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A STUDY OF INHIBITION OF UREASE FROM SEEDS OF THE WATER MELON (*Citrullus vulgaris*)

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Urease from seeds of the water melon was found to be inhibited by various salts of sodium. Sodium fluoride strongly inhibited the activity in the low urea concentration range. The enzyme was also inhibited by a high concentration of urea which was completely abolished in the presence of 10 mM sodium fluoride. Time-dependent inactivation of urease with iodoacetic acid, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate exhibited biphasic kinetics in which half of the initial activity was lost in the fast phase and the remainder in a slow phase. Each phase exhibited first-order kinetics. These observations are suggestive of the existence of half-and-half distribution of sites.

Keywords: Urease; Water melon; Inhibition; Iodoacetic acid; *N*-ethylmaleimide; *p*-hydroxymercuribenzoate

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) has been extensively studied in seeds of various leguminous plants[1–3] where it is an abundant seed protein. Some seeds of cucurbitaceae[4,5] are also known as a rich source of urease. It has been, for the first time, purified from the seeds of the water melon (*Citrullus vulgaris*) to apparent homogeneity and partially characterised.[6]

Earlier studies on urease from various leguminous sources have established that the enzyme interacts with alkali metal ions,[3] fluoride ion[7,8] and

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different group specific reagents.[9] The interaction of this enzyme from other sources with these reagents is not known. The present paper, thus, deals with the influence of various salts of sodium, fluoride ion and –SH reagents on the activity of recently purified water melon urease.

MATERIALS AND METHODS

Materials

Dehusked water melon seeds were purchased from the local market. Tris was obtained from Boehringer Mannheim Gmbh, Germany. *N*-Ethylmaleimide and *p*-hydroxymercuribenzoic acid, sodium salt, were obtained from Sigma Chemical Co., U.S.A. Iodoacetic acid was from Sisco Research Laboratories, Bombay, India. All other reagents were analytical grade chemicals from either BDH or E. Merck, India.

Isolation and Purification

Urease was isolated and purified to apparent homogeneity as described earlier.[6] The enzyme preparation showing a single enzyme and protein band on native PAGE (specific activity 3750 U/mg protein) was employed for the study.

Urease Activity Assay

Enzyme assay was carried out 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 warmed 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. The amount of ammonia liberated was measured at 405 nm in a Spectronic 21 UVD spectrophotometer.

A unit of enzyme activity 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 esimated by the method of Lowry *et al.*,[10] with Folin-Ciocalteau reagent calibrated with crystalline bovine serum albumin.

Inhibition Studies

A number of sodium salts of mineral acids were tested for their effect on urease activity. Stock solutions of these salts were made in 50 mM Tris-acetate buffer (pH 8.5) and diluted as required. The activity of suitably diluted enzyme was determined in the presence of varying concentrations of the salt in a standard assay mixure.

Inactivation Studies

The enzyme and desired reagent at specified concentrations in 50 mM Tris-acetate buffer (pH 8.5) were incubated at 30°C. Aliquots were withdrawn at specified intervals and were immediately transferred to assay mixtures previously warmed to 30°C. The activity was determined as described above.

RESULTS AND DISCUSSION

Inhibition by Sodium Salts

Inhibition of urease activity by various salts of sodium e.g., sodium chloride, sodium fluoride, sodium nitrate, and sodium sulphate was studied in the concentration range 5–750 mM. The desired concentration of the salt solution was added to the standard assay mixture comprising of 250 mM urea, 50 mM Tris-acetate buffer (pH 8.5) and suitably diluted enzyme solution. The results obtained (Figure 1) showed inhibition of the activity by all the sodium salts. The degree of inhibition in the case of sodium chloride, sodium nitrate and sodium sulphate was dependent on the concentration of the salt used being 50% for 750 mM sodium chloride and sodium sulphate and 40% for sodium nitrate. Out of various sodium salts selected, sodium fluoride appeared to be most potent inhibitor of urease, about 85% inhibition occurring at 50 mM concentration.

Ureases of jack bean[11,12] and *Cajanus indicus*[3] have been reported to be inhibited by high concentrations of urea. It was of interest to explore whether this behaviour of sodium fluoride on the activity had any bearing on the inhibition observed at high substrate concentration. This aspect was studied by assaying urease activity in the urea concentration range 5–2000 mM. Two concentrations of sodium fluoride i.e., 10 and 15 mM, were selected for the study since they gave midway inhibition between 45–55%, (Figure 1). The results (Figure 2) showed that sodium fluoride strongly inhibited the urease activity in the lower urea concentration range

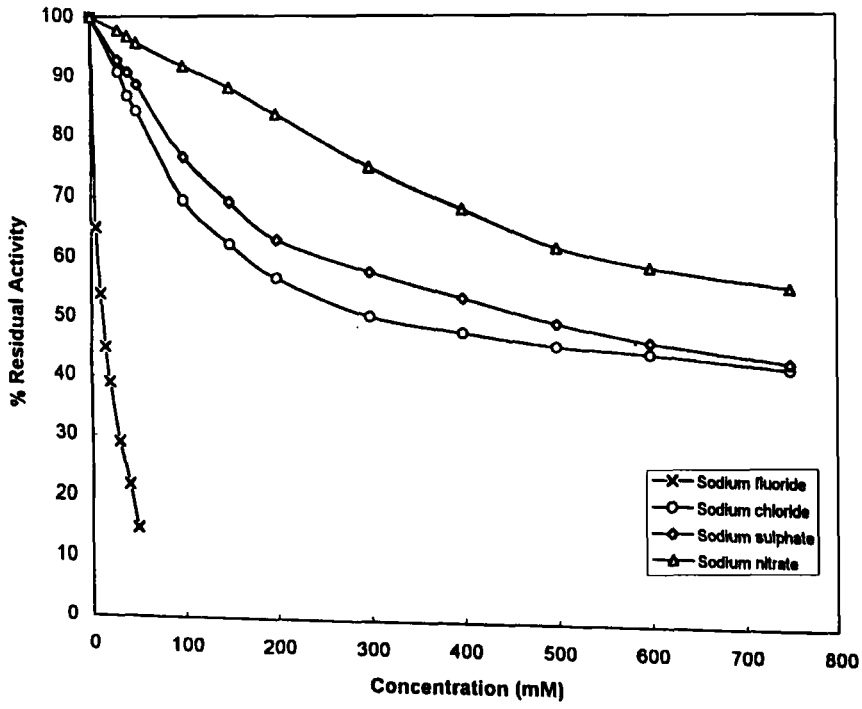

FIGURE 1 Inhibition of water melon urease by sodium salts. Suitably diluted enzyme (10 U/ml, 3.0 μg/ml) was incubated in the presence of varying concentrations of the salts in the standard assay mixture.

(Figure 2, inset). Upon increasing the urea concentration to 500 mM and above, the activity was inhibited even in absence of sodium fluoride, because of the excess substrate effects. However, this inhibitory effect of urea disappeared in the presence of 10 mM sodium fluoride. In fact, the high substrate inhibition was completely abolished after 500 mM and a plateau was obtained for increasing urea concentration (Figure 2). However, the activity pattern with 15 mM sodium fluoride was submaximal with respect to the control and apparently there was no effect of increasing urea concentration.

Inhibition of urease by alkali metal ions like sodium or potassium is known.[3,13] Kistiakowsky and Shaw[14] found that jack bean urease was not inhibited by sodium or potassium ions below pH 7.0; inhibition became apparent only between pH 7.0 and 7.5.

Inhibition by sodium fluoride may be due to the fluoride ion. The rate of urease catalysed urea hydrolysis has been shown to be inhibited by fluoride in the lower urea concentration range (1–100 mM).[15] It appears

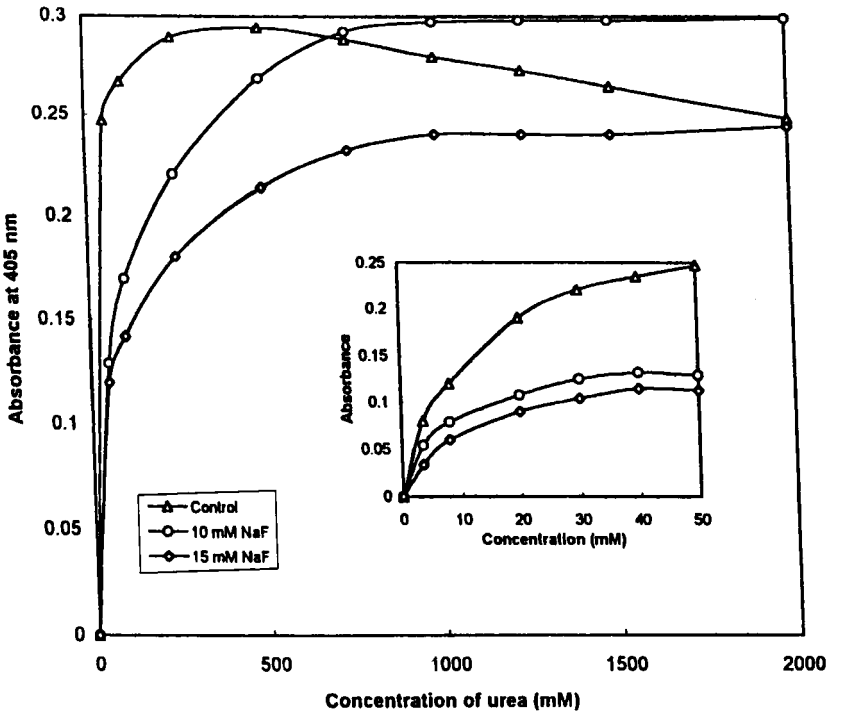

FIGURE 2 Effect of sodium fluoride on the activity of water melon urease in the presence of varying concentrations of substrate. Urea (5–2000 mM) and enzyme (10 U/ml, 3.0 μg/ml) was incubated in the presence of sodium fluoride in 50 mM Tris-acetate buffer, pH 8.5. Inset shows the observations at lower urea concentration range.

that the fluoride apparently binds slowly or reversibly to a form of enzyme that occurs only during catalysis, and so inhibits the urease activity.[7] Moreover, fluoride ion is a known competitive inhibitor of urease.[8] Slow formation of an enzymatically inactive ternary complex (urease–urea–fluoride) has also been proposed.[15] Therefore, disappearance of inhibition by fluoride ion at high substrate concentration is to be expected. Further, it appears that the affinity of water melon urease for the urea molecule is profoundly altered in the presence of sodium fluoride.

Chemical Inactivation by Iodoacetic Acid (IAA)

When water melon urease was incubated with excess IAA, the enzyme activity was lost in two distinct phases (Figure 3). The slow phase showed

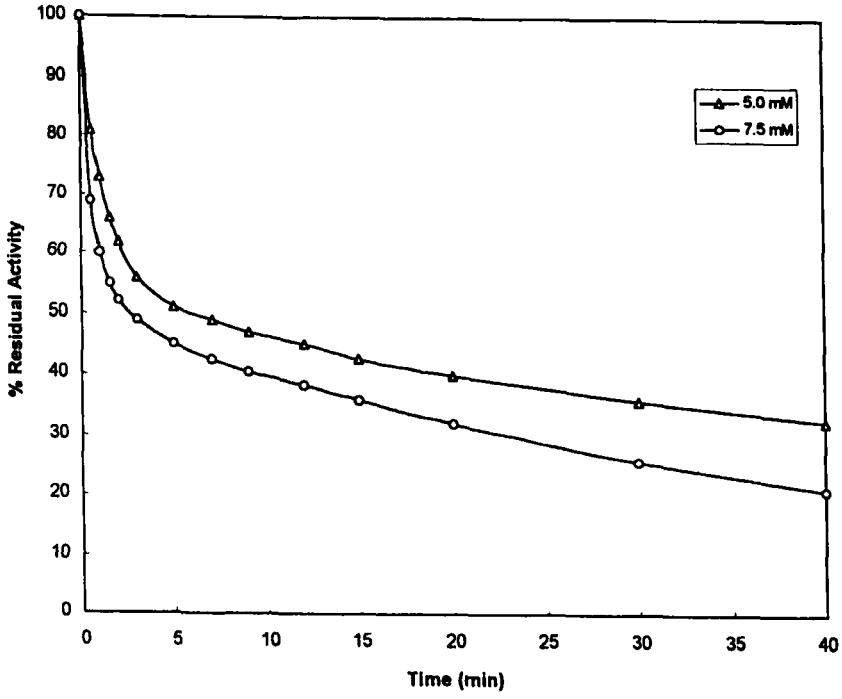

FIGURE 3 Time-dependent inactivation of water melon urease by iodoacetic acid. The enzyme (4.6 μg/ml) and IAA (5.0 and 7.5 mM) were incubated in 50 mM Tris-acetate buffer (pH 8.5) at 30°C. Aliquots were withdrawn at specified intervals and were assayed for activity.

an exponential decay (first-order kinetics), and accounted for half of the initial activity. In order to obtain information about the initial fast phase, the slow phase was extrapolated to zero time in the semi-log plot, and the difference between the experimentally observed activity and the extrapolated slow phase was replotted[16] (Figure 4, inset). The fast phase also showed first-order kinetics and accounted for half of the initial activity. Thus, the inactivation of urease by IAA can be expressed by a biphasic rate equation containing two first-order terms:

$$A_t = A_{fast} \cdot e^{-k_{fast} \cdot t} + A_{slow} \cdot e^{-k_{slow} \cdot t} \quad (1)$$

where A_t is the residual activity at time t, A_{fast} and A_{slow}, k_{fast} and k_{slow} are the amplitudes and the first-order rate constants of the fast and the slow phases, respectively. In the data of Figure 4, $A_{fast} \cong A_{slow} \cong 50\%$ of the initial activity. The values of k_{fast} and k_{slow} obtained with 5.0 and 7.5 mM IAA are shown in Table I.

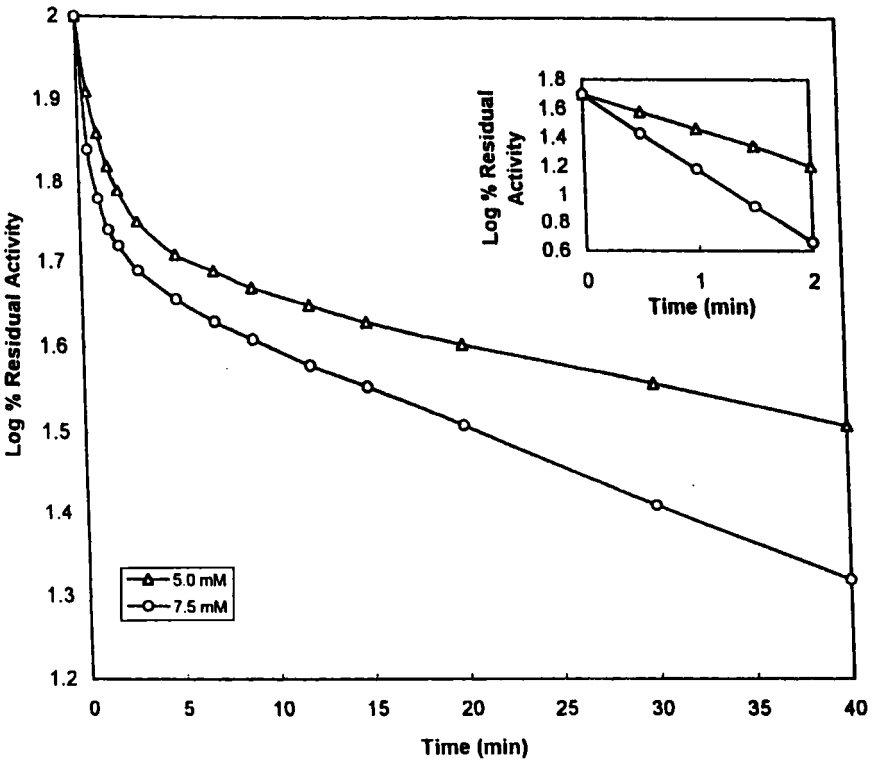

FIGURE 4 Semi-log plot of the data of Figure 3. Inset shows the semi-log plot for the initial fast phase (see text).

TABLE I Rate constants for the biphasic inactivation of water melon urease by IAA, NEM, and PHMB

Reagent	*Concentration* (mM)	*Rate constant* (min^{-1})[a]	
		Fast phase (k_{fast})	*Slow phase* (k_{slow})
IAA	5.00	0.552	0.0111
	7.50	1.232	0.0222
NEM	0.25	0.230	0.0115
	0.50	0.314	0.0265
PHMB	0.10	0.460	0.0098
	0.125	0.575	0.0265

[a] Enzyme concentration was 4.6–6.0 μg/ml in 50 mM Tris-acetate buffer, pH 8.5, at 30°C.

Chemical Inactivation by *N*-ethylmaleimide (NEM)

The time-dependent loss of enzyme activity on incubating water melon urease with excess NEM also exhibited biphasic inactivation kinetics. A semi-log plot of the data showed that about 60 percent of the initial activity was lost rapidly and the remaining 40 percent in a slow exponential decay. Thus, the overall kinetics of inactivation with NEM can be represented by the rate equation (1). The values of k_{fast} and k_{slow} obtained with 0.25 and 0.5 mM NEM are shown in Table I.

Chemical Inactivation by *p*-hydroxymercuribenzoate (PHMB)

The enzyme reacted very rapidly with this reagent. Reasonably low concentrations of PHMB (0.1 and 0.125 mM) had to be employed to obtain a measurable rate of inactivation. Results, when expressed as a semi-log plot, showed a rapid initial loss of enzyme activity to about half of the initial value. The remaining half of the activity was lost slowly. In this case also, the overall kinetics of inactivation can be represented by the rate equation (1). The values of k_{fast} and k_{slow} obtained with 0.1 and 0.125 mM PHMB are shown in Table I.

Inactivation of water melon urease with IAA, NEM, and PHMB occurs in a similar manner and reveals the half-and-half distribution of active sites. All the sites contribute to the activity, but differ with respect to their rate of inactivation with these reagents. One half of the sites undergo inactivation at a much faster rate than the other half. It appears therefore, that the enzyme protein possesses a submaximal symmetry which is a consequence of its unique quaternary structure. Similar site : site heterogeneity has also been reported in the case of other plant enzymes, e.g., spinach leaf glyceraldehyde 3-phosphate dehydrogenase,[17] isocitrate lyase of caster endosperm,[18] and phosphoenolpyruvate-phosphatase from germinating mung beans.[19]

Iodoacetic acid and *N*-ethylmaleimide are highly specific –SH reagents while *p*-hydroxymercuribenzoate is not absolutely specific for –SH groups but may be bound non-covalently to other sites as well.[20] The order of effectiveness of these inhibitors for water melon urease are PHMB > NEM > IAA. This observation is in accordance with those reported in the case of other enzymes, e.g., quinone reductase,[21] β-glycerophosphate dehydrogenase,[22] aconitase,[23] and crotonase,[24] where the order of effectiveness noticed was PCMB > NEM > IAA.

Mahadevan and co-workers[25] have reported that bovine rumen urease was completely inhibited by NEM at 0.1 mM indicating that the thiol

groups on the enzyme are essential for the activity. Similar results were also obtained for jack bean urease.[8,26] There are reports that iodoacetamide irreversibly (though slowly) inactivates urease; iodoacetate has virtually no effect on enzymatic activity.[27] It is evident from the present results that although, water melon urease is being inhibited by iodoacetate, a high concentration is needed to get extensive inactivation.

Acknowledgments

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