSION

CS was extracted from the leaves of *E. crus-galli* and fractionated by ammonium sulfate. The highest activity existed in the 50–60% fraction but the 30–70% fraction was used for the enzyme inhibition trials. The protein was then



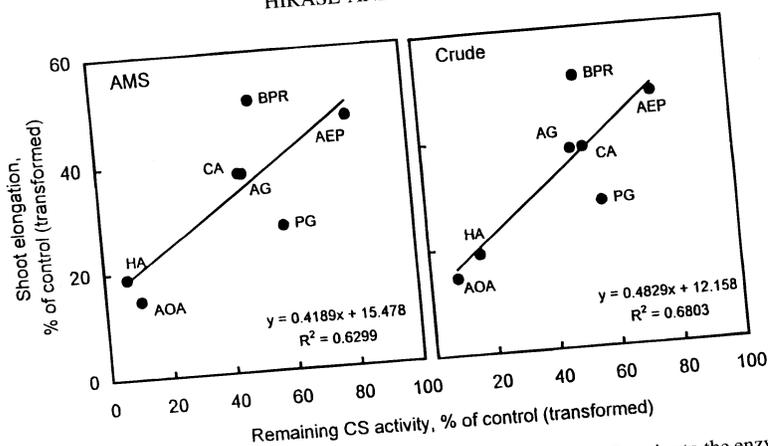
**FIG. 1.** Effect of preincubation time with IPEs on CS activity. The ammonium sulfate fraction was used as the enzyme solution. CS was preincubated with 10 mM AEP (●), 10 mM DL-allylglycine (■), 10 mM DL-propargylglycine (▲), and hydroxylamine (○, 0.1 mM; □, 1 mM; △, 10 mM) at 30°C. Each enzyme assay was conducted with half of the concentration of the IPEs used in the preincubation. The vertical bars represent  $\pm$  standard error.

desalted by Sephadex G-25 column chromatography to remove low-molecular-weight compounds. Therefore, the possibility that the CS assay was affected by low-molecular-weight compounds which might be endogenous inhibitors of CS or reactive with IPEs was minimized. The specific activity of this fraction was 8  $\mu$ mol cysteine/min/mg protein.

In this study, eight IPEs were first chosen to evaluate their inhibitory activity against CS from *E. crus-galli*. These IPEs included AEP (21), DL-allylglycine (22), DL-propargylglycine (15,23), AOA (15,24), hydroxylamine (5,15),  $\beta$ -chloro-L-alanine (15), 3-bromopropionate (15), and 3-bromopyruvate (15). It was found that 3-bromopyruvate reacted with cysteine, thus interfering with the CS assay directly. Because the CS activity was determined by the amount of cysteine produced, it is important to examine the reactivity of IPEs with cysteine before the CS assay to avoid misleading results. Then the CS assay excluded 3-bromopyruvate. IPEs were found to be weak inhibitors of CS because concentrations necessary to inhibit CS were more than 1 mM in most cases (Table 1). All of the compounds had some *in vivo* phytotoxicity against *E. crus-galli* in the shoot growth bioassay (Table 2).

It was important to compare the inhibitory activity of IPEs against CS with their *in vivo* phytotoxicity to determine whether CS could be

a possible target site of herbicides. Unfortunately, there was a very low correlation ( $R^2 = 0.173$ ) when all IPEs were considered together. This is partially due to some IPEs having multiple target sites. For example, AEP inhibited alanine racemase (21) and cysteine desulfhydrase (25), which might explain why AEP had some *in vivo* phytotoxicity without showing CS inhibition. Similarly, DL-propargylglycine, AOA, and hydroxylamine inhibited cystathionine  $\gamma$ -synthase, which is responsible for methionine biosynthesis (23). AOA also inhibited chlorophyll and carotenoid formation in greening leaf segments and serine:glyoxylate aminotransferase (24), aspartate aminotransferase (15), and phenylalanine ammonia-lyase (26). In addition, DL-propargylglycine inhibited many PLP-dependent enzymes such as cystathionase, methionine  $\gamma$ -synthase, alanine aminotransferase, and aspartate aminotransferase (15). These IPEs probably showed relatively high *in vivo* phytotoxicity compared to *in vitro* CS inhibition because of these other inhibitory actions. Therefore, good correlation between enzyme inhibition and phytotoxicity would not be expected for these IPEs. These results suggest that other potential enzymatic sites exist for which herbicides could be developed.



**FIG. 2.** Relationship between inhibition of CS activity (preincubated with IPEs prior to the enzyme reaction) and *in vivo* phytotoxicity in *E. crus-galli*. Transformed data from Table 2 (10 mM) are plotted against transformed data from Table 3 (ammonium sulfate (AMS) fraction, 10 mM, and crude enzyme (Crude)). AEP, 1-aminoethylphosphonic acid; AG, DL-allylglycine; PG, DL-propargylglycine; CA,  $\beta$ -chloro-L-alanine; BPR, 3-bromopropionate AOA, amino-oxyacetate; HA, hydroxylamine. % of control (transformed) =  $\arcsin \sqrt{\% \text{ of control}}$ .

The inhibitory activity of AOA and hydroxylamine on CS increased when CS was preincubated with these compounds (5,14). CS was inhibited by hydroxylamine in a time-dependent manner up to 3 h at 1 and 10 mM (Fig. 1). Incubation of CS from roots of *Raphanus sativas* L. (radish) with hydroxylamine also decreased its enzymatic activity (5). This result indicates that AOA and hydroxylamine may have mechanisms of CS inhibition different from those of other IPEs. Time-dependent inhibition may frequently indicate irreversible inhibition, but does not provide definite evidence of irreversibility (27). AOA was found to complex with the PLP-dependent enzymes via their bound cofactor (24). AOA also forms a 1:1 complex with the monomeric unit of aspartate aminotransferase (15). AOA and hydroxylamine probably inhibited CS through an irreversible reaction with the PLP cofactor during the preincubation. The inhibition degree of DL-allylglycine and DL-propargylglycine was unchanged during the preincubation up to 3 h (Fig. 1), and those of the other IPEs were unaffected by 1-h preincubation. From these results, these compounds appear to be reversible inhibitors of CS. However, different results have been obtained with other PLP-dependent enzymes. For example, AOA inhibits alanine aminotransferase competitively with

amino acid substrates and noncompetitively with oxo acid substrates (16). Irreversible inactivation was caused by 3-bromopropionate in aspartate aminotransferase (15). Further investigation is necessary to reveal the mode of CS inhibition by these compounds.

The correlation coefficient between the remaining activity of CS preincubated with IPEs and the shoot elongation of *E. crus-galli* was  $R^2 = 0.630$  for the ammonium sulfate fraction and  $0.680$  for the crude enzyme (Fig. 2). Inhibition by AOA and hydroxylamine was increased by the preincubation, the result being that CS inhibition and *in vivo* phytotoxicity were more similar. As shown in this test, preincubation is an effective method for comparison of CS inhibition with *in vivo* phytotoxicity.

In this study, the crude or desalted ammonium sulfate fraction were used instead of a purified enzyme. *E. crus-galli* may have isozymes of CS because two or three CS isozymes have been reported in other plants (2-4). The advantage of use of the crude extract for inhibition tests is that overall inhibition of all isozymes can be detected together. This is useful for herbicide screening and for the analysis of the correlation between enzyme inhibition and *in vivo* phytotoxicity.

CS inhibition was compared between the

crude enzyme and the desalted ammonium sulfate fractions. It had been expected that there would be some difference between them because some IPE-metabolizing enzymes and low-molecular-weight compounds that may react to IPEs, if they existed, could have been removed from the desalted ammonium sulfate fraction. We hypothesized that the result of the inhibition test with the crude enzyme was more consistent to the *in vivo* phytotoxicity. However, the difference in the correlation coefficients for the two enzyme preparations was very small, suggesting that the difference in metabolism of the IPEs in both enzyme solutions was perhaps negligible and that the IPEs acted similarly under both conditions.

In conclusion, several IPEs were found to inhibit CS from *E. crus-galli* and their inhibitory activity by preincubation was correlated to *in vivo* phytotoxicity, suggesting that CS inhibition might affect the growth of *E. crus-galli*.

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