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Urease inhibitors: A review

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Urease inhibitors possess a wide application in agriculture, clinical science and understanding of enzyme kinetics. These inhibitors play a significant role in reducing the loss of nitrogen from urea fertilizer in the form of volatile ammonia and, thus, improve the utility of urea based fertilizers. Their impact as an effective drug against urease producing bacterial infection is well documented. The study of urease inhibitors is useful in providing an insight in the catalytic mechanism. This article is an attempt to compile the various urease inhibitors used so far by researchers.

Keywords: Acetohydroxamic acid, inhibitors, urease, N-(n-buty) thiophosphorictriamide

Introduction

Urease and urea are the molecules distinct in the development of Chemistry and Biochemistry. The enzyme urease (urea amidohydrolase; E.C.3.5.1.5) is known to hydrolyze urea to ammonia and carbon dioxide at a rate approximately 10^{14} times the rate of the uncatalysed reaction. It has played a vital role in the development of enzymology and heme protein chemistry. Medically, bacterial ureases are important virulence factors, which are implicated in the pathogenesis of many clinical conditions such as pyelonephritis, hepatic coma, peptic ulceration, and formation of infection-induced urinary stones. Urease inhibitors dissolve crystals and struvite kidney stones and prevent the formation of new crystals in the urine. In agriculture, high urease activity during urea fertilization causes significant environmental and economical problems by releasing abnormally large amounts of ammonia into the atmosphere. This induces plant damage primarily by depriving plants of essential nutrients and secondly through ammonia toxicity. Urea is the most frequently applied nitrogen fertilizer in agriculture[1], which accounts for about 46% of the total world nitrogen fertilizer consumption[2]. Due to action of enzyme urease, urea nitrogen is lost as volatile ammonia. For efficient use of fertilizer nitrogen, urease inhibitor along with the urea fertilizer is one of the available options. Urease inhibitors also provide an insight in understanding the mechanism of enzyme catalyzed reaction, the role of various amino acids in catalytic activity present at the active site of enzyme and the importance of nickel to this metalloenzyme.

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Inhibition Studies of Urease

The inhibitions of urease were extensively studied because of their potential uses like: (i) Therapy against bacterial urease (eg: *Helicobacter pylori*) that induced human pathogenic states, such as, urinary stone formation, peptic ulcer, pyelonephritis and hepatic coma[3], (ii) to protect soil from *p*H elevation and loss of nitrogen after use of urea fertilizer by controlling hydrolysis of urea in soil, and (iii) as an analytical technique for determining substances acting as enzyme inhibitor. Urease inhibitors can be broadly classified into two categories: (i) Substrate structural analogs (hydroxyurea and hydroxamic acid). (ii) Inhibitors that affect the mechanism of reaction (phosphodiamidates). By chemical structure, urease inhibitors can be divided into four major groups. The first group is formed by thiolic compounds, since thiolate anions react directly with the metallocenter of urease. The second group is of hydroxamic acid and its derivatives. Inhibitors of this group compete with urea for binding with the urease active site. The third group is the most effective inhibitors, which include substituted phosphorodiamidates. The fourth group consists of ligands and chelators of nickel, the most notable of which is fluoride ion and certain peptides that exhibit a moderate inhibiting activity ($Ki=3.0\text{-}4.7\times 10^{-5} M$)[4].

Class of Inhibitors Studied

Heavy Metal Inhibition Reports

Urease is found highly sensitive to trace quantities of heavy metals. Reports are available on urease

inhibition by alkali metals[5-7]. Ambrose and coworkers[7] have reported inhibition of jack bean urease by silver ion. Shaw[5] proved that these ions reacted with the sulfhydryl groups on the active site of the enzyme in a manner similar to the formation of metal sulfides. Thus, insoluble sulfides forming metals were reported to be the strongest inhibitors. $Ag^{+1}$and Hg^{2+} inactivated urease completely in the range of 10^{-6}*M*. Metals like Mn^{2+}, Pd^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}were also reported to inhibit urease. The reported series of relative inhibitory efficiency of metal ions was: Ag^{+1} ~ Hg^{2+}> Cu^{2+}> Cd^{2+}> Ni^{2+}> Co^{2+}> Fe^{2+}> Mn^{2+}. Inhibition of urease by heavy metal ions was reported to be non-competitive[6]. Watermelon urease was reported to be inhibited by heavy metal ions with an order of effectiveness of tested metal ion as Cu^{2+}> Pb^{2+}> Ni^{2+}> Co^{2+}. However, the order of inhibition for pumpkin urease[8] was found to be Hg^{2+}> Cu^{2+}> Cd^{2+}> Co^{2+}.

Inhibition of Urease by hydroximate and Its Derivative

Preliminary studies by Fishbein and Carbone[9] on jackbean and bacterial urease have shown hydroxamic acid as a specific, potent and non-competitive inhibitor. Later, hydroxamic acid and its derivatives were reported to be reversible, slow binding inhibitors of both plant and bacterial ureases[10-12]. Acetohydroxamic acid (AHA) was the most widely exploited inhibitor in this class.

AHA binding mode on *Bacillus pasteurii* urease was shown by Benini[13] employing X-ray studies of AHA inhibited crystal at 1.55 Å resolutions. Mishra[11] did a comparative molecular field analysis study on dipeptide hydroxamic acid inhibiting *Helicobacter pylori* urease, which provided an insight in the relationship of three dimensional structure changes and its activity. Prakash and Upadhyay[14] have reported AHA to be an uncompetitive inhibitor of watermelon urease with a Ki value of 2.5 m*M*.

The jack bean urease inhibition of hydroxamic acid was reported to be competitive and the –CONHOH residues of the inhibitor were found to be necessary for inhibition[15]. Kinetic and equilibrium data have established that hydroxamic acid compete for the nickel ion. Acetohydroxamic acids were found to be competitive inhibitor of *Klebsiella aerogenes*[16]. *Anthrobactermobilis* was also known to be inhibited by AHA[17]. Manunza *et al*[18] have reported that hydroxamic acid acted as a monodentate ligand, which bound to the nickel atom in pseudo-tetrahedral coordination geometry with the carbonyl atom in the way as urea binds.

Alkyl hydroxamic acid was tested as an inhibitor for urease in male CAF-1 mice against condition of hepatic coma. The log-dose response curve for inhibition during the first 2 h after intraperitoneal administration was linear over a 20-fold dose range [0.16 gm/kg producing 50% inhibition (ED_{50})]. The half-time for the decline of inhibition was 7 and 11 h for a drug dose of 0.25 gm/kg and 1.0 gm/kg, respectively. Subcutaneous and oral administration of the compound resulted in higher levels of inhibition for longer time periods (half-times being 22 and 17.4 h, respectively for 0.25 gm/kg dose). Oral administration of ED_{50} was being expected to produce cumulative urease inhibition within 3-4 d. The relation of this pharmacologic data to the *in vitro* enzymatic studies of urease inhibition and to the potential clinical usefulness of this compound in the treatment of hepatic coma was reported[19].

Umamaheswari and coworkers[20,21] used AHA as a target drug against *H. pylori* infection. Lipobeads of phosphatidyl ethanolamine liposome anchored polyvinyl alcohol xerobeads and polycarbonated floating microsphere was used as drug carrier system. The growth inhibition studies from gastrointestinal tract have suggested that lipobeads show significant higher inhibitory efficiency and, thus, could be a potent target drug delivery system.

By virtue of urease activity, urease producing species, e.g., *Proteus mirabilis*, increases the urinary *p*H and causes crystallization of calcium and magnesium phosphates in the biofilm within a polysaccharide matrix. This results into encrustation and blockage of indwelling urethral catheters. Morris and Stickler[22] investigated the ability of urease inhibitors to control encrustation. According to their observation, AHA (1.0 mg/mL) and fluorofamide (1.0 μg/mL) restricted the increase of *p*H of *P. mirabilis* infected urine from 9.1 to 7.6. Electron microscopy confirmed significant reductions indeposition of calcium and magnesium salts on silicone catheters in the presence of the urease inhibitors[22].

Philips and coworkers[23] investigated bacteriostatic and bactericidal effects of AHA on *H. pylori* and determined the interaction between AHA and antimicrobial drugs used to treat *H. pylori*. In eight isolates studied by them, the minimum inhibitory concentration (MIC) was found to be 200 mg/L or 400 mg/L. AHA reduced the MIC for colloidal bismuth subcitrate (CBS), tetracycline, metronidazole, and amoxicillin. *In vitro* AHA was active against

H. pylori and interacted with other agents directed against *H. pylori*.

Fluoride Induced Inhibition of Urease

In 1943, fluoride was for the first time demonstrated the inhibitor of bovine rumen urease[24]. Later, it was reported to be a competitive inhibitor of jack bean (Ki=1m*M* at *p*H 7) and non-competitive inhibitor of watermelon urease[25]. Fluoride inhibition has been reported to be time dependent and yield steady state values that are consistent with uncompetitive inhibition[26,27]. It has also been reported to be pseudo-uncompetitive, slow binding inhibitor of *K. aerogenes* urease[28] in the presence and absence of substrate. Interestingly, this inhibition was *p*H dependent but fluoride binding was *p*H independent. Todd and Hausinger[28] proposed that fluoride inhibition involves replacement of water binding site on catalytic site by fluoride ion. Fluoride prevented alkali production from urea by oral bacteria through direct or indirect mechanism. Ureolysis by cells in suspension or mono-organism biofilms of *Staphylococcus epidermidis, Streptococcus salivarius or Actinomycesnaeslundii* was inhibited by fluoride at plaque levels in a *p*H dependent manner[29].

Thiols as Urease Inhibitor

Thiol compounds have been reported as competitive inhibitors of the urease enzyme. Thiols having a positively charged β-amino group, *e.g.*, cysteamine, are the potent inhibitor (Ki=5.0 μ*M*), while thiol compounds containing an anionic carboxyl group, *e.g.*, cysteine and 3-mercaptopropionate, are poor inhibitors (Ki=0.5 and 9.6 m*M*, respectively)[10]. The disadvantage of thiols as an inhibitor is the non-specificity of their action.

Urease Inhibited by Phosphoramide Compounds

Many phosphoramide compounds have been reported as potent inhibitors of urease. Effective inhibition begins from simple compounds like phosphoramidate and diamidophosphate to the substituted phenyl phosphorodiamidates as well as n-acyl phosphoric triamides[30]. Jack bean urease was reported to be inhibited reversibly by phosphoramidate (Ki=1.9m*M*, and a dissociation rate of 8.4×10^{-4}/sec)[26]. N-acyl phosphoric triamides inhibition of jack bean urease and phenyl phosphorodiamidates inhibition of *K. aerogenes urease* showed similar rate constant[10]. Phenyl phosphorodiamidates were found to be slow and tight binding inhibitor with higher affinity for plant than for microbial urease[31].

Phosphoric monoamide and phenyl phosphorodiamidates inhibited urease by forming a chelated complex with the nickel ion in the active site of enzyme[32]. Although no product analysis has been presented in the jack bean urease study. Andrews and colleagues[33] proposed a mechanism in which the diamidophosphate acts as urease inhibitor by coordinating itself to the nickel ions of active site. The parallel nickel bridging mechanism for phenyl phosphorodiamidates was also proposed in case of *K. aerogenes*[10], whereas *B. pasteurii* inhibition by diamidophosphate involves inhibitor oxygen binding to nickel-1 and an amide coordinates to nickel-2, mimicking the substrate-bounded structure[13,30].

Hydroxyurea—An Inhibitor or A Substrate

Hydroxyurea, an antineoplastic agent, has been known to inhibit various ureases of plant and microbial origin, including jack bean and *H. pylori*. Recently, N-substituted hydroxylurea was reported as a new urease inhibitor[34]. Of sixteen derivative of hydroxyurea tested, m-methyl and m-methoxy-phenyl substituted hydroxyurea were the most potent inhibitors. The inhibitory effect of these compounds was observed on jack bean urease and results indicated that they were more potent inhibitor compared to hydroxyurea.

α-Hydroxyketones and α-Diketones—Inhibiting Urease Activity

A variety of α-hydroxyketones and α-diketones were evaluated for their effect on jack bean urease[35]. Among the thirteen α-hydroxyketones tested, 2,2`-thenoin (IC_{50}=0.18 m*M*), furion (IC_{50}=0.36 m*M*), 2-hydroxy-1-phenylethanone (IC_{50}=0.47 m*M*) and acetol (IC_{50}=2.9 m*M*) exhibited potent inhibitory activity against urease. The inhibition depends upon the absence of sulfhydryl compounds and structural feature of individual hydroxyketone. The inhibition was possibly due to the binding of inhibitor to the cysteinyl residues in the active site. But the mechanism of α-hydroxyketones inhibition is yet to be explored[35].

Triketone Oximes—Another Class of Urease Inhibitor

Tarun *et al*[36] reported inhibition of soyabean urease by fifteen triketoneoximes at 36°C in aqueous solution (*p*H 4.95). The studied oximes were found to be chelators for the nickel atom. They compete with the urea for binding with one or two nickel atoms and, thus, block the urea hydrolysis, partially or completely. Inhibition constants varied in the range of

2.7-243.0 μ*M* depending on the oxime structure; for optimal inhibition, carbonyl group at position one, alkyl residue or -NOC_2H_5 group at position two and -$COOCH_3$ at position four of the cycle is preferred[36]. Urease inhibition by oximes of cyclic β-triketones (Ki=2.7-16.2 μ*M*) was also reported. The inhibition efficiency of cyclic β-triketones and triketoneoximes is determined not only by structure but also by the three dimensional (steric) factors[37].

Phosphate Too Inhibits Urease

Competitive inhibition of jack bean urease by phosphate was reported in 1949[38]. Phosphate competitively inhibited *K. aerogenes* urease in a *p*H dependent manner, significantly at low *p*H values[10]. However, the *p*H dependence of inhibition was never reported for plant urease.

Urease Inhibition in Presence of Boric Acid

Boric and boronic acids have been reported as simple competitive inhibitors of *P. mirabilis* (Ki=0.1 m*M*), jack bean (Ki=0.19 m*M*) and *K. Aerogenes* (Ki=0.33 m*M*) urease[10,39,40]. Phosphate buffer influenced the inhibition of boric acid in a *p*H and molarity dependent manner; inhibition constant of the system changed from 0.23 m*M* in 22m*M* phosphate buffer to 0.76 m*M* in 155 m*M* phosphate buffer[41]. Furthermore, the inhibition of urease by boric acid was maximal at acidic *p*H (5.0) and minimal at alkaline *p*H (10.0)[42].

Nature Creates Inhibitors of Urease

A natural inhibitor of urease was isolated from musk melon by Malhotra and Rani[43] in 1978. It specifically inhibited pegion pea and jack bean ureases, non-competitively with almost equal Ki value (0.45 and 0.64 mg per mL, respectively). Another study employed jack bean urease-garlic extract-urea as a model system. Inhibition of urease by garlic extract was reported to be irreversible and incubation time-dependent with biphasic kinetics, where each phase obeyed first-order kinetics. The biologically active component of fresh garlic extract responsible for the inhibition was established to be Allicin-diallylthiosulfinate. Thiol reagents (L-cysteine, 2-mercaptoethanol, glutathione, dithiothreithol) strongly protected the enzyme from the inhibitory effect of garlic extract, while urea and boric acid showed weaker protection. Garlic extract-modified urease could be reactivated with dithiothreitol[44].

Sulfur Compound As Urease Inhibitor

Urease is usually classed as a sulfhydryl enzyme and agents those oxidize the sulfhydryl groups are known to inhibit it. Sulfur compounds were reported to be inhibitor of urease by Ambrose *et al*[45] in 1950. Three sulfur compound investigated by them were sodium sulfate, sodium benezensulfinate and sodium benezen sulfonate. Among them, sulfinate was found to be the stronger inhibitor than sulfite, followed by sulfonate and sulfate.

Inhibitor Influencing Active Site Critical Amino Acid Residues

Reports are available for the presence of cysteine and histidine residues on the active site of plant and microbial urease. Inhibition studies using specific inhibitor of cysteine and histidine residues have been carried out by many workers. These studies have given an insight in the involvement, number and essentiality of these groups in urease catalyzed reaction as well as the active site conformation[46-51].

Iodoacetic acid and N-ethylmaleimide are highly specific cysteine reagents, while p-hydroxymercuribenzoate is not absolutely specific but may attach non-covalently to other site as well[52]. Mahadevan and coworkers[53] have reported that bovine rumen urease was completely inhibited by n-ethylmaleimide at 0.1 m*M*, indicating the essentiality of cysteine residue in the enzyme activity. Similar reports are also available for jack bean urease[54]. Prakash and Bhushan[50] have also reported inhibition of watermelon urease by iodoacetic acid, n-ethylmaleimide and p-hydroxymercuribenzoate. The time dependent inhibition of watermelon urease exhibited biphasic kinetics (a fast and a slow phase), in which each phase exhibited first order kinetic and the order of effectiveness as inhibitors was reported as p-hydroxymercuribenzoate>n-ethylmaleimide>iodoacetic acid[50]. Similarly, p-chloromercuric benzoate was also reported to inhibit urease from *B. ammoniagenes* irreversibly[55].

A New Class of Urease Inhibitor—Biscoumarin

Recently a new class of urease inhibitor, biscoumarin was reported by Khan *et al*[56]. Biscoumarin are naturally occurring compounds but can also be synthesized. A variety of biological activities, such as, anticoagulant, mulloscicid, antianthelmintic, hypnotic and insecticidal are associated with biscoumarin[57]. A variety of biscoumarins (21) with variable substituents were synthesized by Khan and coworkers[56] and their jack bean urease inhibitory activity was determined. The synthesized compounds showed varying degree of

urease inhibitory activity; K_i values ranged from 15.06-91.35 μ*M*. Compound such as 3,3`-methylenebis-4-hydroxycoumarin was found to be the most potent one with IC_{50}=15.01 μ*M*. The size and electron donating or withdrawing effects of substituents influenced the activity, which led to the urease inhibition.

Inhibitory Effect of Organic Solvents

Urease was inactivated by some aqueous-organic interfaces like water with butanol, isopropylether, n-hexane or n-tridecane. Urease was slightly inactivated by 2-octanone and n-butlybenzene. However, the presence of oxygen increased the rate of interfacial inactivation[58]. In more polar solvents, such as, N-methyformamide and N,N-dimethylformamide, urease lost catalytic activity due to the interaction with the solvent molecules. On the other hand, in apolar solvent like cyclohexane and toluene at higher temperature (70°C), urease was found active[59].

Other Inhibition Studies of Urease

In addition to inhibitors discussed above, some other substances have also been reported to act as an inhibitor of urease, which includes buffers[60], excess urea[61-63], benzoquinone (BQ)[64], pyrocatechol[65-66], and some others inhibitors[67-74]. Table 1 summarizes such diverse inhibitors.

Inhibition Mechanism

Hexagonal crystals of urease were obtained by vapour diffusion (293 K, 20 m*M* Tris-HCl, neutral *p*H, 50 m*M* Na_2SO_3) from *B. pasteurii*. Isomorphous crystals of urease inhibited with ß-mercaptoethanol

Table 1—List of some other inhibitors reported to inhibit urease activity

Inhibitor	Study	Reference
Buffers	Product inhibition was competitive in citrate buffer and noncompetitive in phosphate buffer.	60
Excess urea	Jack bean, *Cajanus indicus* and watermelon urease were inhibited by high concentrations of urea.	61-63
1,4-Benzoquinone (BQ) and 2,5-dimethyl-1,4-benzoquinone (DMBO)	Inhibitors of jack bean, reaction progress curve indicated time dependent slow inhibition. Inhibition constants were found to be 5.1 m*M* for BQ and 0.98 m*M* for DMBQ; inhibition involved sulfhydryl groups of enzyme. BQ was proved to be a strong inhibitor than DMBQ.	64
Pyrocatechol	Promising inhibitor of soil urease. It was a time and concentration dependent irreversible inhibitor, which showed non-pseudo-first order reaction. Thiol-compounds protected pyrocatechol inactivation.	65,66
Triacontanyl palmitate	Inhibited jack bean and *Bacillus pasteurii* ureases. It was a non-competitive inhibitor; K_i values were found to be 60.03±1.72 and 88.23±0.31 μ*M* against jack bean and *B. pasteurii*ureases, respectively.	68
N-(n-Butyl) thiophosphorictriamide and thymol	Controlled the coilform bacteria, odour production and ammonia loss from cattle waste. It had the potential to reduce odour in cattle manure and could increase the fertilizer value.	69
Hydroquinone (HQ), phenyl phosphorodiamidate (PPD) and N-(n-butyl) thiophosphorictriamide (NBPT)	All three inhibitors showed mixed inhibition on soil urease. Kinetic characteristic of soil urease under normal moisture and waterlogged conditions under the influence of inhibitor was tested; results indicated the presence of inhibitor and controlled soil moisture could feasibly increase fertilizer N use efficiency. Thus, it would be helpful in agricultural management practices. Among test inhibitors, PPD and NBPT were more effective in influencing the kinetic and thermodynamic behaviors of urease in black soil.	70,71
Barbituric and thiobarbituric acid	Inhibitor of jack bean urease. Coordinating sites, such as, O=C-C=C-N present in these compounds played crucial role in anti-urease activity. These compounds could add value to urease inhibitor based drugs.	72
Triazoles	Akhtar and coworkers had synthesized 3-substitute-4amino-5-thioxo-1H,4H-1,2,4-triazoles and tested their anti-urease activity using thiourea as standard urease inhibitor.	73
Ethanol and methanol extract from different medicinal plant	Anti-urease activity of eleven ethanol and methanol extract from different medicinal plant was investigated against stomach infection associated with pathogenic strains of *Helicobacter pylori.* Extracts of *Taraxacum officinale, Achillea millefolium, Aristolochia bracteata, Eucalyptus globulus, Adhatoda zeylanica, Cuscuta reflexa* and *Mentha longifolia* were stronger inhibitor with IC_{50} values of 33.33, 94.24, 68.62, 66.91, 83.33, 89.19 and 57.47 μg/5 mL, respectively.	74

were also obtained employing 4 m*M* inhibitor in the enzyme solution. Crystals of the native and inhibited enzyme were diffracted respectively to 2.00Å (96.7% completeness) and to 1.65 Å (98.7% completeness) using synchrotron X-ray cryogenic (100 K) conditions[67]. The differaction studies revealed that, in native enzyme, the coordination sphere of each of the two nickel ions was completed by a water molecule and a bridging hydroxide. The enzyme crystallized in the presence of phenylphosphorodiamidate contained the tetrahedral transition-state analogue diamidophosphoric acid bound to the two nickel ions in an unprecedented mode[13]. Both the studies signify the important role played by the nickel ions in catalytic activity of urease. Addition to this, it also provide an insight into the mechanism of enzymatic urea hydrolysis and the mode of binding of the inhibitor to enzyme.

Conclusion

The urease inhibition studies done till date are many but only some of them have given promising results in controlling the loss of soil nitrogen in the form of ammonia. The best studied inhibitor is NBPT (commonly available as agrotain). Incorporation of urease inhibitor is recommended as a preferable management practice for efficient use of urea fertilizer, reduce nitrate runoff and release of ammonia and green house gases. In pharmaceutical applications, only acetohydoxamic acid and its derivates have gained the attention to be used as a potent drug element till date. Still there is a need and scope to explore extensively the potential of other inhibitors in agricultural and pharmaceutical scenario. There is also a need for synthesis of potent urease inhibitors and screening of nature urease inhibitors from biotic factors.

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