Kinetics of thermal inactivation and inhibition studies of pigeonpea urease (*Cajanuscajan*)

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Abstract

Urease (urea amidohydrolase; EC 3.5.1.5) was isolated from mature seeds of pigeonpea (*Cajanuscajan*). The optimum pH and optimum temperature was found to be 7.3 and 70°C, respectively. Further, the K_m and V_{max} were 20.4 mM and 0.625 $\mu\text{mol NH}_3/\text{min/mg}$, respectively. The half-life ($t_{1/2}$) of urease was found to be 70 days and 18 days when stored at 4 °C and 37 °C respectively. Thermal inactivation studies revealed a variation in the kinetic pattern. At 50°C and 60°C, the enzyme showed almost no loss in activity over a period of 35 min. However, at higher temperatures (60°C and 70°C), a biphasic pattern was observed. These studies strongly favour the oligomeric nature of urease. The I_{50} value for boric acid was 7.6 mM and the K_i was 1.10 mM; thereby revealing the competitive type of inhibition. Next, NaCl showed poor inhibition potency ($I_{50} = 205 \text{ mM}$), whereas the I_{50} value for Hg^{2+} was recorded at 6.10 nM.

Keywords: urease, boric acid, pigeonpea, kinetics, inhibition, *Cajanuscajan*

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Introduction

Jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized (Sumner, 1926) and it played an important historical role as proof of the proteinaceous nature of enzymes. Also jack bean urease was the first nickel-containing enzyme to be described (Dixon, *et al.*, 1975) and it is the only nickel-containing metalloenzyme identified so far in plants (Polacco and Holland, 1993). Its rapid catalysis for the hydrolysis of urea to ammonia and carbon dioxide plays an essential role in agriculture and human health (Mulvaney and Bremner, 1981; Mobeley *et al.*, 1995). There are several different varieties of urease, found in plants, animals and microbes, with different chemical properties and compositions in each type. It is found in moist soils as well as several plants including pigeonpea, jack beans and soybeans. Urease plays diverse roles and mainly involved in the nitrogen metabolism.

Bacterial ureases have been shown to be important virulence determinants in the pathogenesis of many clinical conditions in human and animals. Urease is directly

involved in the formation of infection stones and contributes to the pathogenesis of urolithiasis, pyelonephritis, ammonia and hepatic encephalopathy, hepatic coma and urinary catheter encrustation. Urease is known to be the major cause of pathologies induced by *Helicobacter pylori*, which allows this pathogen to survive at the low pH of the stomach during colonization and therefore plays an important role in the pathogenesis of gastric and peptic ulcers, which in some cases may progress to cancer (Mobeley *et al.*, 1995). Studies on inhibitors are useful to learn about how enzymes interact with their substrates; role of inhibitors in enzyme regulation; drugs if they inhibit aberrant biochemical reactions; understanding the role of biological toxins; insecticides etc. Urease inhibitors restrict the conversion of urea and urine to ammonium, and hence to nitrate, in soils.

In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization (Mobeley *et al.*, 1995;

Mobeley and Hausinger, 1989; Sirko and Brodzik, 2000). This further induces plant damage primarily by depriving plants from their essential nutrients and secondly through ammonia toxicity and carbon dioxide release that increases the pH of the soil (Mobeley and Hausinger, 1989). Accumulation of ammonium ions, loss of urea N as ammonia, nitrite as well as ammonia toxicity damages germinating seeds, seedlings, and young plants (Gasser, 1964; Tomlinson, 1970; Radelet *et al.*, 1988). Unfortunately, neither the complete three-dimensional structure of jack bean nor that of any other plant urease has been determined. Therefore, most of our knowledge about the molecular mechanism of ureolytic catalysis by plant ureases is based on the 3-D structures of bacterial ureases. Elevated levels of soil microbial urease have been known to decrease the efficiency of urea fertilizers. It is speculated that the low concentration used for the inhibitors can also inhibit any of the soil ureases, which is important in plant agronomy.

Pigeonpea is an excellent source of organic nitrogen, to increase soil organic matter and improve soil structure and quality. They contain high levels of protein and the important amino acids such as methionine, lysine, and tryptophan. In present studies the urease from pigeonpea has been isolated and characterized biochemically along with thermal inactivation studies.

Materials and methods

Chemicals and enzyme

Urea (Enzyme grade), Tris buffer, Nessler's reagent (NR), Trichloro acetic acid (TCA) and HgCl_2 were from HiMedia, India. Glacial acetic acid, NaCl and boric acid were purchased from Merck, India. All solutions were prepared in double distilled water.

Extraction of enzyme

Approximately 20 g seeds of pigeonpea (*Cajanus cajan*) were soaked overnight in 20 ml of 25 mM Tris-acetate buffer, pH 6.5 and stored at 4°C. Next day, the same were grinded in a kitchen blender to form slurry. The slurry was filtered through a muslin cloth (cheese cloth) and the filtrate was

then centrifuged at 12,000 rpm for 15 min at 4°C. All the operations were carried out at 4°C unless stated otherwise. The clear supernatant (crude preparation of urease) was obtained and stored at 4°C till further use.

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 (0.9 ml) and properly diluted 0.1 ml enzyme solution were pre incubated at 37°C. This is sufficiently high temperature to affect rapid enzymic hydrolysis, but not high enough for inactivation of the enzyme. Urea solution (1.0 M; 0.2 M urea) was added and incubated at 37°C, for 10 min. Next, 1.0 ml of 10% TCA was added to stop the reaction. One ml of reaction mixture was transferred to 50 ml volumetric flask, which already contained 10-15 ml of double distilled water. Nessler's reagent (1.0 ml) was then added to the volumetric flask. The volume was made up to 50 ml with double distilled water and absorbance was measured at 405 nm on spectrophotometer. 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 as stated earlier. 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, 50 mM Tris-acetate buffer, pH 7.3, 37°C).

Steady state kinetics

Determination of optimum pH: The optimum pH was determined by preparing assay buffer of varying pH values (pH 4-9). The activity assay was performed at the different pH and percent relative activity was determined.

Determination of optimum temperature: The effect of temperature was studied by varying the temperature from 20° to 90°C in a multi-temperature (Pharmacia, Sweden) water bath during activity assay. The percent relative activity was plotted against temperature.

Effect of substrate concentration on urease activity: For effect of substrate concentrations, the activity assay was performed in different

substrate concentrations as discussed above. The stock solution of substrate (1.0 M urea) was prepared in assay buffer and different concentrations of the same were made. Initial rates were measured and plotted against corresponding substrate concentrations. The Lineweaver-Burk plot was drawn to determine the K_m .

Storage stability studies: The temperature stability of the pigeonpea urease was determined by storing urease, separately at two different temperatures and assayed at regular intervals. The enzyme was stored at 4°C and 37°C, separately for 80 days.

Time dependent thermal inactivation studies: For time thermal inactivation studies, 0.2 ml urease was incubated in water bath at the indicated temperatures for a fixed period of time. The aliquots drawn at the different time intervals were rapidly cooled in ice water and checked for residual activity. The activity measurement of the sample was executed after 5 minutes storage in ice water. The experiments were carried out at four different temperatures, like 50°C, 60°C, 70°C and 80°C.

Inhibition studies

Urease inhibition with boric acid: The inhibition studies were initiated with boric acid. The stock solution of the inhibitor was prepared in 50 mM Tris-acetate buffer, pH 7.3 and were suitably diluted for experiments. The activity assay was carried out at standard conditions as described earlier in the presence of varying concentration of inhibitor. At first, the I_{50} value of inhibitor was determined. Appropriately diluted urease was mixed with varying concentration of the inhibitor and in presence of either 0.1 M or 0.3 M urea, during the activity assay. The K_i value was determined from Dixon plot.

Inhibition with NaCl and HgCl₂: The 5M stock solution of NaCl was prepared. Different concentrations of inhibitor were made by adding different volumes of NaCl to assay buffer and the final volume was made to 0.9 ml. Urease (0.1ml) was added to 0.9 ml of

assay buffer containing inhibitor and the volume was made up to 1 ml. Next, urea (1.0 ml) was added and incubated with 10 min at 37°C. After stopping reaction with TCA the absorbance was recorded at 405 nm for each sample. The I_{50} value of inhibitor was determined. Next, the solution of 1.0mM HgCl₂ was prepared. The activity assay was carried out at standard conditions as described earlier in the presence of varying concentration of inhibitor and I_{50} value of the inhibitor was determined.

Results and discussion

Steady state kinetics

The rate of hydrolysis of urea by pigeonpea urease at 37°C showed optimum pH at 7.3 in the pH range 4.0-9.0 (Fig.1). It is clear that pigeonpea urease is only 50% efficient at pH 4.0 as compared to 100% activity at pH 7.3; whereas the jack bean urease (Blakeley and Zerner, 1984) has been reported to retain 68% activity at pH 5.2 compared to 100% activity at pH 7.0. 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, the highest value among the ureases purified so far (Hirayama *et al.*, 2000). With the exception of a small group of acid ureases (Kakimoto *et al.*, 1989; Kakimoto *et al.*, 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.

A bell shaped curve was obtained when urease was assayed at different temperatures (20°-90°C). It is clear from the Fig.2, that the activity of urease at first increases with rise in temperature till 70°C and then starts declining until its denaturation. It is evident that the optimum temperature of pigeonpea urease is 70°C. *Lactobacillus reuteri* urease was found to have a maximum activity and stability at 60°C – 70°C (Kakimoto *et al.*, 1989). Palinska and coworkers (Palinska *et al.*, 2000) have shown that *Prochlorococcus marinus* urease was stable

at least for 15 min, at temperature between 40⁰ - 60⁰C.

Furthermore, urease was assayed in different concentrations of urea to determine the effect of substrate (Fig.3); Lineweaver-Burk plot was used to calculate the K_m and the value was 20.4 mM (Fig. 4). The V_{max} for pigeonpea urease was found to be 0.625 μ mol NH_3^3 / min/ mg protein. Kinetic studies of jack bean urease showed a K_m of 2.9 to 3.3 mM (Blakeley and Zerner, 1984; Blakeley *et al.*, 1969). 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 the soybean seed urease depending on the buffer systems chosen has been reported (Talsky and Klunker, 1967). From mulberry leaf urease K_m of urea was reported to be 0.16 mM; this is the lowest value among the plant ureases purified so far (Hirayama *et al.*, 2000).

The stability temperature of the pigeonpea urease was determined by storing the urease at 4⁰C and 37⁰C. The plot of percent residual activity versus the number of days gave a $t_{1/2}$ of 70 days for urease stored at 4⁰C and 18 days for urease that stored at 37⁰C (Fig. 5). *U. urealyticum* urease was fully stable for more than 20 days when stored at 4⁰C in pH 7.2 buffer containing 1% bovine serum albumin (Stemkeet *et al.*, 1987); it is not clear whether the added albumin was required.

Fig.1: The effect of pH on the activity of pigeonpea urease.

Fig. 2: The effect of temperature on the activity of pigeonpea urease.

Fig. 3: Effect of substrate concentration on the activity of pigeonpea urease.

Fig. 4: Lineweaver-Burk plot to determine the value of K_m and V_{max} .

Fig.5: Comparison of storage stability of pigeonpea urease at 4 °C and 37°C

Fig.6: Time dependent thermal inactivation studies of pigeonpea urease.

Time dependent thermal inactivation

Thermal inactivation of pigeonpea urease was investigated at four different temperatures (50°C, 60°C, 70°C and 80°C). The graph shows percent loss in catalytic potential with time while incubating urease for the indicated time intervals (Fig. 6). Enzyme was assayed for residual activity. A variation in the kinetic pattern was observed at different temperatures. At 50°C and 60°C, the enzyme showed almost no loss in its kinetic potential over a period of 35 min. Clearly, thermal inactivation of soluble pigeonpea urease at 60°C and 70°C shows biphasic kinetics, in which the enzyme activity decreases in two

phases, i.e. half of the initial activity was destroyed more rapidly than the remaining half. Earlier similar studies were carried out by several workers (Godjevargova and Dimov, 1997; Rejikumar and Devi, 1998; Chen and Chiu, 2000), where only slight deviation from first-order kinetics was detected. Thermal inactivation studies strongly support the oligomeric nature of urease, which is also suggested by earlier workers (Polacco and Havir, 1979).

Fig.7: Dixon plot for the determination of K_i of boric acid inhibition of pigeonpea urease.

Urease inhibition

The inhibition studies of urease are important due to many factors. Urease is known to be a major cause of pathologies induced by *H. pylori*, which allows the bacteria to survive at the low pH of stomach during colonization and, therefore, plays an important role in the pathogenesis of gastric and peptic ulcers cancer also (Mobeleyet *et al.*, 1995). In infections with *P. mirabilis* and *Y. enterocolitica*, urease has been implicated in urolithiasis (stone formation) and contributes to the development of acute phelonephritis and infection-induced reactive arthritis, respectively (Gripenberget *et al.*, 2000; Li *et al.*, 2002).

Boric acid was investigated for its inhibitory effect on pigeonpea urease. The I_{50} value was determined by performing the activity assay in the presence of boric acid with varying concentrations. The I_{50} value of boric acid inhibition for pigeonpea urease was 7.6 mM. Furthermore, the inhibition constant (K_i) was determined by Dixon plot and the value was found to be 1.10 mM (Fig. 7) and type of inhibition was competitive type as evident from the figure. Boric acid has been reported as a potent competitive inhibitor of soybean urease, with a K_i value of 0.20 ± 0.05 mM at pH 7.0 (Kumar and Kayastha, 2010a). It has also been reported to be a strong competitive inhibitor in the case of jack bean, *P. mirabilis*, and *K. aerogenes* ureases. The detailed mechanism of urease inhibition by boric acid cannot be established by kinetic studies alone. Benini *et al.*, (2004) reported the structural details of the *Bacillus pasteurii* urease–boric acid complex and clarified the molecular details of the inhibition and the unique binding mode for this inhibitor, and provided insights into the role of nickel ions in enzymatic urea hydrolysis.

Next, the NaCl was investigated for its inhibitory effect on pigeonpea urease. The objective was to investigate the effect of constituent anion, as sodium has been reported as poor inhibitory properties for urease. The activity assay was carried out in the presence of varying concentrations of NaCl and the I_{50} value was found to be 0.20 M. The I_{50} value for Hg^{2+} ion was shown to be 6.10 nM. It is well known from the literature that some heavy metal ions are strong inhibitors of urease (Ambrose *et al.*, 1951; Shaw and Raval, 1961; Zaborska *et al.*, 2004). The I_{50} values for Ag^+ , Hg^{2+} and Cu^{2+} metal ions have been reported to be 2.3×10^{-11} M, 7.1×10^{-8} M and 3.3×10^{-6} M respectively (Kumar and Kayastha, 2010b).

Conclusion

The physiological role of pigeonpea or other plant ureases in the cellular economy is not well known. However, it is apparent from the present studies that under physiological

conditions, its activity will be strongly inhibited by several inhibitors. It is speculated that the low concentration used for the inhibitors can also inhibit any of the soil ureases, which is important in plant agronomy. Due to the similar catalytic mechanism exhibited by all ureases, the inhibitors studied can be successfully used in conjunction with the ureases of any origin for controlling / inhibiting urease activity in soil and pathogenic microbes to solve the various problems as stated earlier.

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References:

1. Ambrose, J.F, Kistiakovsky, G.B. and Kridl, A.G. (1951). Inhibition of urease by silver ions. *Journal of the American Chemical Society*, 73: 1232-1236.
2. Benini, S., Rypniewski, W.R., Wilson, W.S., Mangani, S. and Ciurli, S. (2004). Molecular Details of Urease Inhibition by Boric Acid: Insights into the Catalytic Mechanism. *Journal of the American Chemical Society*, 126: 3714-3715
3. Blakeley, R.L. and Zerner, B. (1984). Jack bean urease: The first nickel enzyme. *Journal of Molecular Catalysis*, 23: 263-292.
4. Blakeley, R.L., Webb, E.C. and Zerner, B. (1969). Jack bean urease (EC 3.5.1.5). A new purification and reliable rate assay. *Biochemistry Journal*, 8: 1984-1990.
5. Chen, J. and Chiu, S. (2000). A poly (N-isopropylacrylamide-co-N-acryloxysuccinimide-co-2-hydroxyethyl methacrylate) composite hydrogel membrane for urease immobilization to enhance urea hydrolysis rate by temperature swing.

- Enzyme and Microbial Technology*, 26: 359-367.
6. Dixon, N.E., Gazzola, C., Blakeley, R. and Zerner, B. (1975). Jack bean urease: A metalloenzyme. A simple biological role for nickel? *Journal of the American Chemical Society*, 97: 4131-4133.
 7. Gasser, J.K.R. (1964). Urea as a fertilizer. *Soil Fertilizer*, 27: 175-180.
 8. Godjevargova, T. and Dimov, A. (1997). Immobilization of urease onto membranes of modified acrylonitrile copolymer. *Journal of Membrane Science*, 135, 93-98.
 9. Gripenberg-Lerche, C., Zhange, L., Ahtonen, P., Toivanen, P. and Skurnik, M. (2000). Construction of urease-negative mutants of *Yersinia enterocolitica* serotypes O: 3 and O: 8 Role of urease in virulence and arthritogenicity. *Infection and Immunity*, 68: 942-947.
 10. Hirayama, C., Sugimura, M., Saito, H. and Nakamura, M. (2000). Purification and properties of urease from leaf of mulberry (*Morus alba*). *Phytochemistry*, 53: 325-330.
 11. Kakimoto, S., Sumino, Y., Akiyama, S.I. and Nakao, Y. (1989). Purification and characterization of acid urease from *Lactobacillus reuteri*. *Agricultural and Biological Chemistry*, 53: 1119-1125.
 12. Kakimoto, S., Sumino, Y., Kawahara, K., Yamazaki, E. and Nakatsui, I. (1990). Purification and characterization of acid urease from *Lactobacillus fermentum*. *Applied Microbiology and Biotechnology*, 32: 538-543.
 13. Kerr, P.S., Bievin, D.G., Rapp, B.J. and Randall, D.D. (1983). Soybean leaf urease: comparison with seed urease. *Physiologia Plantarum*, 57: 339-342.
 14. Kumar, S. and Kayastha, A.M. (2010a). Acetohydroxamic acid- A competitive inhibitor of urease from soyabean "*glycine max*". *Journal of Proteins and Proteomics*, 1: 3-8.
 15. Kumar, S. and Kayastha, A.M. (2010b). Inhibition studies of soybean (*Glycine max*) urease with heavy metals, sodium salts of mineral acids, boric acid, and boronic acids. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 25: 646-652.
 16. Li, M., Zhang, G. and Su, Z. (2002). Dual ion exchange chromatography improved refolding yield of lysozyme. *Journal of Chromatography A*, 959: 113-120.
 17. Mobeley, H.L.T. and Hausinger, R.P. (1989). Microbial Ureases: Significance, Regulation, and Molecular Characterization. *Microbiological reviews*, 53: 85-108.
 18. Mobeley, H.L.T., Island, M.D. and Hausinger, R.P. (1995). Molecular Biology of Microbial Ureases. *Microbiological reviews*, 59: 451-480.
 19. Mulvaney, R.L. and Bremner, J.L. (1981). Control of urea transformations in soils. *Biochemistry*, 5: 153-196.
 20. Palinska, K.A., Jahns, T., Rippka, R., Tandeau, D. and Marsac, N. (2000). *Prochlorococcus marinus* strain PCC 9511, a picoplanktonic cyanobacterium, synthesizes the smallest urease. *Microbiology*, 146: 3099-3107.
 21. Polacco, J.C., and Havir, E.A. (1979). Comparisons of soybean urease isolated from seed and tissue culture. *Journal of Biological Chemistry*, 254: 1707-1715.
 22. Polacco, J.C. and Holland, M.A. (1993). Roles of urease in plant cell. *International Review of Cytology*, 145: 65-103.
 23. Prakash, O. and Bhushan, G. (1997). Isolation, purification and partial characterization of urease from seeds of watermelon (*Citrullus vulgaris*). *Journal of Plant Biochemistry and Biotechnology*, 6: 45-47.
 24. Radel, R.J., Gateney, J. and Peters, G.E. (1988). Urease inhibitor

- Developments. In: American volatilization from urea fertilizers. *Tennessee Valley Authority in Muscle Shoals, AL*, 111-136.
25. Rejikumar, S. and Devi, S. (1998). Preparation and characterization of urease bound on crosslinked poly (vinyl alcohol). *Journal of Molecular Catalysis B: Enzymatic*, 4: 61-66.
 26. Shaw, W.H.R. and Raval, D.N. (1961). The inhibition of urease by metal ions at pH 8.9. *Journal of the American Chemical Society*, 83: 3184-3187.
 27. Sirko, A. and Brodzik, R. (2000). Plant ureases: Roles and regulation. *Acta Biochimica Polonica*, 47: 1189–1195.
 28. Stemke, G.W., Robertson, J.A. and Nhan, M. (1987). Purification of urease from *Ureaplasma urealyticum*. *Canadian Journal of Microbiology*, 33: 857-862.
 29. Sumner, J. B. (1926). The Isolation and Crystallization of the Enzyme Urease. *Journal of Biological Chemistry*, 69: 435–441
 30. Talsky, G. and Klunker, G. (1967). On the temperature dependency of enzymatic reactions, IV. Hydrolysis of urea by urease. *Hoppe-Seyler's Zeitschrift für Physiologische Biologische Chemie*, 348: 1372-1376.
 31. Tomlinson, T.E. (1970). Urea agronomic applications. *Proceeding of Fertilizer Society*, 113: 1-76.
 32. Yamazaki, E., Kurasawa, T., Kakimoto, S., Sumino, Y. and Nakatsui, I. (1990). Characteristics of acid urease from *Streptococcus mitior*. *Agricultural and biological chemistry*, 54, 2433-2435.
 33. Zaborska, W., Krejewska, B. and Olech, Z. (2004). Heavy Metal Ions Inhibition of Jack Bean Urease: Potential for Rapid Contaminant Probing. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 19: 65–69.