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Effects of thiols on the activity of soybean (*Glycine max*) urease

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Abstract

The various thiols namely, β-ME, DTT and L-cysteine were studied for their effect on soybean urease. All thiols were shown to enhance urease activity at low concentration, while at higher concentration the inhibitory effects were observed. The β-ME and DTT exhibited the enhancement effects over a wide range of concentration while L-cysteine showed some enhancements only at low concentrations. All thiols showed two or three peaks of activity enhancement at low concentrations (0.01-3 mM). The β-ME was found to be the most effective enhancer of urease activity, being two times more efficient than DTT and three times than L-cysteine. L-cysteine showed inhibition beyond 3 mM. From these studies it was observed that the order of effectiveness as enhancer of urease activity followed the sequence β-ME > DTT > L-cysteine. The observed activation of urease by various thiols was suggestive of the protection of the SH groups of the enzyme by added thiol. Further with time dependent studies the enhancement effects were shown to occur in two different phases; the initial fast phase, in which the rate increases almost instantly and linearly (first-order kinetics) and the later slow phase, in which no significant enhancements were observed (zero-order kinetics).

Key words: Urease, soybean, thiols, β-mercaptoethanol, dithiothreitol, L-cysteine

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Introduction

Urease was first reported from the leaves of legume, soybean (Takeuchi, 1909). The soybean seed and tissue culture urease showed a molecular mass of 480 kDa on analytical Agarose A-15 column as well as on polyacrylamide gel analysis (Polacco and Havir, 1979). Urease sub-unit has a molecular mass of 90 ± 10 kDa. Urease had been found to have a high specificity for its primary substrate, urea (Smith and Douglas, 1993), although it is known that acetamide, formamide, *N*-methyl urea, semicarbazide and hydroxy urea all serve as substrate (*albeit* poorly) for jack bean urease (Blakeley and Zerner, 1984). The amino acid profiles of urease from jack bean and soybean are very similar with high methionine content (Milton and Taylor, 1969; Polacco and Havir, 1979). The presence of two isozymes for urease has been reported in soybean (Holland *et al*., 1987). Ubiquitous urease is synthesized in all organs (constitutively expressed) but it appears to be most active in young tissues (Polacco and Winkler, 1984; Holland *et al*., 1987; Polacco *et al*., 1985; 1989). Embryo-specific urease is synthesized exclusively in the developing embryos, although roots of young soybean plant retain considerable embryo-specific urease derived from the embryonic axis (Torisky and Polacco, 1990).

Thiols play a principal role in maintaining the appropriate oxidation-reduction state of proteins, cells and organisms. However, the susceptibility of thiols to oxidation can lead to the formation of disulfides and higher

oxidation products, often with loss of biological activity. In proteins, thiol groups (also called mercaptans or sulfhydryls) are present in cysteine residues. Thiols can also be generated by selectively reducing cystine disulfides with reagents such as dithiothreitol (DTT) or 2-mercaptoethanol (β-mercaptoethanol), each of which must then be removed by dialysis or gel filtration before reaction with the thiol-reactive probe. The common thiol-reactive functional groups are primarily alkylating reagents, including iodoacetamides, maleimides, benzylic halides and bromomethylketones. Arylating reagents such as NBD halides react with thiols or amines by a similar substitution of the aromatic halide by the nucleophile. Reaction of any of these functional groups with thiols usually proceeds rapidly at or below room temperature in the physiological pH range (pH 6.5–8.0) to yield chemically stable thioethers.

The objective of the present investigation is to study the relative effectiveness of mercaptoethanol, cysteine, and DTT in the reactivation of urease and some characteristics of the reaction process.

Materials and methods

Chemicals: Bovine serum albumin, Dithiothreitol, β-Mercaptoethanol, L-cysteine, Tris and Urea (Enzyme grade), were purchased from Sigma Chemicals Co., USA. Nessler's reagent, Folin-Ciocalteau reagents and TCA were from Hi Media, India. All other chemicals were of analytical grade obtained from either SISCO or Merck, India. All the solutions were prepared in triple distilled MQ water.

Enzyme: The enzyme was isolated from the soybean seeds procured from the local market as per the protocol described by Polacco and Havir (1979) with some modifications. The specific activity varied 350-366 units/mg protein from batch to batch.

Urease Activity Assay: Urease was assayed by determining the amount of ammonia liberated in a fixed time interval on incubating the enzyme and urea. Assay buffer (0.05M Tris-acetate beffer, pH 7.0, 0.9 ml) and properly diluted enzyme solution (0.1 ml) was pre-incubated at 37ºC. Reaction was started by adding 1 ml of 0.2 M urea (in assay buffer) also pre-incubated at 37 ºC. After incubation for the required time (10 min), 1 ml of 10% TCA was added to stop the reaction. An aliquot of the test mixture was transferred to a 50 ml volumetric flask. Nessler's reagent 1.0 ml was added to the test solution. The volume was made up to 50 ml with distilled water. Absorbance was measured at 405 nm (path length: 1 cm) on a Spectronic 1001 Spectrophotometer (Das and Kayastha, 1998). A blank without enzyme was run side by side and correction was applied for the same. The Nessler's reagent was calibrated with standard ammonium chloride solution. An enzyme unit has been defined as the amount of enzyme required to liberate 1 μmol of ammonia per min under our test conditions (0.1 M urea, 0.05 M Tris-acetate buffer, pH 7.0, 37ºC).

Protein Estimation: Protein content of urease preparation was estimated by the method of Lowery et al. (Lowry *et al.,* 1951) using bovine serum albumin as standard.

Effect of Thiol on the Activity of Urease: The stock solutions of various thiols (β-ME, DTT and L-cysteine) were prepared in 0.05 M Tris acetate buffer, pH 7.0 and diluted with the same buffer, when required. The urease activity was determined in the presence of varying concentrations of thiols (0.01-16 mM) and a comparison was made. Further urease was subjected to time dependent studies with various thiol. The purified urease, suitably diluted (0.87 μg/ml) in 0.1 M Tris acetate buffer, pH 7.6, was incubated with various thiols (1.5 M each) separately, for certain period at 30 ^{o}C. Aliquots were drawn at the specified time intervals and assayed immediately.

Results and Discussion

The effect of various thiols namely, β-ME, DTT and L-cysteine were studied by varying the thiol concentration (0.01-16 mM) in the assay mixture and the activity was determined as stated earlier. It was observed that all thiols were efficient activity enhancer at low concentrations, while at higher concentration the

inhibitory effects were more pronounced (Fig 1). The β-ME and DTT enhanced the activities over a wide range of their concentrations, ranging from 0.01-14 mM, while with L-cysteine the enhancement was observed only at lower concentrations (0.01-3 mM). Also it was observed (Fig. 1 & 3) that almost all thiols showed two or three peaks of activity enhancement at low concentrations (0.01-3 mM) of thiols and thereafter an inhibition of urease activity was observed.

By contrast, the β-ME was found to be the most effective enhancer of urease activity, being two times more efficient than DTT and three times than L-cysteine, whilst assay was performed at low concentrations. The data from Fig 1 for β-ME was re-plotted as Fig. 3, over a low concentration range to see the effects more clearly. Also as mentioned earlier and can be seen from Fig 2 that the β-ME exhibits two peaks of activity enhancement at two different concentrations; namely 0.2 mM and 1 mM. Furthermore, it was noticed that the activity enhancement decreased beyond 13 mM of each of these thiols and inhibition of urease activity became more pronounced thereafter. Clearly, L-cysteine was the least effective enhancer of urease activity among various thiols investigated and indeed showed strong inhibition beyond 3 mM.

Urease is known to contain one or more sulfhydryl groups as integral part of its catalytically active site (Malhotra and Rani, 1970; Srivastava and Kayastha, 2000). Earlier β-ME has been shown to be a simple competitive inhibitor of jack bean urease catalyzed hydrolysis of urea (Dixon *et al.,* 1980; Blakeley and Zerner, 1984). Blakeley and Zerner (1984), on the basis of spectral studies at 25 ^{o}C in oxygen-free 0.05M *N*-ethylmorpholium chloride buffer (pH 7.12, 1 mM in EDTA) further concluded that β-ME and urea compete for the same binding site on jack bean urease. However, from our studies it is evident that β-ME, DTT and L-cysteine are activators of soybean urease and the order of effectiveness is β-ME > DTT > L-cysteine. Similar studies have been reported for watermelon urease and the thiols were found to follow the similar trend of activation (Prakash and Upadhyay, 2003).

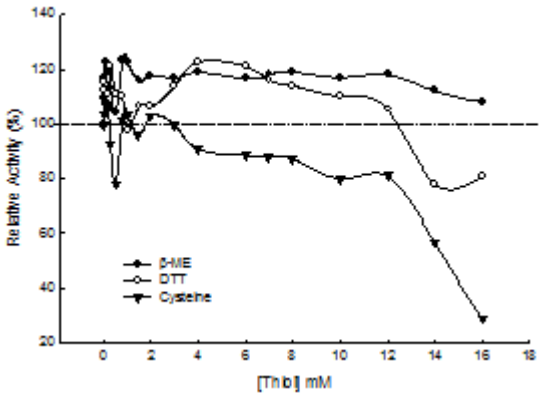

Fig. 1: Effect of various thiols on the activity of urease

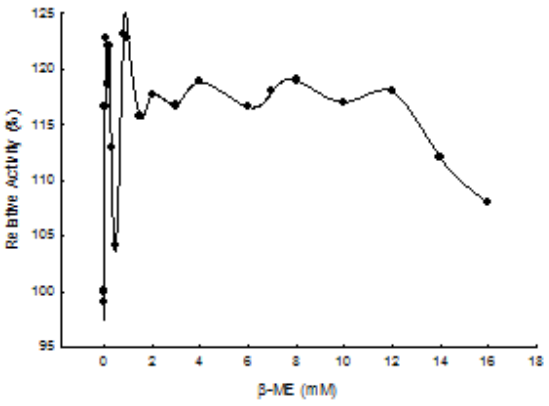

Fig. 2: Effect of β-ME on the activity of urease.

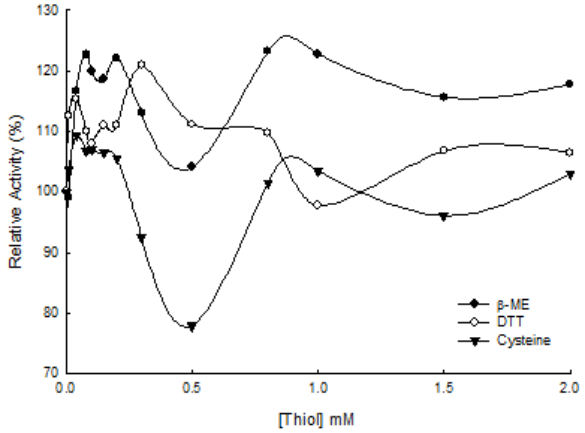

Fig. 3: Effect of thiols on the activity of urease.

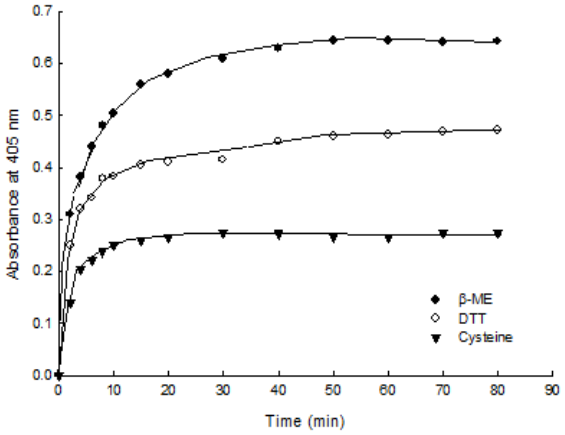

Fig. 4: Time dependent activation of urease with desired concentration of thiols

It has been established that the slow loss of jack bean urease activity in presence of oxygen is due to the formation of mixed disulfide involving a thiol located at the active site (Riddles *et al.,* 1983). The observed activation of the soybean urease reported above is suggestive of the protection of the SH groups of the enzyme by added thiol. It is likely that the thiol reagent itself is consumed by oxygen, leaving the SH group of the enzyme intact for the catalytic functions.

Furthermore, the various thiols were investigated for their effects on urease activity in time-dependent manner (Fig. 4). The purified urease in 0.1 M Tris acetate buffer, pH 7.6 was incubated at 37 ^{o}C with 1.5 M of each of the thiols separately for a specified time interval. The aliquots drawn were immediately checked for residual activity. When the data was plotted and analyzed (Fig. 4), it was observed that all thiols showed time-dependent rate enhancement in the activity of urease. The enhancement effects were exhibited in two different phases; the initial fast phase, in which the rate increases almost instantly and linearly (first-order kinetics) and the later slow phase, in which no significant enhancements were seen (zero-order kinetics).

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