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Acetohydroxamate Inhibition of the Activity of Urease from Dehusked Seeds of Water Melon (*Citrullus vulgaris*)

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Urease from the seeds of watermelon (*Citrullus vulgaris*) was purified to apparent homogeneity, using two acetone fractionation steps, heat treatment at 48°C and gel filtration through Sephadex G-200. Effect of acetohydroxamic acid (AHA) on the activity of the homogeneous enzyme preparation (sp. act. 3000 ± 550 U/mg protein) was investigated. AHA exhibited a concentration-dependent inhibition both in the presence and absence of the substrate. The inhibition was uncompetitive and the K_i was 2.5 mM. Binding of AHA with the enzyme was reversible, as 63% activity could be restored by dialysis. Time-dependent inhibition revealed a monophasic inhibition of the activity. Addition of β-mercaptoethanol (ME) gradually abolished the inhibition. Pre-treatment of native enzyme with 8.0 mM ME for 5 min at 30°C exhibited protection against AHA-induced inhibition. The significance of these observations is discussed.

Keywords: Urease; *Citrullus vulgaris*; Acetohydroxamate; β-Mercaptoethanol; Watermelon

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbon dioxide. In 1926, urease was the first protein-enzyme to be crystallized in pure form from jack bean seeds by Sumner.[1] Nearly 50 years later, this plant protein was demonstrated to possess nickel.[2] Since then, the interest in the plant urease has continued, but recently crystal structures of *Klebsiella aerogens* and *Bacillus pasteurii* ureases have been solved at low resolutions.[3,4] However, the most extensively studied plant urease is from the seeds of jack bean[5] where it is an abundant seed protein.

The study of urease inhibitors has been useful in providing insight into the catalytic mechanism. Several classes of urease inhibitors are known and some have been examined for their agricultural value.[6] The control of urea hydrolysis by use of urease inhibitors would lead to a reduction in environmental pollution, enhanced efficiency of urea nitrogen uptake by plants, and improved therapeutic strategies for the treatment of infections due to ureolytic bacteria.[7]

Acetohydroxamate (AHA) and its derivatives are reported to be reversible, slow binding inhibitors of both plant and microbial urease.[8,9] Recently, Benini *et al.*[10] have grown the crystals of AHA-inhibited *Bacillus pasteurii* urease and demonstrated the binding mode of AHA to the active site by X-ray data at 1.55 Å resolution. The AHA-bound structure revealed a chelate interaction similar to those seen in other metallo-enzymes. Similarly, purified enzyme from *Anthrobacter mobilis* is also known to be inhibited by AHA and heavy metal ions[11] like Hg^{2+} and Cu^{2+}.

Urease from watermelon (*Citrullus vulgaris*) seeds has been purified to apparent homogeneity and partially characterized.[12] We have also presented evidence for the presence of thiol groups on the active site of the enzyme. Its inactivation kinetics with thiol-specific reagents showed biphasic kinetics in that half of the initial activity was destroyed more rapidly than the remainder.[13] This unique phenomenon of molecular asymmetry or more specifically half-site reactivity in watermelon urease, reported for the first time, was further substantiated by its thermal inactivation studies.[14] Earlier studies on jack bean urease have established

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that thiols are inhibitors; β-mercaptoethanol (ME) being a competitive inhibitor in the hydrolysis of urea and dithiothreitol (DTT) being a poor inhibitor.[15,16] However, we have recently demonstrated, for the first time, that not only ME but also DTT and *L*-cysteine at 30°C in 50 mM Tris–acetate buffer (pH 8.5) are excellent activators of the watermelon urease and the order of effectiveness as activator was ME > DTT > L-cysteine.[17]

Although the mode of binding and kinetic parameters for AHA inhibition to microbial ureases is well documented, no corresponding detailed kinetic studies on plant urease are available. In the present communication, therefore, we have studied the influence of AHA on the kinetic behaviour of purified watermelon urease which, to our knowledge, is the first detailed report on any plant urease.

MATERIALS AND METHODS

Materials

Dehusked watermelon seeds were purchased from the local market. Tris was obtained from Boehringer Mannheim Gmbh, Germany. Bovine serum albumin, DTT, *L*-cysteine, and ME were obtained from Sigma Chemical Co., USA. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Acetohydroxamic acid was obtained from HiMedia Laboratories Limited, Mumbai, India. Nessler's and Folin–Ciocalteau reagents were from Qualigens Fine Chemicals, Mumbai. All other reagents were analytical grade chemicals either from BDH or E. Merck, India.

Enzyme

Urease was isolated and purified from dehusked seeds of watermelon as described earlier.[12,17] The enzyme preparation (sp. activity 3000 ± 550 U/mg protein), showing a single enzyme and protein band on native 7.5% PAGE (at pH 8.3), was employed for the study.

Urease Activity Assay

Enzyme activity was assayed in 50 mM Tris–acetate buffer (pH 8.5). An aliquot (0.8 ml) of buffer and 1.0 ml of 250 mM urea in the same buffer were brought to 30°C. The reaction was started by adding 0.2 ml of suitably diluted enzyme. After 10 min, 1.0 ml of 10% trichloroacetic acid was added to stop the reaction. The total reaction mixture was transferred to a measuring flask (50 ml) and the volume was made to 50 ml with distilled water after adding 1.0 ml of Nessler's reagent as described earlier.[17] The amount of ammonia liberated was measured at 405 nm in a Spectronic 21 UVD spectrophotometer.

One enzyme unit was defined as the amount of enzyme required to liberate one μmole of ammonia in one minute under the test conditions defined above (30°C, 50 mM Tris–acetate buffer, pH 8.5, 250 mM urea).

Protein was estimated by the method of Lowry *et al.*[18] with Folin–Ciocalteau reagent calibrated with crystalline bovine serum albumin.

Effect of AHA on the Activity

A stock solution of AHA was made in 50 mM Tris–acetate buffer (pH 8.5) and diluted with the same buffer as required. The activity of suitably diluted enzyme was determined in the presence of varying concentrations of AHA added in the standard assay mixture. For the direct effect of AHA, enzyme alone was incubated with the desired concentration of inhibitor for 10 min at 30°C and the treated enzyme was assayed for activity. The inhibition pattern and inhibition constant (K_i) was calculated from a Lineweaver–Burk plot.[19]

The effect of thiols was simultaneously examined in another set of experiments where suitably diluted urease was first treated with the desired concentrations of AHA for 10 min at 30°C and then the reaction mixture was exposed to varying concentrations of ME. The activity was assayed as described above.

The results reported are the mean of 5–8 replicate experiments carried out with a fresh batch of purified enzyme.

RESULTS AND DISCUSSION

The effect of AHA on the activity of watermelon urease was studied in the concentration range of 0.05–1.0 mM. The desired concentration was added to the standard assay mixture (comprising of 250 mM urea, 50 mM Tris–acetate buffer, pH 8.5, at 30°C) and after adding suitably diluted enzyme solution (10.0–11.0 U/ml, 6–7 μg protein/ml) and incubating for 10 min, the activity was determined. The results (Figure 1) revealed a concentration-dependent inhibition of the activity. About 50% inhibition was observed at 700 μM, which increased to about 75% at 1 mM of AHA.

In order to explore the direct effect of AHA, watermelon urease (10.0–11.0 U/ml, 6–7 μg protein/ml) was incubated with AHA (0.05–1.0 mM) in the absence of urea for 10 min at 30°C and the treated enzyme was assayed for activity. There was an instant loss in the activity. Therefore, reasonably low concentration (1–10 μM) had to be employed to

obtain a measurable rate of inhibition. The results (Figure 2) again revealed a rapid and concentration-dependent inhibition in urease activity. There was an initial rapid inactivation followed by a slow and sustained inhibition. About 73% inhibition was observed at 3.0 μM AHA, which increased to 100% at 10.0 μM concentration.

The nature of the inhibition of urease (7.0–9.0 U/ml, 8–10 μg protein/ml) was studied by employing four concentrations of AHA in such a manner that the inhibition was measurable. For the study, urea concentration in the assay mixture was varied from 2 to 125 mM. The results, when expressed by a Lineweaver–Burk double reciprocal plot of substrate concentration versus velocity (optical density at 405 nm), revealed an uncompetitive inhibition (Figure 3). The K_i was found to be 2.5 mM.

In order to assess the interaction of AHA with the enzyme alone, suitably diluted urease (10.0–11.0 U/ml, 6–8 μg protein/ml) was treated with 1.0 μM AHA for 10 min at 30°C in absence of urea. The resulting urease-AHA mixture was subsequently dialyzed against 50 mM Tris–acetate buffer (pH 8.5) overnight at 4–6°C. The enzyme when assayed, exhibited 63% recovery, suggesting a reversible interaction of AHA with the enzyme.

Hydroxamic acids have been shown to be potent inhibitors of jack bean urease.[20] Since then, a wide range of hydroxamic acids and its derivatives has been shown to inhibit microbial ureases competitively, non-competitively, mixed, or irreversibly.[7] AHA is a well-known reversible competitive inhibitor of jack bean, *K. aerogenes*, and pigeon pea ureases with K_i values of 19.8 μM, 2.6 μM, and 41.0 μM respectively.[8,9,21] Here, we find that AHA is a reversible uncompetitive inhibitor of watermelon urease at 30°C in 50 mM Tris–acetate buffer (pH 8.5). The K_i value is also comparatively higher than those reported for other ureases. The inhibitor significantly lowered the apparent V_{max} and K_m values at all concentrations of AHA used. This suggests that AHA presumably distorts the urease active site, thereby rendering the enzyme catalytically inactive. The known binding of AHA to the active site Ni^{2+} ion of jack bean and the cysteine residue of pigeon pea ureases[8,21] support this view.

Time-dependent inhibition of watermelon urease was studied by incubating the enzyme (9.0–10.0 U/ml, 8–9 μg protein/ml) with 0.5, 1.0, and 2.5 μM AHA at 30°C in the absence of urea. Aliquots were withdrawn at specific time intervals and assayed for activity. The results (Figure 4) revealed a monophasic, time-dependent inhibition of the activity. The rate of inactivation was much faster with 2.5 μM AHA (about 90% loss within 12 min of treatment) than with 0.5 μM (90% loss in about 35 min). In contrast to the present observations, a *biphasic* time-dependent inhibition of pigeonpea urease with AHA (75 and 100 μM at 37°C, 50 mM Tris–acetate buffer, pH 7.3) has recently been reported.[21] This difference in the behaviour of urease towards AHA may be due to the nature of the enzyme obtained from a different source.

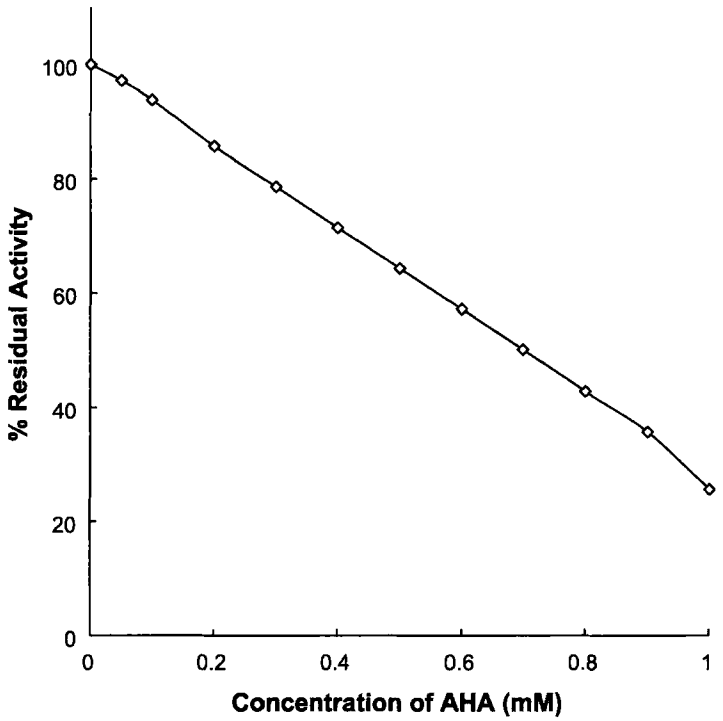

FIGURE 1 Effect of AHA on the activity of watermelon urease. Suitably diluted enzyme (10.0–11.0 U/ml, 6–7 μg protein/ml) was incubated for 10 min in the presence of varying concentrations of AHA in the standard assay mixture.

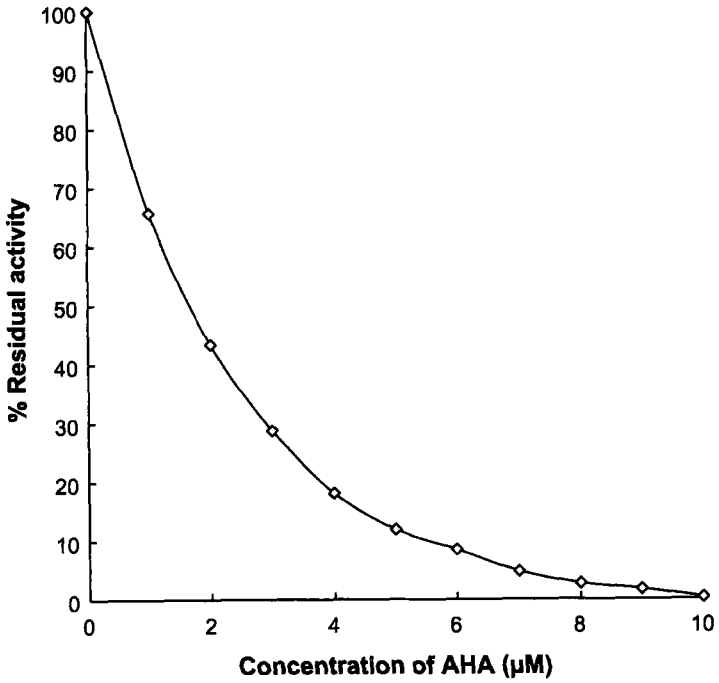

FIGURE 2 Inactivation of watermelon urease by AHA. Suitably diluted enzyme (10.0–11.0 U/ml, 6–7 μg protein/ml) was incubated with varying concentrations (1–10 μM) of AHA for 10 min at 30°C in 50 mM Tris–acetate buffer (pH 8.5) and then assayed for activity.

However, the possibility that the effects are due to the difference in the experimental conditions (viz., nature and composition of buffer, temperature, concentration of AHA, etc.) cannot be completely ruled out.

Thiols have recently been demonstrated to be an excellent activator of watermelon urease.[17] It was of interest to explore whether the AHA-induced inhibition had any bearing on the thiols-induced activation. This was studied by adding the desired concentration of ME (2, 4, and 8.0 mM) in the standard assay mixture containing varying concentrations of AHA (0.1–1.0 mM). The results

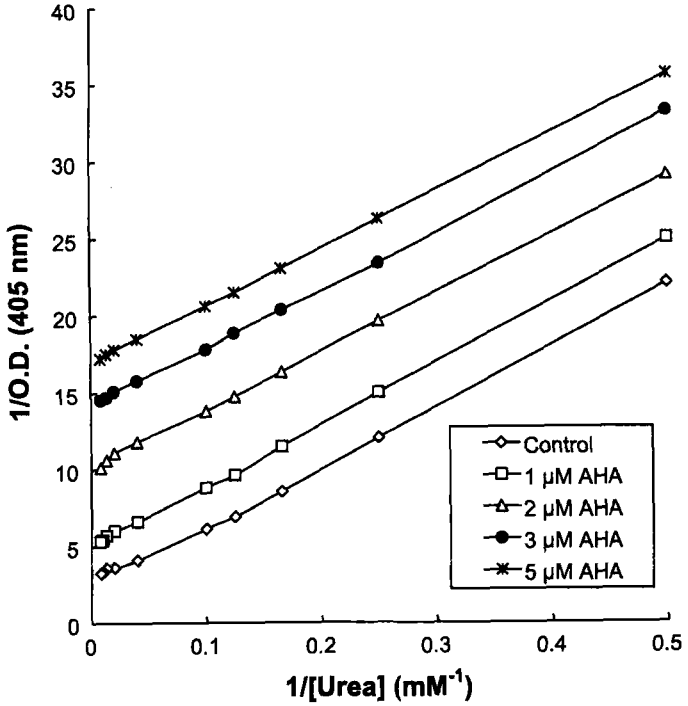

FIGURE 3 Lineweaver–Burk plot of reciprocals of substrate concentration against optical density at 405 nm. The activity of urease (8.0–9.0 U/ml, 5–6 μg protein/ml) was assayed in presence of 1, 2, 3, and 5 μM AHA. The urea concentration in the assay mixture was varied from 2 to 125 mM.

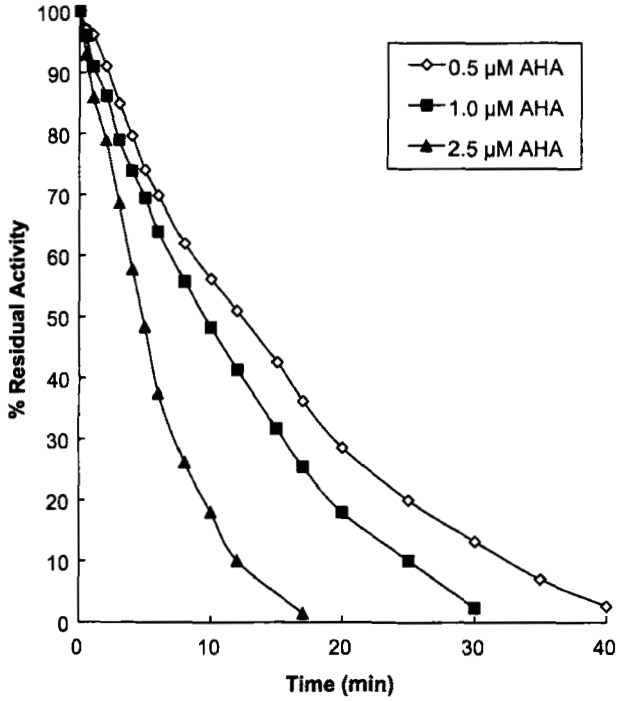

FIGURE 4 Time-dependent inhibition of watermelon urease with AHA. Enzyme (9.0–10.0 U/ml, 8–9 μg protein/ml) was incubated with 0.5, 1.0, and 2.5 μM AHA at 30°C in absence of urea. The aliquots withdrawn at specific time intervals were assayed for activity.

(Figure 5), in the absence of ME revealed AHA-dependent suppression of the activity (a result identical to that of Figure 1). However, upon addition of ME, the extent of the inhibition was decreased. Thus, 2.0 mM ME lowered AHA (1.0 mM)-induced inhibition from 75% to 56% and 4.0 mM ME suppressed it to only 43%. With a further increase in the concentration of ME to 8.0 mM, only 30% inhibition of activity was observed. DTT and *L*-cysteine, on the other hand, were ineffective in lowering the inhibition.

Time-dependent hydrolysis of urea by AHA-treated watermelon urease in the presence of 8.0 mM ME was studied by treating the enzyme first with 1.0 μM AHA for 2, 5, and 10 min at 30°C in 50 mM Tris–acetate buffer (pH 8.5) and then transferring the treated enzyme to a standard assay system containing 8.0 mM ME in addition to 250 mM urea. The samples were withdrawn at different time intervals and assayed for the ammonia formed by Nessler's reagent. The rate of urea hydrolysis by the untreated enzyme progressed with time (Figure 6). The ureolysis by the AHA-treated enzyme progressed initially and then attained a steady state. The initial rate of urea hydrolysis though was faster with urease treated with AHA for 2 and 5 min, but

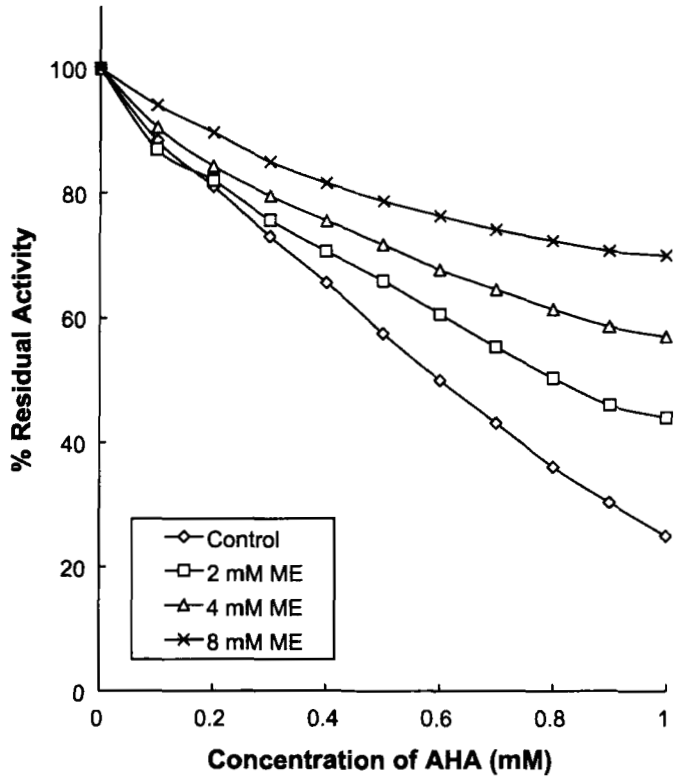

FIGURE 5 Effect of ME on AHA-induced inhibition of watermelon urease. The activity of suitably diluted enzyme (8.5–10.0 U/ml, 5–8 μg protein/ml) was determined at 30°C in presence of varying concentrations of AHA and ME added in 50 mM Tris–acetate buffer (pH 8.5) containing 250 mM urea.

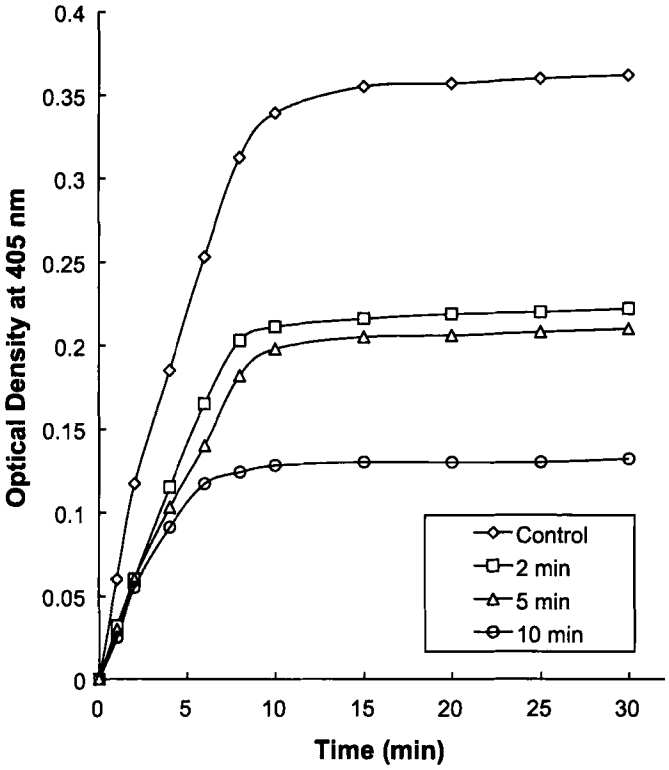

FIGURE 6 Time-dependent hydrolysis of urea by AHA-treated watermelon urease in presence of 8.0 mM ME. Suitably diluted enzyme (9.5–10.0 U/ml, 7–8 μg protein/ml) was first incubated with 1.0 μM AHA for 2, 5, and 10 min at 30°C in 50 mM Tris–acetate buffer (pH 8.5) and was then transferred to standard assay system containing 8.0 mM ME in addition to 250 mM urea. The samples withdrawn at different time intervals were assayed for the ammonia formed using Nessler's reagent.

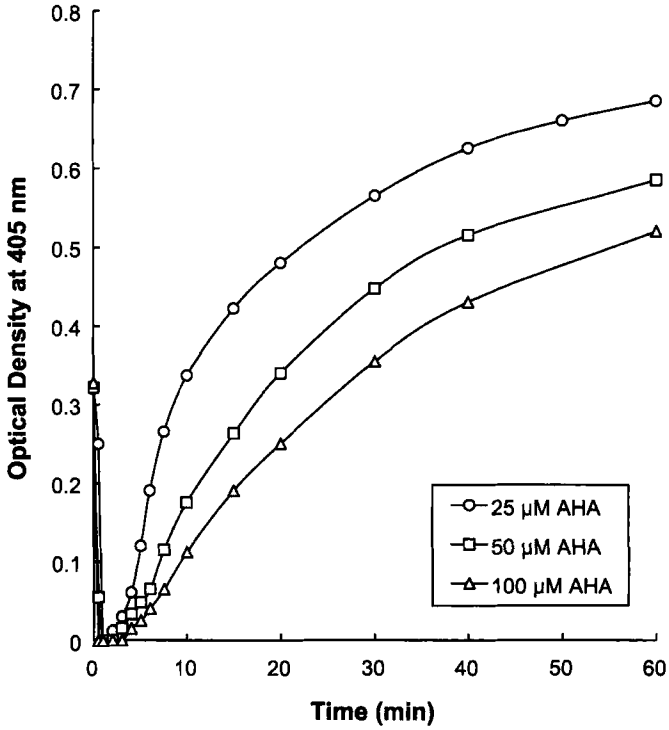

FIGURE 7 Time-dependent reversal of AHA induced inhibition of watermelon urease by ME. Suitably diluted enzyme (9.0–10.0 U/ml, 8–9 μg protein/ml) was first treated with 25, 50, and 100 μM AHA and then treated with 8.0 mM ME. The aliquots withdrawn at specific time intervals were assayed for activity.

was much slower than the untreated enzyme and attained its maximum in about 10 min. Enzyme treated for 10 minutes on the other hand, could hydrolyze urea at about half the rate when compared to the enzyme treated for 2 min and attained its maximum in about 6 min.

Time-dependent reversal of AHA-induced inhibition by ME was studied next by treating urease with 25, 50, and 100 μM AHA at 30°C in 50 mM Tris–acetate buffer (pH 8.5). This high concentration of AHA was selected to ensure instant and complete inhibition of the enzyme. The AHA was added to the suitably diluted urease solution and the mixture was assayed. When the enzyme was completely inhibited (in less than 2 min), ME (8.0 mM) was added to the urease-AHA mixture and samples withdrawn at various time intervals were assayed by transferring into standard assay medium. The results (Figure 7) revealed a reversal of the inhibition. In fact, inhibition was completely abolished in the presence of ME and the activity was significantly increased. The rate of reversal of 25 μM AHA treated enzyme was much faster than that for a 100 μM treated one. Thus, for the former, the activity adjusted to its normal rate in less than 10 min while for the latter it took about 20 min. The enzyme activity continued to increase with time and was, in fact, doubled; the effects of AHA were completely abolished. When the enzyme alone was treated with ME (8.0 mM) for 5 min at 30°C and then treated with AHA (25–500 μM) for varying time periods, there was no inhibition of activity (data not reported), suggesting a protective effect of ME on the enzyme.

Urease is a metalloenzyme containing nickel at its active center. AHA, a metal chelator, is known[8] to form a stable bidentate complex with Ni^{2+}. Andrews *et al.*[22] have demonstrated that in jack bean urease the binding of hydroxamates might involve the interaction with active site cysteine residues. Recently, Srivastava and Kayastha[23] established binding of AHA to the active Ni^{2+} ion and cysteine residues of pigeonpea urease, suggesting a direct interaction of AHA with thiol residues. This binding to the metallocentre might destroy the active site of the enzyme thereby rendering the enzyme catalytically inactive. The observed uncompetitive inhibition and altered affinity of the enzyme for its substrate support this view. In the presence of ME, however, the thiol groups at the active site are protected, making them unavailable to AHA. Therefore, disappearance of AHA inhibition by ME is expected. Moreover, in each case the concentration of ME was kept much greater than that of the enzyme or AHA, so it appears that the ME replaces the AHA that is bound to the active site cysteine or Ni^{2+} because the affinity of watermelon urease for ME is greater than AHA. However, the possibility of altered affinity of urease for AHA in the presence of ME, also cannot be ruled out. The reversal of AHA-induced inhibition by ME reported above supports this view.

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