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 μmol NH₃/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 ($I_{50} = 205$ mM), whereas the $I_{50}$ value for Hg²⁺ was recorded at 6.10 nM.

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

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Introduction

Jack bean (Canavaliaensiformis) 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 most 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 pyroli, 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 (Mobeleyet 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 (Mobeleyet 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 HgCl2 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°C 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 mMTris-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 HgCl$_2$:** 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 (0.1 ml) 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 HgCl$_2$ 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 (Kakimotoet al., 1989; Kakimotoet 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 (Kakimotoet al., 1989). Palinska and coworkers (Palinskaet al., 2000) have shown that *Prochlorococcus marinus* urease was stable.
at least for 15 min, at temperature between 40\(^0\) - 60\(^0\)C.

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 \(\mu\) mol NH\(_3\)/ 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 4\(^0\)C and 37\(^0\)C. The plot of percent residual activity versus the number of days gave a \(t_{1/2}\) of 70 days for urease stored at 4\(^0\)C and 18 days for urease that stored at 37\(^0\)C (Fig. 5). \textit{U. urealyticum} urease was fully stable for more than 20 days when stored at 4\(^0\)C in pH 7.2 buffer containing 1% bovine serum albumin (Stemkett et al., 1987); it is not clear whether the added albumin was required.
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).

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 (Mobeley 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 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 \( B. \) pasteurii–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 x 10\(^{-11} \) M, 7.1 x 10\(^{-8} \)M and 3.3 x 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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