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ORIGINAL ARTICLE

Inhibition studies of soybean (*Glycine max*) urease with heavy metals, sodium salts of mineral acids, boric acid, and boronic acids

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Abstract

Various inhibitors were tested for their inhibitory effects on soybean urease. The K_i values for boric acid, 4-bromophenylboronic acid, butylboronic acid, and phenylboronic acid were 0.20 ± 0.05 mM, 0.22 ± 0.04 mM, 1.50 ± 0.10 mM, and 2.00 ± 0.11 mM, respectively. The inhibition was competitive type with boric acid and boronic acids. Heavy metal ions including Ag^+, Hg^{2+}, and Cu^{2+} showed strong inhibition on soybean urease, with the silver ion being a potent inhibitor ($IC_{50} = 2.3 \times 10^{-8}$ mM). Time-dependent inhibition studies exhibited biphasic kinetics with all heavy metal ions. Furthermore, inhibition studies with sodium salts of mineral acids (NaF, NaCl, $NaNO_3$, and Na_2SO_4) showed that only F^- inhibited soybean urease significantly ($IC_{50} = 2.9$ mM). Competitive type of inhibition was observed for this anion with a K_i value of 1.30 mM.

Keywords: *Urease; inhibition; soybean; boric acid; boronic acid; metals*

Introduction

Soybean (*Glycine max*) has been economically very important in its use as a source of various proteins for industrial purposes. Among the various proteins present in soybean is urease, which is present in abundance in its seeds. Biochemically, the best-characterized plant urease is that from jack bean (*Canavalia ensiformis*)[1–4]. The best genetic data concerning plant ureases are available for soybean[5,6]. Urease (urea amidohydrolase, EC 3.5.1.5) occurs throughout the animal and plant kingdoms. Many microorganisms use this enzyme to provide a source of nitrogen for growth, and it plays an important role in plant nitrogen metabolism during the germination process[7,8]. The presence of urease activity in soils is exploited in the widespread agricultural practice of urea-based fertilizer application for enhancing crop yields. Unfortunately, excessive levels of soil urease can degrade the fertilizer urea too rapidly, and result in phytopathic effects and loss of volatilized ammonia[9]. Of medical and veterinary interest, urease is a virulence factor in certain human and animal pathogens; it participates in the development of kidney stones, pyelonephritis, peptic ulcers, and other disease states[10].

Strategies based on urease inhibition are now considered as the first line of treatment for infections caused by urease-producing bacteria, as well as for preventing the huge losses of urea from agricultural fields. The kinetics of inhibition of urease has been extensively studied[7]. It has been found that the inhibitor mechanism of action and the kinetics of inhibition for bacterial urease and jack bean urease are similar[11]. Therefore, urease from any source, be it bacterial or plant, can be used as a model system for inhibition studies, and results would be equally applicable for any system or field of application. Four major classes of urease inhibitors have been investigated, namely hydroxamic acids[12–14], phosphoroamide compounds[13], boric and boronic acids[15], and heavy metal ions[16,17]. The first three classes have been investigated mainly as potential therapeutic agents against certain bacterial urease-induced human pathogenic states, and the fourth class for analytical purposes. Urease inhibitors inactivate the enzyme in a variety of ways. Hydroxamic

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acids and phosphoroamide compounds create a tetrahedral intermediate with a structural similarity to the tetrahedral intermediate postulated to occur during urea hydrolysis[12,13]. Heavy metal ions react with the active site sulfhydryl group. The reaction is analogous to the formation of metal sulfide[18]. Boric and boronic acids are suggested to form a complex with nickel ion(s)[15]. Several thiol compounds have been shown to be competitive inhibitors of *Klebsiella aerogenes* urease. Previously, a number of synthetic and natural inhibitors of urease have been reported, and their inhibition kinetics and structure–activity relationships have been studied[7,10–13,15,18].

In the present study, the inhibiting effects of various chemicals, namely heavy metals, boric and boronic acids, and sodium salts of mineral acids, on soybean urease have been investigated in order to elucidate the kinetics and mechanism of inhibition. Also, the present study will have practical significance in solving the problems as mentioned above in medical and agricultural agronomy.

Materials and methods

Chemicals and enzyme

Bovine serum albumin (BSA), β-mercaptoethanol, Tris, urea (enzyme grade), and dialysis tubing were purchased from Sigma Chemical Co., St. Louis, USA. Sodium salts of mineral acids (NaF, NaCl, $NaNO_3$, and Na_2SO_4), salts of heavy metals ($HgCl_2$, AgCl, and $Cu(CH_3COO)_2$), Nessler's reagent, and trichloroacetic acid (TCA) were obtained from HiMedia, Mumbai, India. All other chemicals were of analytical grade and obtained from SRL or Merck, Mumbai, India. All the solutions were prepared in Milli-Q (Millipore, USA) water. Urease was purified from the mature seeds of soybean to apparent homogeneity by the method of Polacco and Havir[19] with minor modifications.

Urease activity assay

For routine assay of urease activity, the ammonia liberated in a fixed time interval while incubating the enzyme with saturating concentrations of urea was determined using Nessler's reagent, as described earlier[20]. The yellow-orange colored solution was measured at 405 nm on a Unicam UV-2 spectrophotometer. The amount of NH_3 liberated in the reaction mixture was estimated by calibrating Nessler's reagent with standard NH_4Cl solution. One enzyme unit is defined as the amount of urease required to liberate 1 μmol of ammonia per minute under our test conditions (0.1 M urea, 0.05 M Tris-acetate buffer, pH 7.0, 37°C).

Protein estimation

The protein content of the urease preparation was estimated by the method of Lowry *et al.*[21] using bovine serum albumin as standard.

Inhibition studies

The inhibition studies of soybean urease were initiated with boric acid and boronic acids (butylboronic acid, 4-bromophenylboronic acid, and phenylboronic acid). Also, heavy metal ions ($HgCl_2$, AgCl, and $Cu(CH_3COO)_2$) and sodium salts of mineral acids (NaF, NaCl, $NaNO_3$, and Na_2SO_4) were investigated for their inhibitory effects. Stock solutions of inhibitors, except for 4-bromophenylboronic acid, were prepared in 0.05 M Tris-acetate buffer, pH 7.0, and were suitably diluted for experiments, whereas a stock solution of 4-bromophenylboronic acid was prepared in absolute ethanol and subsequently diluted in respective buffer. The activity assay was carried out at standard conditions as described earlier in the presence of varying concentrations of inhibitors. First, the IC_{50} values of inhibitors were determined, and the compounds with more inhibition potency were selected for further studies. Appropriately diluted urease was mixed with varying concentrations of the inhibitors and in the presence of either 0.1 M or 0.3 M urea, during the activity assay. The K_i values were determined from a Dixon plot. For time-dependent inhibition studies, suitably diluted urease was incubated with the desired concentration of inhibitor in 0.1 M Tris-acetate buffer, pH 7.6, for a certain period. Aliquots of treated urease drawn at regular intervals were checked for residual activity.

Analysis of kinetics data

With time-dependent inhibition, the data were collected and plotted as log % residual activity versus time. The time-course of inhibition for all the inhibitors studied was found to be consistent with Equation (1), and therefore the data were processed and analyzed in accordance with the following:

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

where A_t is the fraction residual activity at time t, A_{fast} and A_{slow} are amplitudes (expressed as percent of starting activity), and k_{fast} and k_{slow} are rate constants of the fast and slow phases, respectively. Initial estimates of the rate constants and amplitudes were obtained from semi-log plots, as described earlier[22].

Results and discussion

Inhibition with boric acid and boronic acids

Boric and boronic acids were earlier reported to be competitive inhibitors of *Proteus mirabilis* urease[15], and are also shown to inhibit *K. aerogenes* urease in a similar manner[13]. Boric acid and boronic acids were investigated for their inhibitory effect on soybean urease. Initially, IC_{50} values were determined by performing the activity assay in the presence of the respective inhibitors with varying concentrations. The IC_{50} values for boric acid, 4-bromophenylboronic acid, butylboronic acid, and phenylboronic acid were found to be 0.7, 1.0, 2.9, and 4.1 mM, respectively (Figure 1). Furthermore, the inhibition constant (K_i) was determined by Dixon plot in each case and the respective values were found to be 0.20 ± 0.05 mM, 0.22 ± 0.04 mM, 1.50 ± 0.10 mM, and 2.00 ± 0.11 mM (Figure 2). A comparison of K_i values of boric acid and boronic acids for soybean urease along with urease from other sources is presented in Table 1.

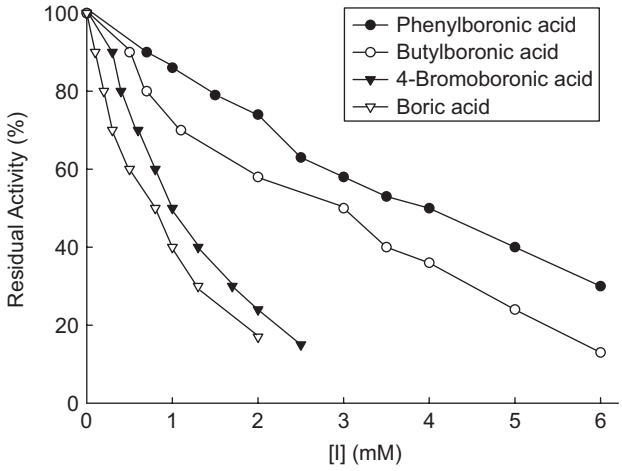


Figure 1. Effect of boric acid and boronic acids on the activity of soybean urease. Suitably diluted urease (0.87 µg/mL) was assayed in the presence of varying concentrations of respective inhibitors. Each experimental point represents the mean of three determinations.

It is clear that boric acid is a potent competitive inhibitor of soybean urease, with a K_i value of 0.20 ± 0.05 mM at pH 7.0 (Table 1). It has also been reported to be a strong competitive inhibitor in the case of jack bean[23,24], pigeonpea (*Cajanus cajan*)[25], *P. mirabilis*[15], and *K. aerogenes*[13] ureases. Boric acid acts as a competitive inhibitor for many enzymes including prostate specific antigen[26] and *Streptomyces griseus* proteinase[27], and also reversibly inhibits the second step of pre-mRNA splicing[28]. Three boronic acids (4-bromophenylboronic acid, butylboronic acid, and phenylboronic acid) were examined for inhibitory action on soybean urease, and all were found to inhibit competitively. Among the above tested boronic acids, 4-bromophenylboronic acid was found to be the most potent competitive inhibitor. A similar trend has also been reported for *P. mirabilis*[15], *C. cajan*[25], and *K. aerogenes* ureases[24]. However, phenylboronic acid was found to be a weak inhibitor, similar to the case of *K. aerogenes* where the K_i was 10 mM[24]. Also, *P. mirabilis* urease appears to be more sensitive to these inhibitors among all

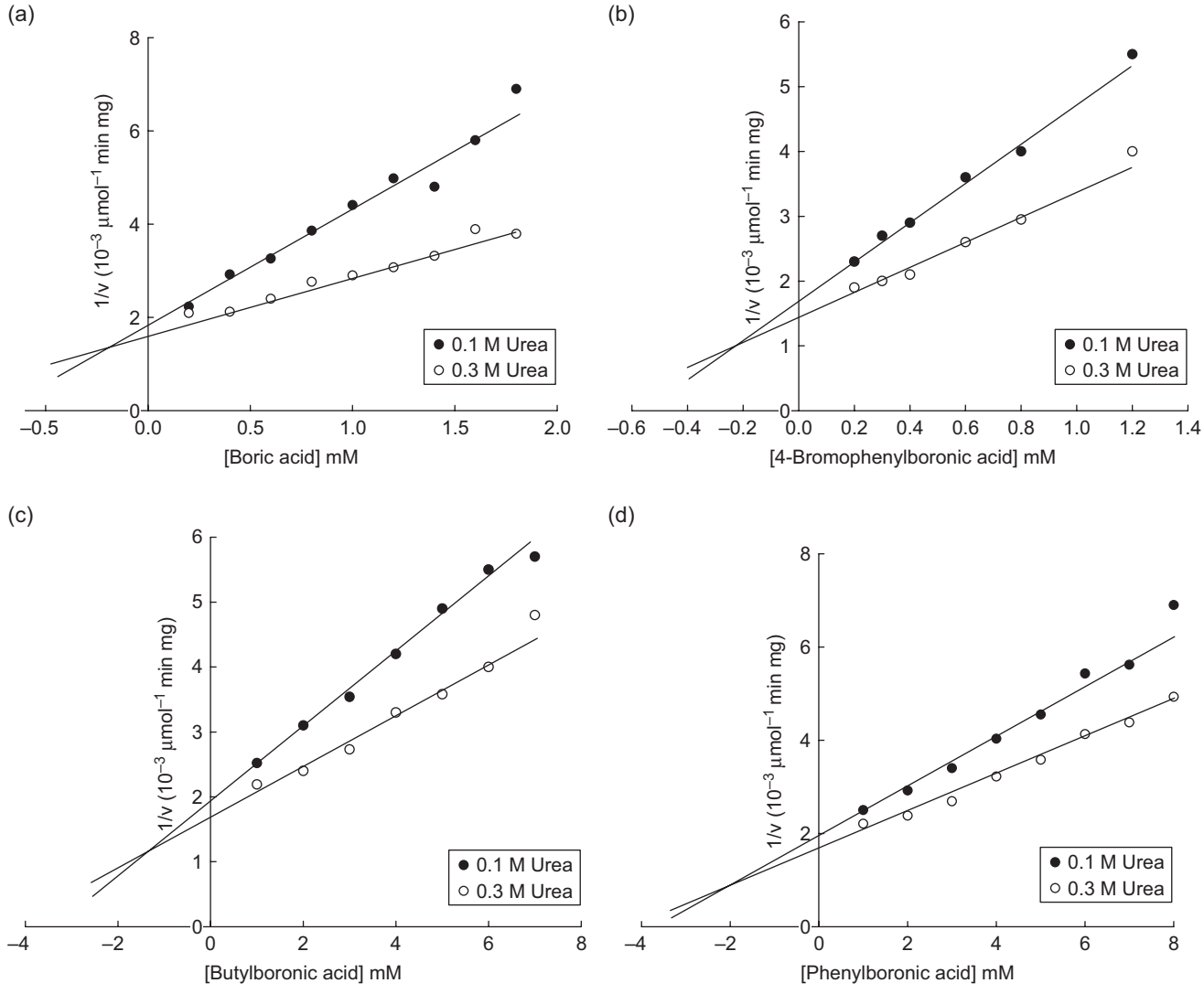


Figure 2. Dixon plots for boric and boronic acids. Enzyme (0.87 µg/mL) was assayed in the presence of varying concentrations of respective inhibitors and in 0.1 M or 0.3 M urea as described in "Materials and methods." (a) Boric acid, (b) 4-bromophenylboronic acid, (c) butylboronic acid, (d) phenylboronic acid. Each experimental point represents the mean of three determinations.

Table 1. Comparision of K_i values for boric and boronic acids for urease from different sources.

Urease source	K_i (mM)			
	Boric acid	Butylboronic acid	4-Bromophenyl-boronic acid	Phenyl-boronic acid
G. max	0.20 ± 0.05	1.50 ± 0.10	0.22 ± 0.04	2.00 ± 0.11
C. ensiformis[a]	0.23	—	—	—
K. aerogenes[b]	0.33	—	0.37	10
P. mirabilis[c]	0.099 ± 0.008	0.547 ± 0.069	0.124 ± 0.048	1.26 ± 0.32
C. cajan[d]	0.35 ± 0.15	1.8 ± 0.2	0.3 ± 0.1	2.5 ± 0.4

[a]Krajewska *et al.*, 1999[24].
[b]Todd and Hausinger, 1989[13].
[c]Breitenbach and Hausinger, 1988[15].
[d]Reddy and Kayastha, 2006[25].

the ureases listed in Table 1. As in the case of several proteases, boronic acids are thought to inhibit by reacting with an active site serine group[29].

The pH-dependent inhibition of boric and boronic acid has been reported earlier for *P. mirabilis*[15], *C. ensiformis*[24], and *C. cajan*[25], and was shown to be consistent with trigonal $B(OH)_3$ being the inhibitor rather than the tetrahedral $B(OH)^-_4$. The inhibition is maximal between pH 6.2 and 9.3, suggesting that only the neutral trigonal $B(OH)_3$, and not the $B(OH)^-_4$ anion, is an inhibitor of urease. Soybean urease is supposed to follow a similar mechanism of boric acid inhibition to that proposed above for urease from other sources, due to the comparable K_i value as well as resemblance of active site structure. The detailed mechanism of urease inhibition by boric acid cannot be established by kinetic studies alone. Benini et al.[30] 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.

Inhibition with heavy metals

It is well known from the literature that some heavy metal ions are strong inhibitors of urease[31–34]. Therefore, heavy metal ions (Ag^+, Hg^{2+}, and Cu^{2+}) were investigated for their inhibitory effects on soybean urease, for better understanding of urease action and also for inhibition-based metal detection that could be exploited in the construction of biosensors and other bio-sensing systems. Initially, the IC_{50} values for the different heavy metal ions were determined. It was found that all the heavy metal ions were strong inhibitors of soybean urease, though their potencies differed. The IC_{50} values for Ag^+ (Figure 3a), Hg^{2+}, and Cu^{2+} metal ions were found to be 2.3×10^{-8} mM, 7.1×10^{-5} mM, and 3.3×10^{-3} mM, respectively. Clearly, the Ag^+ ion with IC_{50} value 2.3×10^{-8} mM was the most potent inhibitor. Time-dependent inhibition studies were carried out with Ag^+ (Figure 3b), Hg^{2+}, and Cu^{2+} ions and biphasic kinetics were observed. The values of amplitudes and rate constants of slow and fast phases for the metal ions were determined from the semi-log plots (Table 2). It is clear from Table 2 that each metal ion follows biphasic kinetics: fast and slow phases and each phase with equal amplitude (50%). Evidently, the metal ions inactivate

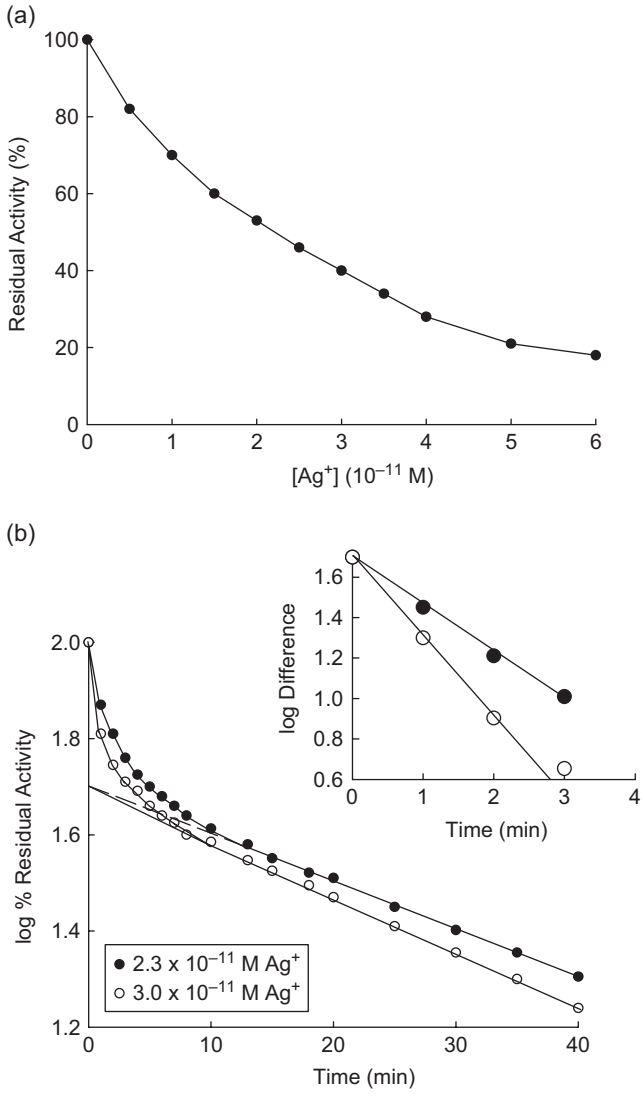


Figure 3. Inhibition of soybean urease with Ag^+ ion. (a) Effect of Ag^+ ion on the activity of soybean urease. Enzyme (0.87 μg/mL) was assayed in the presence of different concentrations of AgCl. (b) Time-dependent inhibition studies of soybean urease with Ag^+ ion. Enzyme (2.28 μg/mL) in 0.1 M Tris-acetate buffer, pH 7.6, was incubated separately in specified concentrations of AgCl at 37°C. Aliquots withdrawn at specified intervals were assayed for percent residual activity. Inset shows the semi-log plot for the fast phase. Each experimental point represents the mean of three determinations.

Table 2. Values of amplitudes (A) and rate constants (k) determined during time-dependent inhibition studies of soybean urease.

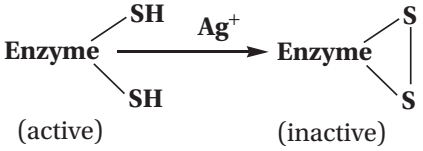
		Fast phase		Slow phase	
	Inhibitor (M)	A_{fast} (%)	k_{fast} (min^{-1})	A_{slow} (%)	k_{slow} (min^{-1})
Ag^+	2.3×10^{-11}	50.2	0.5433 ± 0.0023	49.8	0.0226 ± 0.0010
	3.0×10^{-11}	49.1	0.6852 ± 0.0017	50.9	0.0269 ± 0.0021
Hg^{2+}	8.0×10^{-8}	51.1	0.2482 ± 0.0019	48.9	0.0138 ± 0.0022
	8.7×10^{-8}	50.1	0.3632 ± 0.0023	49.9	0.0163 ± 0.0015
Cu^{2+}	4.0×10^{-6}	49.3	0.1314 ± 0.0007	50.7	0.0059 ± 0.0010
	5.0×10^{-6}	50.8	0.2041 ± 0.0013	49.2	0.0091 ± 0.0015

the urease at very low concentrations, which therefore indicates that they have extremely high affinities for soybean urease. Time-dependent inhibition studies clearly established the half-site reactivity of the active sites of soybean urease,

indicating half of the active sites behaving differently from the other half. Similar results were reported for the inhibition of pigeonpea urease by heavy metal ions, and molecular asymmetry of the active site was established[35].

In the case of the Ag^+ ion, inactivation of soybean urease activity occurred at a very low concentration (pM range). The Ag^+ ion concentration was much lower than that of the enzyme in the incubation mixture. This suggests a catalytic rather than a stoichiometric role of Ag^+ ions, and therefore inactivation requiring stoichiometric reaction between Ag^+ ions and the enzyme, e.g. Equation (2), may be ruled out. These ions probably act as a catalyst in some other reactions involving SH groups for which Ag^+ ions have a high affinity, for example the formation of disulfide bonds (Equation (3)). A similar reaction has been proposed by Shaw[32]. Hellerman *et al.*[36] also postulated the formation of disulfide bonds in the reaction of jack bean urease with Cu(II)/oxygen. The other heavy metal ions, Cu^{2+} and Hg^{2+}, may possibly also inhibit soybean urease by a similar mechanism, i.e. through Equation (3). However, in their case the possibility of inactivation through Equation (3) cannot be ruled out, because in these cases the enzyme concentration is less than that of the metal ions.

$$\mathbf{E} + \mathbf{I} = \mathbf{E.I}\ (\text{inactive complex}) \tag{2}$$



(3)

The relative effectiveness of the heavy metal ions as inhibitors of jack bean urease has been reported to decrease in the following approximate order: $Hg^{2+} > Ag^+ > Cu^{2+} >> Ni^{2+} > Cd^{2+} > Zn^{2+} > Co^{2+} > Fe^{3+} > Pb^{2+} > Mn^{2+}$ [18,34], with Hg^{2+}, Ag^+, and Cu^{2+} ions nearly always listed as the most effective inhibitors[18,32–34,37]. This inhibition has been habitually ascribed to the reaction of the ions with the thiol groups of cysteine residues of the enzyme, resulting in the formation of mercaptides[18,31–34,36,37]. This was supported by a conclusion of Shaw[32,33] that the order of effectiveness of heavy metal ions as urease inhibitors correlated with the solubility product constants of the corresponding metal sulfides. However, very importantly, heavy metal ions can also bind to functional groups in proteins other than thiols. These mainly include nitrogen- (histidine) and oxygen- (aspartic and glutamic acids) containing functional groups[38], and in fact, the relative frequency of sites reported as utilized by metals in metalloproteins follows the order: His > Cys > Asp > Glu.

More recently, Krajewska[39] studied the effect of monovalent (Ag, Hg) and divalent (Cu, Hg) metal ions on the activity of jack bean urease and reported through time-dependent inhibition and titrimetric studies with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) that the heavy metal ions react with the thiol groups of cysteine residues of urease. Through their observations, they also supported the notion that the reaction of urease with the metal ions is not restricted to cysteine residues in the active site, but involves more functional groups. A combination of effects may be responsible for distortion of the architecture of the active site, the mechanism of which remains to be elucidated.

Inhibition with sodium salts of mineral acids

The fluoride ion was first demonstrated to inhibit bovine rumen urease in 1943[40]. Several groups have described the inhibition of jack bean urease by fluoride as being competitive[41,42]. The sodium salts of mineral acids (NaF, NaCl, $NaNO_3$, and Na_2SO_4) were investigated for their inhibitory effects on soybean urease. The objective was to investigate the effect of constituent anions, as sodium was the common cation among all the salts. The activity assay was performed in the presence of varying concentrations of inhibitor and the IC_{50} was determined. It was found that only the F^- ion showed significant inhibition, with IC_{50} at 2.9 mM (Figure 4a), and therefore needed further investigation. The activity assay was carried out in the presence of varying concentrations of NaF with either 0.1 M or 0.3 M urea, and K_i was determined by Dixon plot. Competitive type of inhibition was observed for this anion, and K_i was 1.30 mM (Figure 4b).

Furthermore, in order to assess the interaction of fluoride with enzyme, suitably diluted urease (2.28 µg/mL) was incubated with sodium fluoride (3 mM) for 30 min at 37°C in the absence of urea. The activity assay showed complete loss of activity. The urease–fluoride mixture was subsequently dialyzed overnight against 0.1 M Tris-acetate buffer, pH 7.6, at 4°C. The next day, the enzyme, when assayed, showed a regain of 82% of the original activity. These observations suggest a reversible interaction of fluoride with the urease. Todd and Hausinger[43] demonstrated that the fluoride-inhibited *K. aerogenes* urease complex, when diluted into buffer that lacked substrate, could be reverted to uninhibited enzyme. Similarly, after complete removal of fluoride by dialysis, jack bean urease could also be activated to about 88.5% of its original activity, in both the absence and the presence of β-mercaptoethanol[41]. Kaneshiro and Reithel[44] showed that fluoride apparently binds slowly and reversibly to jack bean urease and inhibits the urease activity. Our active site studies showed that soybean urease is protected strongly by the fluoride ion against the *N*-ethylmaleimide (NEM) inactivation of cysteine residues (data not shown). This suggests that fluoride does bind to the active site and decreases the reactivity of active site thiol in the vicinity of the nickel ion. Kinetic studies showed that fluoride is a reversible, competitive inhibitor of soybean urease, with a K_i of 1.30 mM at pH 7.0 and 37°C.

It has also been shown to be a competitive inhibitor of jack bean urease, with a K_i of 1 mM at pH 7.0, 38°C[41,45]. Srivastava *et al.*[35] have also shown the reversible and competitive nature of the F^- ion for pigeonpea urease. Prakash and Upadhyay[46] reported that fluoride is a non-competitive inhibitor of watermelon urease at 30°C in 50 mM Tris-acetate buffer (pH 8.5), and suggested that the fluoride binds to a site distinct from the substrate binding site. Todd and Hausinger[43] reported that the fluoride ion is a slow-binding, pseudo-uncompetitive inhibitor of *K. aerogenes*, using steady state and pre-steady state kinetic studies. According to their studies, steady-state

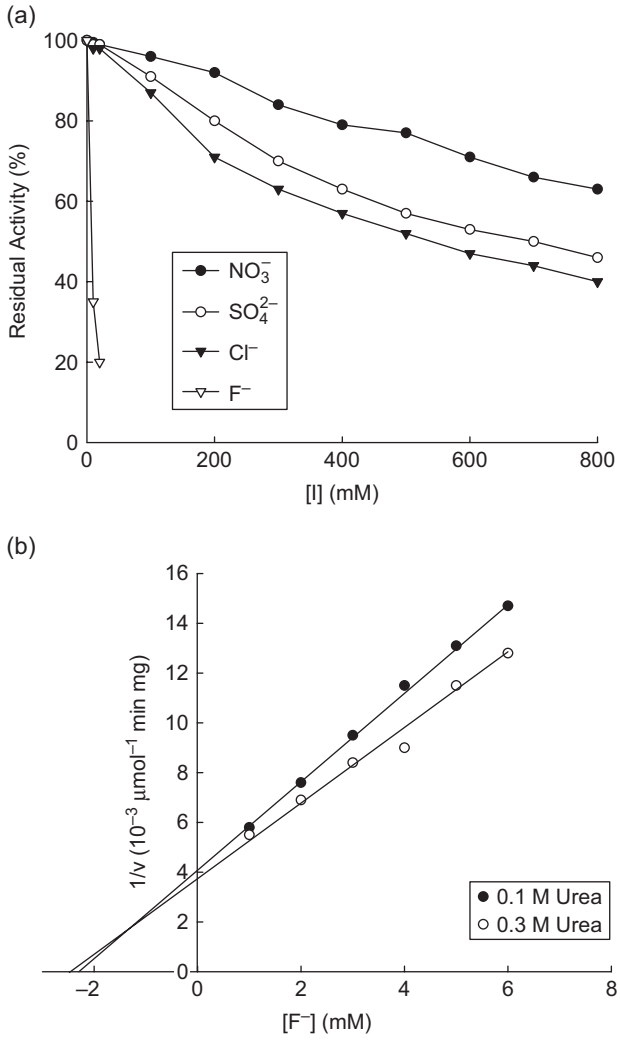


Figure 4. Inhibition of soybean urease with sodium salts of mineral acids. (a) Effect of salts on the activity of soybean urease. Enzyme (0.87 µg/mL) was assayed in the presence of varying concentrations of respective inhibitors. (b) Dixon plot for the inhibition of soybean urease by F^- ion. Enzyme (0.87 µg/mL) was assayed in the presence of varying concentrations of F^- ion and with 0.1 M or 0.3 M urea as described in "Materials and methods." Each experimental point represents the mean of three determinations.

kinetics data that exhibit parallel lines in double reciprocal plots (1/v vs. 1/[Urea]) do not conclusively demonstrate the existence of an enzyme–substrate–inhibitor species arising from uncompetitive inhibition, as commonly invoked. The data obtained from the microbial enzyme show many features that are similar to jack bean urease inhibition[43].

Conclusion

The physiological role of soybean or other plant urease 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 metal ions. Most of the inhibitors studied showed significant inhibition of soybean urease with a competitive type of mechanism; the K_i values were in mM ranges, which are close enough to the physiological values. 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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Declaration of interest

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