

## Research Article

# SOYBEAN (*GLYCINE MAX*) UREASE: STEADY STATE KINETICS, STABILITY AND THERMAL INACTIVATION STUDIES

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**Abstract:** The soybean (*Glycine max*) urease was characterized with respect to kinetic parameters, stability studies and thermal inactivation. The stability temperature and stability pH of the purified urease was found to be 4 °C and 7.6, respectively. The optimum pH and optimum temperature were 7.0 °C and 65 °C, respectively. The energy of activation ( $E_a$ ) was 15.40 kJ/mol. Further, the  $K_m$  and  $V_{max}$  were determined by Lineweaver Burk plot and the values were  $2.70 \pm 0.10$  mM and  $2.85 \times 10^2$   $\mu\text{mol NH}_3/\text{min}/\text{mg}$  protein, respectively. Thermal inactivation studies at 65 °C, revealed the mono-phasic kinetics, which indicated the loss in activity in single phase. However, at higher temperatures (70 °C, 75 °C and 77 °C), the kinetic pattern was mainly bi-phasic. At 80 °C, there was complete loss in activity thereby showing the denaturation of enzyme. Thermal inactivation studies strongly support the oligomeric nature of urease.

**Key words:** *Glycine max*; soybean; thermal inactivation; urease

## Introduction

Urease (EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and  $\text{CO}_2$ . A variety of ureases are found in bacteria, fungi, higher plants, and in soil as a soil enzyme (Mobley and Hausinger, 1989). Urease was first reported from the leaves of legume, soybean (Takeuchi, 1909). Urease has been found to have a high specificity for its primary substrate, urea (Smith *et al.*, 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 (Andrews *et al.*, 1984; 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; Polacco *et al.*, 1985; Holland *et al.*, 1987; Polacco *et al.*, 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).

The plant and fungal ureases are homo-oligomeric proteins (consist of identical subunits), while the bacterial ureases are multimers formed from a complex of two or three subunits (Mobley *et al.*, 1995; Tange and Niwa, 1997). Significant amino acid similarities have been observed between all known ureases. Amino-terminal residues of the monomers of plant and fungal enzymes are similar to the small subunits of bacterial enzymes (e.g., UreA of *H. pylori*). The large subunits of bacterial ureases (e.g., UreB of *H. pylori*) resemble the carboxy-terminal portions of plant and fungal subunits. The high sequence similarity indicates that

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all ureases are variants of the same enzyme and are likely to possess similar tertiary structures and catalytic mechanisms (Mobley *et al.*, 1995). This conclusion is supported by the available biochemical and structural data obtained for the best-characterized ureases, *e.g.*, from jack bean (Hirai *et al.*, 1993) and *K. aerogenes* (Jabri *et al.*, 1995). Jack bean urease exists as a homotrimer able to aggregate to a homo-hexamer (Hirai *et al.*, 1993). Bacterial ureases possess structures similar to the jack bean urease. They are either trimers or hexamers of subunit complexes. They can also exist in aggregated forms. The stoichiometry of subunits (1:1 for ureases from *Helicobacter* sp. or 1:1:1 for most bacterial ureases, including the urease from *K. aerogenes*) is always maintained. The catalytic site is located in subunit UreC of the *K. aerogenes* enzyme and in the respective regions of ureases from other organisms. Each catalytic subunit contains the active site with two nickel ions that, in the case of crystallized *K. aerogenes* urease, were shown to be 3.5 Å apart (Jabri *et al.*, 1995). In this enzyme, one nickel atom is bound to two histidine residues (His<sup>246</sup> and His<sup>272</sup>), while the second nickel atom is bound to three residues: two histidines (His<sup>134</sup> and His<sup>136</sup>) and aspartic acid (Asp<sup>360</sup>). Additionally, a carbamate ligand derived from Lys<sup>217</sup> bridges the two nickel ions (Mobley *et al.*, 1995).

The objective of the present study was to characterize soybean urease biochemically including its kinetic aspects and stability and thermal inactivation studies, which have not been investigated as of yet. Most of the work on ureases has been reported from microbes and very few reports are available on ureases from plant sources. Therefore in that direction, the current studies were undertaken to further refine the enzyme's catalytic mechanism and other kinetic details for a better understanding of plant ureases in general. Our previous reports on soybean and pigeonpea urease, along with the current investigation, have further added and refined the overall details of catalytic mechanism and role of various inhibitors in medicine, agriculture and environment (Kumar and Kayastha, 2010a; 2010b; 2010c; 2012; Kumar, 2015; Kumar *et al.*, 2015; Kumar, 2016, Lehari *et al.*, 2015).

## Materials and methods

### Chemicals and enzyme

Bovine serum albumin (BSA), Tris, and urea (enzyme grade) were purchased from Sigma

Chemicals Co., St. Louis, USA. Nessler's reagent, Na<sub>2</sub> EDTA, and trichloroacetic acid (TCA) were from HiMedia, India. All other chemicals were of analytical grade obtained from either SRL or Merck, India. All the solutions were prepared in Milli Q (Millipore, USA) water. The urease was purified from the mature seeds of soybean to apparent homogeneity by the method of Polacco and Havar (1979) with minor modifications. The specific activity of the final preparation was 270 units/mg of protein.

### Urease activity assay

Urease (0.87 µg/mL) was assayed by determining the amount of ammonia liberated in a fixed time interval on incubating the enzyme and urea (Das *et al.*, 2002). 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 urease 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, 37 °C).

### Protein estimation

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

### Stability studies

The stability temperature of purified urease was determined by storing the urease (0.87 µg/mL) at 4 °C and 37 °C, separately and performing the activity assay for residual activity at the regular intervals. Furthermore, the stability pH was determined by incubating urease at various pH (7.0, 7.6 and 8.0) overnight at 4 °C and then assayed next day for the residual activity. 0.1 M Tris-acetate buffer was used for stability studies. The purified urease was always stored in 50 mM Tris acetate buffer, pH 7.6 at 4 °C containing 1 mM β-ME.

### Steady state kinetics

The effect of temperature was studied by varying the temperature from 15 °C to 80 °C in a multi-temp (Pharmacia, Sweden) water bath during activity assay. The percent relative activity was plotted against temperature. The data of optimum temperature was selected in the temperature range 20-60 °C and were replotted in the form of Arrhenius

graph to determine the energy of activation ( $E_a$ ). The optimum pH was determined by using the three buffer systems, *viz.*, citrate-phosphate buffer (pH 4.6-6.6), phosphate buffer (pH 6.6-7.6) and Tris buffer (pH 7.2-9.5). The activity assay was performed at the different pH and percent relative activity was determined. For studying the effect of substrate concentrations, the activity assay was performed at different substrate concentrations (1-40 mM urea). The velocities were determined and plotted against respective substrate concentrations. The Lineweaver-Burk plot was drawn to determine the  $K_m$ .

### Thermal inactivation studies

Urease (0.5 mg/mL) in 0.1 M Tris acetate buffer, pH 7.6, was incubated in a water bath at desired temperatures for a fixed period. The enzyme aliquots withdrawn at different time intervals were rapidly cooled in ice water and were assayed for residual activity. The activity measurement of the sample was executed after 5 min storage in ice water. The experiments were carried out at four different temperatures, such as 65 °C, 70 °C, 75 °C and 77 °C. Equation (1) was used to analyze the data and the values of  $t_{1/2}$ ,  $k$  and amplitude were determined at the indicated temperatures.

### Analysis of the kinetic data

With time-dependent inactivation, the data was plotted as log percent residual activity versus time. The time-course of inactivation was found to consistent with Equation (1) and therefore the data was processed and analyzed in accordance with the following equation:

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

where  $A_t$  is the fraction residual activity at time  $t$ ,  $A_{fast}$  and  $A_{slow}$  are amplitudes (expressed as percent of the starting activity) and  $k_{fast}$  and  $k_{slow}$  are the rate constants of the fast and the slow phases, respectively. Initial estimates of the rate constants and amplitudes were obtained from the semi-log plots as described earlier (Kayastha and Gupta, 1987).

## Results and Discussion

### Enzyme stability studies

Ureas from several sources have been found to be inactivated by heavy metals (Magana-Plaza *et*

*al.*, 1971; Mahadevan *et al.*, 1977; Nakano *et al.*, 1984; Glemzha *et al.*, 1986; Blanchard *et al.*, 1988) and by oxidation (Lister, 1956; Magana-Plaza *et al.*, 1971; Mahadevan *et al.*, 1976); therefore, during the purification process 1 mM each of EDTA and  $\beta$ -ME were found most appropriate to preserve the enzyme activity. The purified urease was always stored in 0.1 M Tris-acetate, pH 7.6 at 4 °C containing 1 mM  $\beta$ -ME, and it was observed that it retained nearly 50% of the original activity after 20 days. *Ureaplasma urealyticum* urease was fully stable for more than 20 days when stored at 4 °C in pH 7.2 buffer containing 1% bovine serum albumin; it is not clear whether the added albumin was required (Stemke *et al.*, 1987). Further, purified urease was stored in 0.1 M Tris-acetate buffers, pH 7.6 separately at two different temperatures, 4 °C and 37 °C for 40 days. The semi-log plot of percent residual activity versus number of days shows the  $t_{1/2}$  value at 20 days for urease at 4 °C and 5 days for that stored at 37 °C (Figure 1). The stability pH was determined by incubating the urease in 0.1 M Tris acetate buffer, 4 °C at three different pHs, *viz.*, 7.0, 7.6 and 8.0. Maximum urease activity was observed when enzyme was stored in 0.1 M Tris acetate buffer, pH 7.6. The pH and temperature stabilities for several ureases have been reported. Activity was irreversibly lost when ureases from *Arthrobacter oxydans* and *Bacillus pasteurii* were exposed to pH values below 5.0 and 5.2, respectively (Larson and Kallio, 1954; Schneider and Kaltwasser, 1984). Full activity was retained after 5 h at 30 °C for *Brevibacterium ammoniagenes* for pH values between

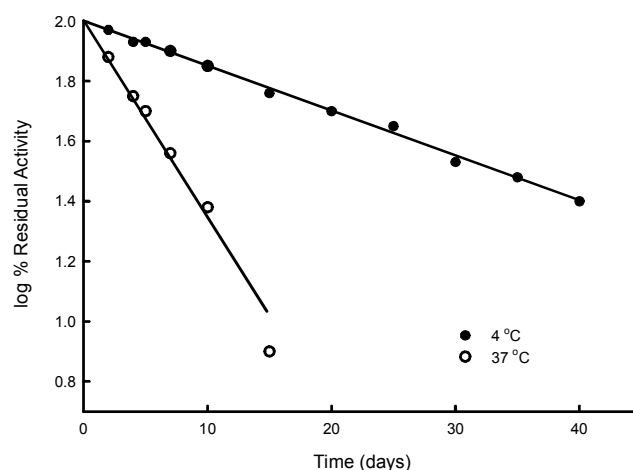


Figure 1: Comparing the storage stability of soybean urease at 4 °C and 37 °C. Suitably diluted urease (0.87  $\mu$ g/mL) was assayed at standard conditions after incubating at the indicated temperatures. Each experimental point represents the mean of three determinations

5 and 10, with sharp decreases in activity at each pH extreme (Nakano *et al.*, 1984). In general, ureases are quite stable in the presence of EDTA and thiol protectants when they are not subjected to pH extremes or high temperature.

### Steady state kinetics

The optimum pH was found to be 7.0 (Figure 2). It has been shown that the rate of hydrolysis of urea by pigeonpea urease was maximum at pH 7.3 among various pH in the range 5.0-8.5 (Das *et al.*, 2002). The soybean urease retained only 50% of the activity at pH 5.3 as compared to that observed at pH 7.0. Pigeonpea urease was shown to be only 50% as efficient at pH 5.0 as compared to its activity at pH 7.3 whereas, jack bean urease (Blakeley and Zerner, 1984) has 68% activity at pH 5.2 compared to its activity at pH 7.0. Evidently from the Figure 2, it is clear that soybean urease shows broad optimum pH ranging from 6.5 to 8.0. Watermelon urease showed a sharp pH optimum at 8.0 (Prakash and Bhushan, 1997), while the optimum pH for mulberry leaf urease is 9.0 (Hirayama *et al.*, 2000). With the exception of a small group of acid ureases (Kakimoto *et al.*, 1989; 1990; Yamazaki *et al.*, 1990), most ureases possess an optimum pH of near neutrality and are often irreversibly denatured by exposure to pH values below 5. An important point to be made with regard to examination of the pH optima for these enzymes is that urease is inhibited by several common buffers, including phosphate (Todd and Hausinger, 1989) and boric acid (Breitenbach and Hausinger, 1988).

The results of effect of temperature on urease catalyzed reaction and Arrhenius plot are shown in Figure 3 and 4, respectively. The temperature optimum was at 65 °C and beyond that there was drop in activity. The  $E_a$  and  $Q_{10}$  values were found to be 15.40 kJ/mol and 1.32, respectively. The Lineweaver-Burk plot was drawn to calculate the  $K_m$  and the value was  $2.70 \pm 0.10$  mM (Figure 5). The  $K_m$  value of pigeonpea urease for its substrate, urea has been reported at  $3.0 \pm 0.2$  mM (Das *et al.*, 2002). The kinetic studies on jack bean urease have shown the  $K_m$  at 2.9 - 3.3 mM (Blakeley *et al.*, 1969; Blakeley and Zerner, 1984). An apparent  $K_m$  of 0.85 mM for soybean leaf urease (Kerr *et al.*, 1983) and a  $K_m$  of 19-476 mM for soybean seed urease has been reported depending on the buffer systems chosen (Talsky and Klunker, 1967). From mulberry leaf urease  $K_m$  of urea was reported to be 0.16 mM; the value being the lowest among the plant ureases

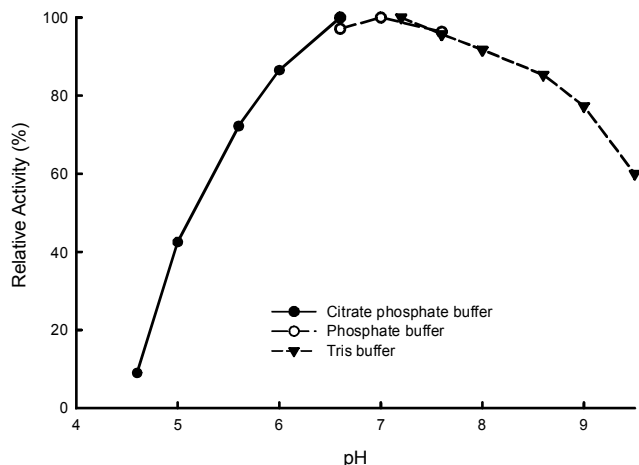


Figure 2: Determining the optimum pH of soybean urease. Suitably diluted urease (0.87  $\mu\text{g/mL}$ ) was assayed at various pH under standard assay conditions. Each experimental point represents the mean of three determinations

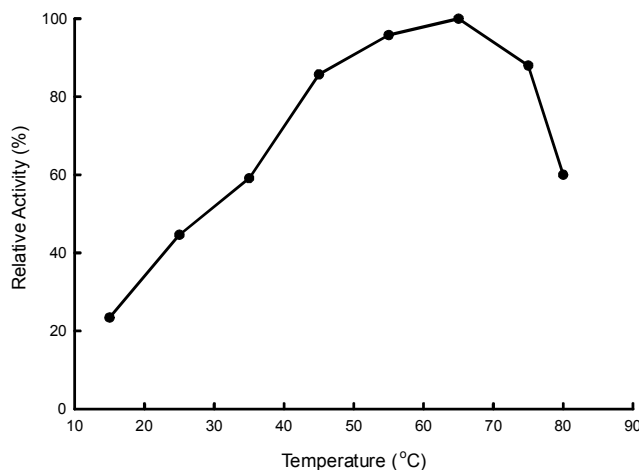


Figure 3: Determining the optimum temperature of soybean urease. Suitably diluted urease (0.87  $\mu\text{g/mL}$ ) was assayed at various temperatures. Each experimental point represents the mean of three determinations

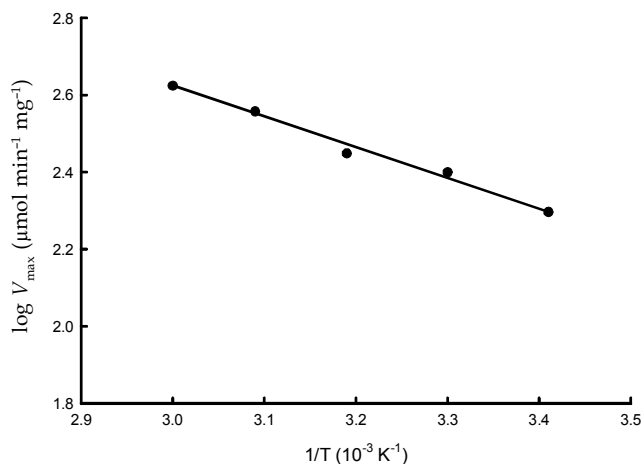


Figure 4: Determining the activation energy ( $E_a$ ) from Arrhenius plot. Data from Figure 3 was replotted in the temperature range 20-60 °C

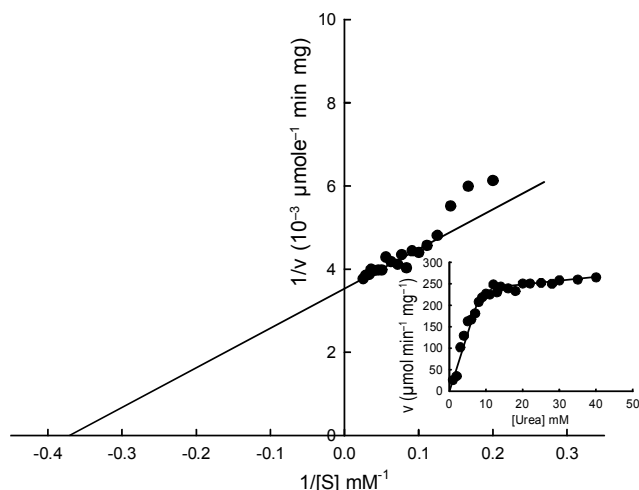


Figure 5: Determining the kinetic parameters,  $K_m$  and  $V_{max}$  from Lineweaver-Burk plot. Inset shows the effect of substrate concentration on the activity of soybean urease. Suitably diluted urease ( $0.87 \mu\text{g/mL}$ ) was assayed in the presence of varying concentrations of urea (1-40 mM) and at standard conditions. Each experimental point represents the mean of three determinations

purified so far (Hirayama *et al.*, 2000). The  $V_{max}$  for soybean urease was found to be  $2.85 \times 10^2 \mu\text{mol NH}_3/\text{min}/\text{mg}$  of protein at standard assay conditions as mentioned. Individual bacterial ureases possess  $K_m$  values ranging from 0.1 to  $>100$  mM urea (Mobley and Hausinger, 1989). A range of  $K_m$  values have been reported for *Bacillus pasteurii* (Larson and Kallio, 1954), *Brevibacterium ammoniagenes* (Nakano *et al.*, 1984), and *Providencia rettgeri* (Magana-Plaza *et al.*, 1971); because the  $K_m$  values reported varied with buffer and pH conditions. In contrast, ureases from *K. aerogenes* (Todd and Hausinger, 1987) and *Proteus mirabilis* (Breitenbach and Hausinger, 1988) exhibited nearly pH-independent  $K_m$  values within the pH range of 5 to 11, when non-inhibitory buffers were used. Different strains of the same species may possess very different  $K_m$  values; for example, various strains of *Proteus mirabilis* exhibited  $K_m$  values ranging from 13 to 60 mM (Breitenbach and Hausinger, 1988).

It is known that acetamide, formamide, *N*-methylurea, semicarbazide, and hydroxyurea all serve (*albeit* poorly) as substrates for jack bean urease (Andrews *et al.*, 1984; Blakeley and Zerner, 1984). It has been shown that the urea  $K_m$  values of purified ureases agree quite well with the values observed for crude cell extracts (Mobley and Hausinger, 1989). The different urea concentrations were prepared and activity assay was performed.

The initial velocities were determined and plotted against respective urea concentrations. Soybean ureases exhibited simple Michaelis-Menten kinetics as is evident from the velocity versus substrate concentration curve (Figure 5; inset). No substrate inhibition or allosteric behavior was observed.

### Thermal inactivation studies

The results of thermal inactivation are shown in Figure 6. At  $65^\circ\text{C}$ , the enzyme exhibited first order kinetics (monophasic) with single exponential decay in enzyme activity (Figure 6a). The fifty percent loss in activity ( $t_{1/2}$ ) was observed after 73 min of incubation at the indicated temperature. The result of thermal inactivation at  $70^\circ\text{C}$  has been shown in Figure 6a. Interestingly, at  $70^\circ\text{C}$ , a transition from monophasic to biphasic kinetics was observed (Figure 6b). From the figure, it is clear that the time course of loss in activity occurs in two distinct phases namely, fast phase and slow phase. The amplitude of the fast phase was only 37%, while that of the slow phase was 63%; thereby indicating that most of loss occurs in the slow phase.

Further at higher temperatures *viz.*,  $75$  and  $77^\circ\text{C}$  (Figure 6 b,c), biphasic kinetics was observed in both the cases. At temperature  $75^\circ\text{C}$ , the rate constant for the fast phase was 5.5 times greater than that of the slow phase. The amplitude of fast phase was also higher (68%). There was increase in amplitude (76%) of fast phase when the urease was incubated at  $77^\circ\text{C}$ . When studies were carried out at  $80^\circ\text{C}$ , the kinetic pattern was in first-order (monophasic; fast phase only) and the respective values of rate constant and amplitude were much higher ( $k = 0.690 \text{ min}^{-1}$ , amplitude = 100%) (Figure 6c). Evidently it is clear from all the figures (6a,b,c)

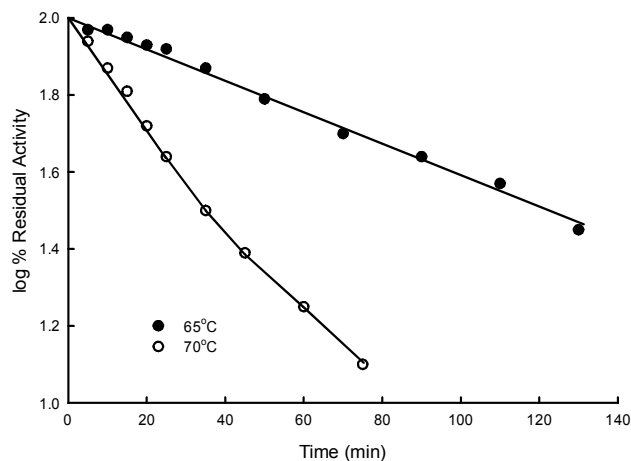


Figure 6(a)

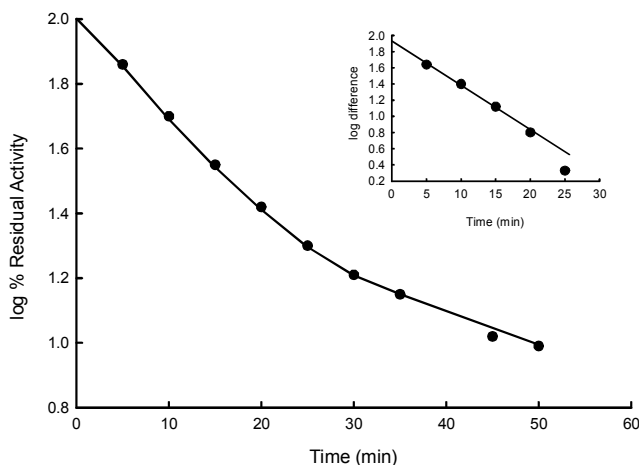


Figure 6(b)

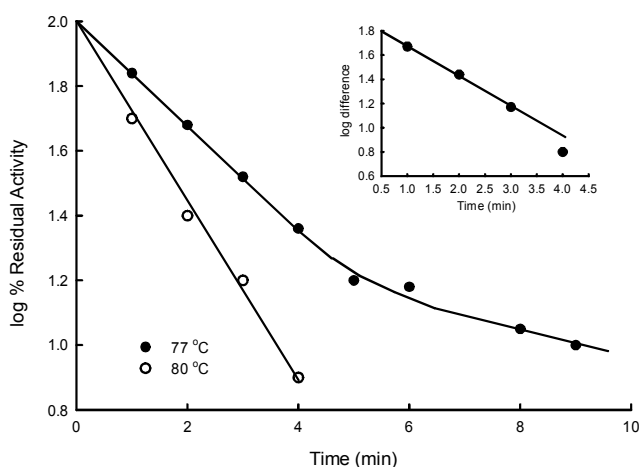


Figure 6(c)

Figure 6: (a, b, c). Thermal inactivation studies of soybean urease at various temperatures. Suitably diluted urease (0.87  $\mu\text{g/mL}$ ) was assayed at standard conditions after incubating at the indicated temperatures. Each experimental point represents the mean of three determinations

and the Table 2.1 that with temperature the amplitude of fast phase increases until it reaches 100% at 80 °C and the slow phase is missing. During these studies a variation in the kinetic pattern

(monophasic to biphasic and then again to monophasic) was observed at various temperatures. At 65 °C the activity was lost in single phase (slow phase only) and the kinetics was monophasic. At temperatures between 70 °C to 77 °C the pattern of kinetics was biphasic with two distinct phases namely, fast and slow phase. At higher temperatures (above 80 °C) the kinetics was again monophasic but with much higher rate (fast phase only; slow phase diminishes). Earlier similar studies were carried out by various workers (Godjevargova and Dimov, 1997; Rejikumar and Devi, 1998; Chen and Chiu, 2000), where only slight deviation from first-order kinetics was detected. They observed that the acceleration of inactivation process with temperature led to disguised first-order kinetics. It is well known that first-order kinetics corresponding to a one-step irreversible transition of native form is suitable for small, monomeric enzymes whereas, for oligomeric enzymes having a more complex quaternary structure and high molecular weight, the existence of intermediate forms and deviation from first-order kinetics can be expected.

Thermal inactivation studies strongly support the oligomeric nature of urease, which is also suggested by earlier workers (Polacco and Havir, 1979). It is possible that soybean urease dissociates into smaller aggregates when incubated at different temperatures during thermal inactivation studies and exhibits variations in their kinetic pattern may be due to their complex quaternary structure and high molecular weight. Several oligomer-to-monomer pathways of the native hexamer have been suggested for urease. Some authors declared that the hexamer can dissociate into two active trimers and further into six monomers (Lencki *et al.*, 1992; Hirai *et al.*, 1993). Lencki *et al.*, (1992) assumed that the hexamer was transformed in two series reversible reaction steps into a stable trimer

Table 1  
The values of amplitudes ( $A$ ) and rate constants ( $k$ ) determined for both fast and slow phases during time-dependent thermal inactivation studies

| Temperature (°C) | Kinetic pattern | Fast phase     |                                  | Slow phase     |                                  |
|------------------|-----------------|----------------|----------------------------------|----------------|----------------------------------|
|                  |                 | $A_{fast}$ (%) | $k_{fast}$ ( $\text{min}^{-1}$ ) | $A_{slow}$ (%) | $k_{slow}$ ( $\text{min}^{-1}$ ) |
| 65               | Monophasic      | -              | -                                | 100            | 0.0094                           |
| 70               | Biphasic        | 37             | 0.0408                           | 63             | 0.0209                           |
| 75               | Biphasic        | 68             | 0.1277                           | 32             | 0.0230                           |
| 77               | Biphasic        | 76             | 0.3943                           | 24             | 0.0793                           |
| 80               | Monophasic      | 100            | 0.6900                           | -              | -                                |

that then similarly dissociated into an inactive monomer. Omar and Beauregard (1995), who investigated the unfolding of jack bean urease by fluorescence emission spectroscopy, found that native urease dissociated into an active, folded dimer. The dimer, depending on the conditions, could then be further dissociated either into an active or inactive monomer. Possibly, a similar mechanism may be affecting the decay of soybean urease too.

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### Abbreviations

LB plot, Lineweaver-Burk plot; *H. pylori*, *Helicobacter pylori*; *K. aerogenes*, *Klebsiella aerogenes*; BSA, bovine serum albumin; Tris, Tris-(hydroxymethyl) aminomethane; NR, Nessler's reagent; Na<sub>2</sub>EDTA, disodium ethylenediaminetetraacetate dehydrate; TCA, Trichloro acetic acid; β-ME, β-mercaptoethanol; SH, sulphhydryl;  $V_{max}$ , Maximal velocity;  $K_m$ , Michaelis-Menten constant.

### Conflict of interest

The author declares that there is no conflict of interest.

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