Proteus mirabilis urease

Partial purification and inhibition by boric acid and boronic acids

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Urease was purified 800-fold and partially characterized from *Proteus mirabilis*, the predominant micro-organism associated with urinary stones. Boric acid is a rapid reversible competitive inhibitor of urease. The pH-dependence of inhibition exhibited pK_a values of 6.25 and 9.3, where the latter value is probably due to the inherent pK_a of boric acid. Three boronic acids also were shown to inhibit urease competitively.

INTRODUCTION

The enzyme urease catalyses urea hydrolysis to yield NH_3 and CO_2 [1]. Ureolytic microbial infections of the urinary tract result in elevated pH, which in turn can lead to deposition of urinary salts known as stones [2]. It has been estimated that 20–40% of urinary stones arise as a result of infection by ureolytic micro-organisms [3]. The most common micro-organism associated with urinary stone deposits is *Proteus mirabilis* [3], yet the only description of urease purification from this microbe is an abstract reporting 200-fold partial purification [4]. In contrast, the present paper provides a detailed procedure for routine 800-fold purification of *P. mirabilis* urease.

Urease inhibitors have been proposed as a potentially effective method to combat urease-induced stone formation [3]. Two major classes of urease inhibitors have been investigated, namely hydroxamic acids and phosphoramidate compounds [3]. For example, acetohydroxamic acid prevented alkalinization of urine in rats with *P. mirabilis* urinary-tract infections [5]. Alternatively, fluorofamide [N-(diaminophosphinyl)-4-fluorobenzene] was reported to be 1000-fold more potent than acetohydroxamic acid against the *P. mirabilis* enzyme [6]. In addition to their potential pharmaceutical value, urease inhibitors also can provide insight into the detailed mechanism of catalysis. Here we describe the effect of an unexplored class of urease inhibitors, namely boric acid and boronic acids, on *P. mirabilis* urease.

EXPERIMENTAL METHODS

Bacterial growth conditions

Proteus mirabilis (A.T.C.C. 29906) was cultured at 37 °C in a minimal medium containing 5 g of glucose, 2.54 g of NaH_2PO_4 , 1.5 g of KH_2PO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $CaCl_2$, 10 mg of nicotinic acid, 1.2 mg of ferric ammonium citrate, 2.5 ml of trace mineral solution and 1.2 g of urea (filter-sterilized) per litre. The trace mineral solution contained, per 100 ml, 20 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of $NiCl_2 \cdot 6H_2O$, 5 mg of $CuSO_4 \cdot 5H_2O$, 2 mg of $AlK(SO_4)_2 \cdot 12H_2O$ and 1 ml of H_2SO_4 . Large-scale aerobic batch culture was performed by using a New Brunswick 25L Microferm fermenter with

rapid agitation. The culture was grown to an absorbance (600 nm) of at least 1.5 and harvested by using a Pellicon cassette-system concentrator (Millipore). The cells (85–150 g wet wt. per 22 litres) were washed with PEB buffer (20 mM-potassium phosphate/1 mM-EDTA/1 mM-2-mercaptoethanol buffer, pH 7.0) and either used immediately or stored at –20 °C.

Assays

Urease was assayed by measuring the rate of release of NH_3 from urea. The released NH_3 was converted into indophenol, whose absorbance was monitored at 625 nm [7]. The urease specific activity was defined as μmol of urea hydrolysed/min per mg at 37 °C in 25 mM-Hepes/0.5 mM-EDTA buffer, pH 7.5, containing 50 mM-urea. Protein was assayed as described by Lowry *et al.* [8], with bovine serum albumin as the standard. All u.v.-visible absorbance determinations were made by using a Gilford Response spectrophotometer.

Enzyme purification

P. mirabilis urease enzyme was highly purified by slight modifications of the methods described for urease isolations from *Klebsiella aerogenes* [9] and *Selenomonas ruminantium* [10]. Cells (approx. 100 g wet wt.) were suspended in an equal volume of PEB buffer containing 1.0 mM-toluenesulphonyl fluoride and disrupted by two passages through a French pressure cell at 125 MPa (18000 lbf/in²). Streptomycin sulphate was added (final concentration 1.5%) and the suspension was stirred on ice for 30 min. Cellular debris was removed by centrifugation at 100000 g for 60 min at 4 °C. Urease was enriched from the soluble cell extracts by chromatography on a series of resins manufactured by Pharmacia. Extracts were applied to a column (2.5 cm × 20 cm) of DEAE-Sepharose, and a 400 ml linear gradient from 0 M- to 0.5 M-KCl in PEB buffer was used to recover urease as a single peak of activity at approx. 0.2 M-KCl. Fractions containing peak urease activity were adjusted to 1 M-KCl and chromatographed on a column (2.5 cm × 15 cm) of phenyl-Sepharose by using step elution. The column was washed with 100 ml of 1 M-KCl in PEB buffer and activity was eluted with 100 ml of PEB buffer containing 10% (v/v) dimethyl sulphoxide.

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The active fractions were pooled, dialysed and chromatographed on a Mono Q HR 10/10 column by eluting with a 100 ml linear gradient from 0 M- to 0.5 M-KCl in PEB followed by a 10 ml linear gradient to 1 M-KCl in PEB. Two pools of activity were obtained, namely a highly enriched fraction eluted at 0.15 M-KCl and a smaller, lower-specific-activity, fraction eluted at 0.3 M-KCl. The fractions containing the major enzyme peak were pooled, adjusted to 2 M-KCl and chromatographed on a phenyl-Superose HR 5/5 column. A 40 ml linear gradient from 2.0 M- to 0.0 M-KCl in PEB buffer was used to recover urease activity at approx. 0.8 M-KCl.

pH-stability studies

The enzyme pH-stability was assessed by incubating the enzyme in various buffers overnight at 0 °C and at 37 °C and assaying the activity remaining under standard pH conditions. The test buffers included acetic acid (pH 3.5–5.5), Mes (pH 5.0–7.0), Hepes (pH 6.5–9.0), Ches (2-cyclohexylaminoethanesulphonic acid) (pH 8.5–10.5) and Caps (3-cyclohexylaminopropanesulphonic acid) (pH 9.5–11.5), all containing 50 mM buffer and 1 mM-EDTA.

Kinetic analysis

The effects of pH on K_m and V_{max} were established by assaying urease activity in buffers containing 25 mM buffer, 0.5 mM-EDTA and 5–100 mM-urea at the indicated pH values. Similarly, the effects on K_m and V_{max} were established for inhibitors at several inhibitor concentrations. Under all conditions described in the present paper the rate of urea hydrolysis was linear with time. The data were analysed by the method of Wilkinson [11].

RESULTS AND DISCUSSION

Urease purification

Partial purification of *P. mirabilis* urease is summarized in Table 1. The enzyme was enriched 800-fold with an overall recovery of 26%. *P. mirabilis* urease was estimated to be approx. 50% homogeneous at this stage of purification on the basis of gel-electrophoresis analysis, e.g. only about half of the protein detected on native polyacrylamide gels was associated with urease activity by using a modification of the urease staining procedure of Blattler *et al.* [12]. Furthermore, three *P. mirabilis* urease polypeptides, which are presumably analogous to

the three *K. aerogenes* urease subunits, accounted for half of the protein staining intensity on SDS/polyacrylamide gels [9]. Further isolation efforts were inconsistent from preparation to preparation and are not described here. The routine protocol detailed above provides highly enriched enzyme with a final specific

Fig. 1. Effect of pH on *P. mirabilis* urease catalysis

The V_{max} and K_m values for urea hydrolysis were calculated over a range of pH values. $\log V_{max}$ and $\log(V_{max}/K_m)$, in arbitrary units, and $-\log K_m$, in mM concentration units, were each plotted as a function of pH. The buffers included acetate (●), Mes (○), Hepes (■), Ches (▲) and Caps (□).

Table 1. Summary of the purification of *Proteus mirabilis* urease

Cells were disrupted by using a French pressure apparatus, and debris was removed by ultracentrifugation after addition of streptomycin sulphate. Purification details are provided in the text.

Step	Specific activity (μ mol of urea/min per mg)	Total activity (μ mol of urea/min)	Purification (fold)	Yield (%)
Cell extracts	2.57	8450		100
DEAE-Sepharose	8.20	7150	3.2	85
Phenyl-Sepharose	18.6	6120	7.2	72
Mono Q	78.6	2420	30.6	29
Phenyl-Superose	2057	2160	800	26

Fig. 2. Inhibition of *P. mirabilis* urease by boric acid

Urease activity was determined at urea concentrations of 5, 10, 20, 40 and 100 mM, and at boric acid concentrations of 0.0 (●), 0.1 (○), 0.2 (■), 0.3 (□), 0.4 (▲) and 0.5 mM (△) under standard conditions described in the text. The Lineweaver-Burk plot shows inverse velocity in arbitrary units and inverse substrate concentration as mM^{-1} . The inset is a replot of the slopes versus the inhibitor mM concentration.

activity of over 2000 μmol of urea/min per mg and represents a significant improvement over the only other *P. mirabilis* urease purification reported (680–800 μmol /min per mg), for which no details were provided [4]. Purified urease exhibited a K_m for urea of 13 ± 3 mM, consistent with the value of 10 mM reported by others [4].

pH studies

P. mirabilis urease was stable over the pH range 7–10 when incubated for 24 h at 0 °C. At 37 °C long-term stability was observed over the narrower pH range 8–9. The enzyme activity was assayed under uniform pH 7.5 conditions in these experiments. The pH-dependence of V_{max} , V_{max}/K_m and K_m is depicted in Fig. 1. The data suggest that two groups are required for activity. One group, which must be deprotonated for activity, possesses a $\text{p}K_a$ of about 6.25 both in the free enzyme and in the enzyme-substrate complex. A second group must be protonated for activity and possesses a $\text{p}K_a$ of 9.2–9.4 in the free enzyme and the enzyme-substrate complex.

Boric acid inhibition

As shown in Fig. 2, boric acid is a competitive inhibitor of *P. mirabilis* urease and exhibits a K_i of 0.1 mM. This inhibition was rapid and readily reversible by dialysis. To define more clearly the mechanism of boric acid inhibition of urease, the K_i value was determined over a range of pH values. As shown in Fig. 3, boric acid inhibition was associated with two $\text{p}K_a$ values of approx. 6.2 and 9.3. These values correspond closely to the uninhibited $\text{p}K_a$ values observed in the $\log V_{\text{max}}$ or $\log(V_{\text{max}}/K_m)$ plots (Fig. 1). However, boric acid has a $\text{p}K_a$ of approx. 9 [13], which may give rise to the observed increase in K_i at high pH. These results

Fig. 3. pH-dependence of boric acid inhibition of *P. mirabilis* urease

The urease inhibition constant for boric acid, in mM concentration units, was determined over a range of pH values and plotted as $-\log K_i$. Buffers included Mes (○), Hepes (■), Ches (▲) and Caps (△).

Table 2. Studies on the inhibition of *P. mirabilis* urease

The competitive inhibition constant was determined for each inhibitor by using standard assay conditions as described in the text. 4-Bromobenzenboronic acid and butaneboronic acid were obtained from Aldrich Chemical Co., and benzenboronic acid was from ICN Biomedicals.

Compound	K_i (mM)
Boric acid	0.099 ± 0.008
4-Bromobenzenboronic acid	0.124 ± 0.048
Benzenboronic acid	1.26 ± 0.32
Butaneboronic acid	0.547 ± 0.069

can be interpreted to suggest that only trigonal $\text{B}(\text{OH})_3$ is an inhibitor of urease, and not the $\text{B}(\text{OH})_4^-$ anion. A similar pattern for borate inhibition was reported for *Streptomyces griseus* proteinase 3 [13]. In that case trigonal borate competed with substrate when an active-site group was deprotonated ($\text{p}K_a$ 6.6), whereas ionized borate was inactive as an inhibitor.

Boronic acid studies

The demonstration of boric acid inhibition of urease raised the possibility that boronic acids may also inhibit the enzyme. Three boronic acids were tested as urease inhibitors, and each was shown to exhibit competitive inhibition (Table 2). Boronic acids have been investigated by others as competitive inhibitors in a variety of serine hydrolases, including chymotrypsin [14–16], subtilisin [16,17], acetylcholinesterase [18], pig pancreatic lipase [19], elastase [20], cathepsin G [20], cholesterol esterase [21] and lipoprotein lipase [21]. Two examples, subtilisin and chymotrypsin, were examined by crystallographic methods [22,23]; inhibitory boronic acids were shown to be bound to the active-site serine residue, apparently isosteric with the substrate transition state. In contrast with these enzymes, *P. mirabilis* urease does not appear to be a serine hydrolase, on the basis of the ineffectiveness of typical serine-proteinase inhibitors. Rather, this enzyme is likely to possess a bi-nickel active site, as in the

case of jack-bean [1] and other microbial ureases [24]. At least one other metallohydrolase, the zinc-containing *Aeromonas* aminopeptidase, also has been shown to be inhibited by boronate compounds [25,26]. The detailed mechanism of bacterial urease inhibition by boric acid and boronic acids is not established, but it is likely to involve the metallocentre. In analogy with serine hydrolases, the inhibitors may form a reversible covalent bond with a urease active-site group. Alternatively, the boronate group may form a metal chelate at the ligand position normally occupied by substrate. In addition to their potential pharmaceutical value in minimizing urinary stone formation, boric acid and boronic acids may provide useful probes for the study of the active site of urease and other hydrolytic enzymes.

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