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CYSTEINE-AUGMENTED INHIBITION OF *AVENA* INVERTASE BY MERCURIC ION

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Key Word Index—*Avena sativa*; Gramineae; oat; invertase; inhibition; mercuric ion; cysteine.

Abstract—One of the invertases (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) isolated from *Avena* seedlings was very sensitive to mercuric ion, with 60% inhibition by 0.4 μM $HgCl_2$, but concentrations of $HgCl_2$ as high as 2 mM did not increase the inhibition. The enzyme and mercuric ion formed a complex that was stable to dialysis and gel filtration and to the addition of cyanide and EDTA. Cysteine had no effect on the activity of the invertase, but when added to the mercuric ion–invertase, it increased the inhibition to 93%. Other thiols and cysteine derivatives exerted effects ranging from augmentation to reversal of the inhibition. Of all the reagents tested, cysteine and *N*-acetyl cysteine were the most effective in increasing the inhibition. However, the effectiveness of cysteine was destroyed by removal of either the amino group or the carboxyl group and by increasing the carbon chain by one unit (homocysteine).

INTRODUCTION

We recently demonstrated that the soluble invertase activity in oat seedlings is due to two enzymes (designated invertase I and invertase II) which can be separated by chromatography on DEAE-cellulose [1]. Both enzymes appear to be acid invertases, with pH optima between 4 and 5, but they differ in other properties. The MW of invertase II is almost twice that of invertase I (108000 vs 59000). The isoelectric points are 8.6 and 4.4 for invertase I and invertase II, respectively. Invertase I has a higher affinity for the substrates sucrose, raffinose and stachyose than invertase II. The enzymes further differ in their reactivity with a number of inhibitors but especially with $HgCl_2$. Invertase II is completely inhibited by 2 μM $HgCl_2$. Invertase I is also very sensitive to mercuric ion but the activity is only partially inhibited even by high levels of $HgCl_2$.

Similar partial inhibition has been reported for pig muscle lactate dehydrogenase [2], rabbit muscle phosphoglucomutase [3], and potato phosphorylase [4]. Webb [5] provided an explanation for the partial inhibition of an enzyme by assuming that, even though combination of the enzyme with a mercurial is complete, the groups reacted are sufficiently far from the active site so that the introduced group would modify only the catalysis. This explanation may apply to the partial inhibition of oat invertase by mercuric ion, but the results of my studies suggest that the ionic and steric properties of the introduced group can have a large effect on the inhibition. Attempts to reverse the inhibition with thiols led to the discovery that some of these reagents increased the inhibition, presumably by reacting with the mercuric ions on the enzyme. This paper describes how structural changes in cysteine affect the inhibition of oat invertase by $HgCl_2$.

RESULTS AND DISCUSSION

Oat invertase I was very sensitive to mercuric ion: 28% of its activity was inhibited by 0.2 μM $HgCl_2$; 45%, by 0.3 μM $HgCl_2$; and 59%, by 0.4 μM $HgCl_2$ (Fig. 1). But as the $HgCl_2$ concentration was further increased to as high as 2 mM, the level of inhibition remained at *ca* 60%. The assay conditions were first varied in an attempt to find an explanation for the partial inhibition. Increasing the period of pre-incubation of the enzyme with $HgCl_2$ beyond 10 min did not increase the inhibition. Similarly,

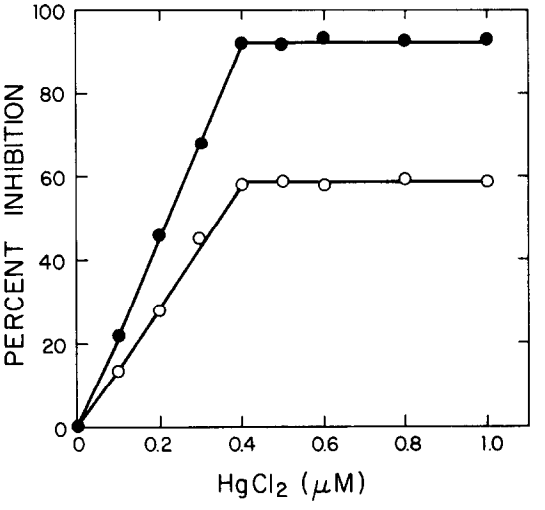

Fig. 1. Effect of $HgCl_2$ concentration on the inhibition of oat invertase I. The assay conditions were standard and $HgCl_2$ and cysteine were added as described in the text. ○—○, Absence of cysteine; ●—●, presence of 40 μM cysteine.

the nature of the buffer and the pH of the reaction solution did not affect the degree of inhibition, with *ca* 60% inhibition on both sides of the pH optimum at 4.5. The extent of inhibition by $HgCl_2$ was essentially independent of substrate size, with 60, 63 and 66% inhibition for sucrose, raffinose and stachyose, respectively.

Many enzyme inhibitions by heavy metals are reversible by the addition of metal-complexing agents such as cyanide, EDTA and thiols [6]. In preliminary studies on reversing the inhibition of oat invertase by Hg^{2+}, cyanide and EDTA were ineffective. But I was surprised to find that addition of cysteine to the reaction solution increased rather than reversed the inhibition (Table 1). Where 5 μM $HgCl_2$ alone inhibited the invertase 60%, the addition of 40 μM cysteine increased the inhibition to over 90%. The effect of cysteine on the inhibition was a linear function of $HgCl_2$ concentration up to 0.4 μM, the concentration for maximum inhibition by $HgCl_2$ alone, and then remained constant at *ca* 93% inhibition as the concentration of $HgCl_2$ increased further (Fig. 1).

Cysteine enhanced the inhibition of the invertase by Hg^{2+} only when it was added after the $HgCl_2$ to the enzyme solution. Addition of cysteine (40 μM) before $HgCl_2$ (5 μM) completely prevented inhibition of the enzyme. This could be the result of Hg^{2+} reacting preferentially with the excess of cysteine rather than with the enzyme. However, when invertase was first treated with 40 μM cysteine and then dialysed to remove the excess cysteine, it was also inhibited *ca* 60% by 5 μM $HgCl_2$. Thus, the reaction of the enzyme with Hg^{2+} was a prerequisite for the cysteine effect on the inhibition. The product formed by Hg^{2+} with the enzyme was quite stable: invertase treated with 5 μM $HgCl_2$ was not reactivated by prolonged dialysis against 0.15 M NaCl or by gel filtration on Sephadex G-100. The mercuri-invertase complex obtained after dialysis, which removed the excess $HgCl_2$, was not further inhibited by $HgCl_2$ added to the assay solution (Table 2). However, cysteine added to the mercuric ion–enzyme decreased its activity to about a fourth of the original level. The cysteine–mercuric ion–invertase complex, prepared by treating the enzyme for 15 min periods first with 5 μM $HgCl_2$ and then with 200 μM cysteine, was also stable to dialysis and gel filtration. The activity of the dialysed or filtered complex was not decreased by additions of $HgCl_2$ and cysteine to the assay solution.

Some of the properties of the mercuric ion–invertase and the cysteine–mercuric ion–invertase were compared with those of the native enzyme. Identical elution volumes were obtained for the three preparations on chromatography through Sephadex G-100, demonstrating that the Stokes' radius of the enzyme was unchanged by the Hg^{2+} and cysteine. The pH optimum and stability to heat of the invertase were similarly not affected. The effect of sucrose concentration on the three enzyme samples was also determined. The Lineweaver–Burk plots were linear for all three enzymes over the range of 3–146 μM sucrose. The K_ms calculated from the plots were 2.6, 1.7 and 3.7 for the native invertase, mercuric ion–invertase, and cysteine–mercuric ion–invertase, respectively.

A variety of cysteine derivatives and aliphatic thiols were tested on the mercuric ion–invertase to determine the molecular requirements for augmentation of the inhibition (Table 3). All reagents were tested at 200 μM

Table 1. Effect of cysteine concentration on the inhibition of oat invertase by Hg^{2+}

$HgCl_2$ (μM)	Cysteine (μM)	Invertase activity (μmol reducing groups)
0	0	3.85
5	0	1.54
5	4	1.40
5	10	0.91
5	20	0.39
5	40	0.28
5	100	0.23
5	200	0.25
0	200	3.90

Table 2. Effects of $HgCl_2$ and cysteine on the activity of dialysed mercuri-invertase

Addition	Invertase activity (μmol reducing groups)
None	2.85
5 μM $HgCl_2$	2.81
4 μM cysteine	2.75
10 μM cysteine	2.42
20 μM cysteine	1.60
40 μM cysteine	0.89
100 μM cysteine	0.85
200 μM cysteine	0.76

Table 3. Effects of cysteine derivatives and aliphatic thiols on the activity of the mercuri-invertase

Addition	Relative invertase activity
None	100
Mercaptoethanol	89
Thioglycolate	77
Cysteine	23
Serine	98
Cysteic acid	96
β-Mercaptoethylamine	112
β-Mercaptopropionic acid	150
β,β-Dimethylcysteine	87
N-Acetyl-L-cysteine	26
Homocysteine	100
S-2-Aminoethylcysteine	103
S-Ethyl cysteine	101
Glutathione	116
Cysteine ethyl ester	162
Cysteine methyl ester	158
Thioproline	105
Cystine	97

concentration, and the results represent the means of at least three assays. Cysteine increased the inhibition more than any other thiol studied, and the D- and L-forms of this amino acid were equally effective. Cysteine derivatives with the sulfhydryl group modified (serine and cysteic acid) or bonded (*S*-ethyl cysteine, *S*-2-aminoethyl cysteine, cystine and thioproline) were not effective. This suggests that a free sulfhydryl group is a primary requirement for interaction with the mercuric ion–enzyme. However, even with a free sulfhydryl group, the remaining structure of the thiol molecule determines its effect on the activity of the mercuric ion–invertase complex. Cysteine was the most effective inhibitor, and the maximum effect was dependent on the presence of both the amino and carboxyl in the molecule. Removal of the carboxyl group (thus forming β-mercaptoethylamine) resulted in slight activation of the complex, and esterification of the carboxyl group (forming cysteine ethyl ester and cysteine methyl ester) increased the activation. Similarly, removal of the amino group to form β-mercaptopropionic acid led to a pronounced activation of the mercuric ion–invertase. On the other hand, *N*-acetyl cysteine was almost as effective an inhibitor as cysteine, indicating that substitution on the amino group did not affect the inhibition. Chain length of the molecule is another requirement for inhibition by cysteine derivatives: homocysteine, which has one additional carbon in its chain, did not block enzyme activity. The effectiveness of cysteine was also markedly decreased by substitution of two methyl groups on the carbon adjacent to the sulfhydryl, forming β,β-dimethyl cysteine.

Webb [7] has proposed that the failure of thiols to achieve reversal of enzymes inhibited by mercuric ions may be due to one of several reactions: (1) the thiols reduce enzyme disulfide groups to SH groups and increase the binding of Hg^{2+} to the enzyme; (2) the Hg^{2+} reacts with both enzyme SH groups and the thiols to form E–S–Hg–S–R complexes which are less active than the simple E–S–Hg^{+} complex; (3) the R–S–Hg–X or R–S–Hg–S–R mercaptides formed are inhibitory by a mechanism unrelated to enzyme SH groups. The results of the present study are consistent with reaction (2). Invertase reacted with Hg^{2+} to form a stable complex. Pretreatment of the enzyme with cysteine did not increase the inhibition by Hg^{2+}. This suggests that cysteine did not release new SH groups in the enzyme, thus ruling out reaction (1). Similarly, reaction (3) does not appear to apply because the thiols reacted only with mercuric ion–invertase complex. Presumably the thiols react with the mercuric ions on the enzyme. However, the actual effect of thiol may vary from augmentation to reversal of the inhibition, depending on the structure of the thiol. The different effects may be due to electrostatic and/or steric factors. That cysteine was the most effective inhibitor of the complex suggests that the charge effects of the amino and carboxyl groups are important in increasing inhibition. But steric effects are also important as illustrated by the loss of effectiveness when the chain length of cysteine was increased by one carbon (homocysteine). Further work is needed to determine how interactions of Hg^{2+} and thiols with the enzyme affect the active site in reducing the activity without abolishing it.

A similar augmentation of mercurial inhibition by thiols has been reported for yeast invertase [8]. An important difference between the yeast and oat invertases is that the yeast enzyme is completely inhibited by Hg^{2+} alone at relatively high concentrations. But at a concentration of 0.13 μM $HgCl_2$, which inhibited the invertase *ca* 60%, Gemmill and Bowman [8] found that the inhibition was increased by both cysteine and glutathione but not by thioglycolate. When I re-examined the yeast invertase system to determine the structural requirements of the thiol for maximum inhibition (assay conditions as described by Gemmill and Bowman, with yeast invertase obtained from Sigma Chemical Co.), I confirmed that glutathione increased the inhibition by Hg^{2+}. But contrary to the findings of Gemmill and Bowman, cysteine reversed the inhibition. Furthermore, none of the cysteine derivatives listed in Table 3 increased the inhibition of yeast invertase, and most of them decreased the inhibition, especially homocysteine and β-mercaptopropionic acid.

EXPERIMENTAL

Invertase I was extracted from seedlings of *Avena sativa* cv Victory. The seeds were planted in moistened vermiculite and germinated at 22° in the dark. After 5 days, the seedlings were harvested and homogenized in 0.15 M NaCl with VirTis and Polytron homogenizers. The homogenate was centrifuged, and solid $(NH_4)_2SO_4$ was added to the supernatant soln to 80% satn. The resulting ppt. was collected by centrifugation, dissolved in H_2O, and dialysed against 0.01 M Tris (pH 6.5). The enzyme soln was concd to 10 ml by ultrafiltration (Amicon Corp. cell 202 and PM 10 membrane) and applied to a 2.5 × 30 column of DEAE-cellulose pre-equilibrated with 0.01 M Tris (pH 6.5). Elution was conducted with a linear gradient of 0–0.5 M NaCl in 0.01 M Tris (pH 6.5). The chromatography on DEAE-cellulose separated invertase I from invertase II. Invertase I was eluted by approximately 0.05 M NaCl while invertase II was not eluted until the NaCl concn reached 0.25 M. The fractions containing invertase I were pooled and concd to 5 ml by ultrafiltration.

The enzyme was further purified by the following procedure. The conc soln of invertase I was applied to a 2.5 × 90 cm column of Sephadex G-100 pre-equilibrated with 0.05 M NaCl. The eluent was 0.05 M NaCl. The fractions containing invertase activity were pooled and concd to 3 ml by ultrafiltration. This soln was subjected to isoelectric focusing with a Desaga/Brinkman TLE Double Chamber according to the manufacturer's instructions. The soln was mixed with Sephadex G-75 'superfine' and applied to a trough cut out of the middle of a 20 × 20 cm plate coated with Sephadex G-75 containing 2% pH 2–10 pHisolyte. Focusing was conducted at 4° and 200 V for 12 hr and then at 300 V for 2 hr. Narrow strips of the gel were cut parallel to the electrodes, suspended in 0.15 M NaCl, and assayed for invertase. The activity was found in a band approximately 5 cm from the origin on the cathode side (pH 8.6). The soln was dialysed against 0.15 M NaCl to remove the carrier ampholytes. The specific activity of the invertase after the isoelectric focusing was 260 units/mg protein.

Invertase was assayed by adding 0.1 ml 0.73 M sucrose to a soln containing 0.1 ml invertase, 0.1 ml 0.2 M NaOAc, (pH 4.5) and 0.2 ml H_2O. The samples and blanks prepared with heated enzyme were incubated at 30° for 30 min. The reactions were stopped by adding 0.5 ml 0.5 M Na_2HPO_4 followed by heating for 3 min. 0.5 ml of each sample was then analysed for reducing groups by the arsenomolybdate method [9]. A unit of invertase is defined as the amount of invertase that catalyses the release of 1 μmol of reducing groups in the standard assay. When $HgCl_2$ was included in the reaction soln, it was pre-incubated another 10 min before the addition of the substrate.

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