Isolation and characterization of urease from *Aspergillus niger*

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Urease was purified (4126-fold) from *Aspergillus niger* (NRRL 003) to a homologous enzyme preparation with a specific activity of 1341 μmol min⁻¹ (mg protein)⁻¹. One species of urease was detected in *A. niger*, with $K_m = 3.0 \text{ mm}$, native molecular mass 250 000 Da, pH optimum of 8.0 and a high specificity for urea. Hydroxyurea was a strong competitive inhibitor of urease activity, while N-methylurea acted as a weak uncompetitive inhibitor, based on Lineweaver–Burk and Eadie–Hofstee plots. The activity of urease was enhanced by, but not dependent on, the presence of Na,EDTA, DL-dithiothreitol (< 0.1 to 5.0 mm), Ca²⁺, Ba²⁺ and citrate (2 to 20 mm). Urease activity was not affected by Na⁺, K⁺, Cl⁻, Br⁻, acetate or nitrate (2 to 20 mm), but was significantly decreased in the presence of Li⁺, Ni²⁺, Mg²⁺, Zn²⁺ or I⁻. Urease activity decreased 26.0% after 30 min at 65 °C, and 86.5% and 100.0% after 5 and 1 min at 80 and 100 °C, respectively. Urease activity decreased 30.5% after 90 d at 4 °C and 21.0% after 28 d at −20 or −80 °C.

**Introduction**

*Aspergillus niger* has been used for the commercial production of citric acid and cellulolytic enzymes (Friedrich et al., 1989; Gokhale et al., 1991; Lee et al., 1989; Roukas, 1991). The technology for producing large quantities of *A. niger* mycelia has been established, thus alternative uses of *A. niger* are being investigated.

One enzyme of increasing interest is acid urease (urea amidohydrolase, EC 3.5.1.5). Acid ureases obtained from *Lactobacillus* strains have been used to reduce urea levels in wine, thereby preventing the formation of ethyl carbamate, a carcinogen (Famuyiwa & Ough, 1991; Fujinawa et al., 1990; Kakimoto et al., 1989; Ough & Trioli, 1988). However, *Lactobacillus* spp. yield low quantities of urease, and alternative microbial sources of the enzyme are being sought. Urease activity in *A. niger* was first reported in 1903, yet the characteristics of this enzyme have not been determined (Pomar et al., 1970). This strain of *Aspergillus* could be used for the production of an acid urease, for the fungus grows readily under acidic (wine) conditions. Here, we report the first isolation and characterization of urease from *A. niger* and suggest which steps in the purification strategy may be suitable for industrial applications of the purified enzyme.

**Methods**

**Chemicals.** Materials used in the suspension culture medium were obtained from Difco. Other chemicals, prepared solutions (alkaline hypochlorite and phenol-nitroprusside solutions) and Phenyl Sephadex were obtained from Sigma. DEAE Fast Flow Sepharose and Sephacryl S 300 were from Pharmacia. Macro-Prep Q anion exchange media and BioRad Protein Stain were from BioRad.

*Aspergillus niger* culture. The *A. niger* (NRRL 003) strain used for this experiment was obtained from the USDA culture collection at Peoria, Illinois, USA. Thirty strains of *Aspergillus niger*, *Aspergillus aculeatus* and *Aspergillus ficuum* were screened for the presence of urease. The *Aspergillus* species were grown on maltose extract agar at 25 °C in the dark for 7 d and conidia were harvested with sterile water containing 0.05% Tween 80. The growth media used in the production of *Aspergillus* mycelia contained the following: 20% (w/v) glucose, 1.0% (w/v) yeast extract, 1.0% (w/v) yeast nitrogen base without amino acids, 0.5% citrate, 15% (w/v) NaHPO₄, 0.0032% NiSO₄ · 6H₂O and the pH of the growth media was adjusted to 3.0 with tartaric acid. Filter (0.2 μm) sterilized urea was added after autoclaving to obtain a final urea concentration of 0.02% (or 3 mm). The growth medium (1 litre in a 2.8 l Fernbach flask) was inoculated with a suspension of *Aspergillus* conidia and shaken (250 r.p.m.) at 35 °C for 2 d in an Environ-Shaker (Lab-Line Instruments). *Aspergillus* mycelia were harvested by filtering through four layers of cheesecloth, rinsing thoroughly with water, then immediately freezing at −80 °C. The frozen mycelia were lyophilized, ground to a fine powder in a Wily mill and stored at −80 °C until needed. Based on the levels of urease activity, *Aspergillus niger* (NRRL 003) was selected for further analysis.

**Urease assay.** Urease activity was determined with a modified protocol based on Weatherburn (1967). Urease reactions were done in...
Urease activity was purified 4126-fold from *A. niger* with the isolation scheme presented in Table 1. This isolation strategy recovered 0.55% of the original activity and achieved a final specific activity of 1341 pmol min⁻¹ (mg protein)⁻¹. The specific activity of the final enzyme preparation was twice that of previous purifications for enzymes of *Aspergillus nidulans* and *Aspergillus tamarii* (Creaser & Porter, 1985; Mackay & Pateman, 1980, 1982; Zawada & Sutcliffe, 1981).

The first stage of purification, following extraction of urease from ground lyophilized mycelia, involved a direct application of the crude extract to DEAE column chromatography. Purifications of urease are usually initiated with an acetone, ammonium sulphate or ethanol precipitation (Creaser & Porter, 1985; Evans *et al.*, 1991; Mahadevan *et al.*, 1977; Rai, 1989). Increasing the NaCl concentration to 200 mM in the crude extract prior to DEAE chromatography allowed the retention of all of the urease activity by the column while permitting > 95% of the soluble protein to elute directly through the column. This replaced the initial concentrating or precipitation step. Christians *et al.* (1991) also began a purification of urease from *Staphylococcus xylosus* with an anion exchange column, although the salt concentration of the crude extract was not increased before transferring the sample to the column. Elution of > 80% of the urease activity was achieved with the application
Aspergillus niger urease

Fig. 1. Elution of Aspergillus niger urease activity (Δ) and protein (○) from a Macro-Prep Q Anion Exchange column with increasing KCl concentration (——). Fractions (10 ml) with the highest urease activity were pooled (▲) and retained for further purification.

Table 1. Isolation of urease from Aspergillus niger

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol min⁻¹ mg protein⁻¹)</th>
<th>Purification (fold)</th>
<th>Activity recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1066.1</td>
<td>3278.9</td>
<td>0.325</td>
<td>—</td>
<td>100.00</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
<td>361.4</td>
<td>99.863</td>
<td>3.619</td>
<td>11.1</td>
<td>33.90</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>118.5</td>
<td>12.833</td>
<td>9.234</td>
<td>28.4</td>
<td>11.10</td>
</tr>
<tr>
<td>Macro-Prep Q Anion Exchange</td>
<td>24.2</td>
<td>0.0871</td>
<td>277.8</td>
<td>854.8</td>
<td>2.27</td>
</tr>
<tr>
<td>Sephacryl S 300</td>
<td>5.9</td>
<td>0.0044</td>
<td>1340.9</td>
<td>4125.8</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Values are means of four purifications.

of RB containing 350 mM-NaCl. The enzyme was eluted from the DEAE column in a single activity peak. Therefore, direct application of the crude A. niger extract to a DEAE column could be suitable for industrial purification of urease. When fractions with the highest urease activity were retained, 33.9% of the original urease activity was recovered from the DEAE column, while eliminating > 97% of the soluble protein in the enzyme preparation (Table 1). If the entire urease activity peak was retained from the DEAE column, the pooled fractions contained > 80% of the original activity with < 10% of the initial soluble protein. Additionally, a single DEAE column was used for all purifications experiments in this study and elution of urease activity from the column was reproducible. Therefore, a highly purified urease preparation can be achieved with a single predictable and reproducible step.

When the pooled DEAE fractions were transferred to the Phenyl Sepharose column, all urease activity was retained by the column. Previously, Phenyl Sepharose (or hydrophobic) columns have been used effectively in the purification of urease (Dunn et al., 1991). Washing the column with RB containing 350 mM-NaCl and RB did not elute detectable urease activity. The 50% EG solution eluted > 99% (or 33% of the original activity) of the urease activity bound to the Phenyl Sepharose column. Of this urease activity, 11.1% (of the original activity) was retained for further purification.

The pooled Phenyl Sepharose fractions were dialysed overnight without a significant reduction in the total urease activity or an increase in sample volume. Dialysing the sample against 25 mM-potassium phosphate buffer containing 100 mM-KCl did not prevent the Macro-Prep column from retaining the enzyme (Fig. 1). Washing the Macro-Prep column with RB2 containing 180 mM-KCl, then RB2 containing 220 mM-KCl, eluted > 90% of the total (non-urease) protein bound to the column. The application of an increasing KCl gradient (220 to 500 mM) eluted the enzyme in a single activity peak between 250 and 300 mM KCl.

Reducing the pooled Macro-Prep fractions from 3 ml to 500 μl with the Amicon concentrator did not result in a significant loss of enzyme activity. Concentrated sample was transferred to a S 300 column and eluted in a single enzyme peak. Fractions with the highest urease activity were pooled, and a small portion (200 μl) was prepared for SDS-PAGE. A single protein band of 83 kDa was visualized with Coomassie blue and silver stain on the SDS-PAGE from the pooled S 300 fractions (Fig. 2), thus the enzyme preparation appeared to be homo-
DUCTED WITH VARIING LEVELS OF UREA (0 TO 20 mM) AND THREE 
HYDROXYUREA, AND THIOUREA) CAN BE INHIBITORY (MOBLEY 
UNCOMPETITIVELY (CORNISH-BOWDEN, 1979; HAMMES, 1982) BASED ON A LINEWEAVER–BURK PLOT. UNCOMPETITIVE 
INHIBITION OF UREASE HAS BEEN PREVIOUSLY REPORTED FOR HALOGENS BUT SO FAR, NOT FOR AN ANALOGUE OF UREA (MOBLEY 
ACTED AS AN INHIBITOR, BUT THE MODE OF INHIBITION WAS UNCLEAR.

**ESTIMATION OF NATIVE MOLECULAR MASS.** THE NATIVE 
MOLECULAR MASS OF UREASE ELUTED FROM THE S 300 COLUMN 
WAS ESTIMATED AT 250000, WHICH IS SIMILAR TO PREVIOUS 
FINDINGS (CHRISTIANS ET AL., 1991; CREASER & PORTER, 1985; 
MOBLEY & HAUSINGER, 1989). THE SINGLE PROTEIN BAND OF 
83 kDa IN THE FINAL ENZYME PREPARATION INDICATES THAT THE 
PROTEIN COULD BE COMPOSED OF THREE IDENTICAL SUBUNITS 
(FIG. 2). HOWEVER, IT IS UNCLEAR IF THE 83 kDa BAND 
REPRESENTS THREE MONOMERIC SUBUNITS. UREASES ARE LARGE 
(200 TO 600 kDa) PROTEINS COMPOSED OF SIX OR MORE 
SUBUNITS, VARYING FROM 16 TO 60 kDa, AND ARRANGED IN 
PROTEIN COMPLEXES OF (αβ)₆ OR (αβ)₆ (CHRISTIANS ET AL., 
1991; CREASER & PORTER, 1985; DUNN ET AL., 1991; EVANS 
ALTHOUGH THE ENZYME PREPARATION WAS HOMOGENOUS, 
BASED ON THE SDS-PAGE PROTEIN BANDS VISUALIZED WITH 
SILVER STAIN, THE SUBUNIT MAKEUP OF THE ENZYME IS UNCLEAR.

**ESTIMATION OF pH OPTIMUM.** THE PURIFIED ENZYME 
DEMONSTRATED ACTIVITY OVER A WIDE pH RANGE (5 TO 10) 
WITH AN OPTIMAL pH OF 8.0. THE pH OPTIMUM OF THE 
PURIFIED UREASE WAS DETERMINED WITH CITRATE PHOSPHATE BUFFER (pH 3–7) AND HEPPSO (pH 7–9). THE RANGE AT WHICH A. NIGER UREASE ACTIVITY COULD BE DETECTED AND 
ESTIMATION OF OPTIMAL ACTIVITY IS SIMILAR TO PREVIOUS 
REPORTS OF UREASE ACTIVITY (CHRISTIANS ET AL., 1991; CREASER 
& PORTER, 1985; EVANS ET AL., 1991; MOBLEY & HAUSINGER, 

Acid ureases have been used to remove urea from wine (FAMUYIWA & OUGH, 1991; FUJINAWA ET AL., 1990; 
KAKIMOTO ET AL., 1989; OUGH & TRIOLI, 1988). When purified A. NIGER urease was immobilized on a Phenyl 
Sepharose column, the enzyme reduced urea concentrations in solutions containing ethanol levels (15% v/v) 
higher than wine (Table 2). However, decreasing the pH of the ethanol solution to 3.5 eliminated the urease’s 
ability to metabolize urea. Therefore, the urease from A. niger would not be suited for the commercial scale 
removal of urea from wine. However, if the A. niger urease was immobilized on a matrix, this immobilized 
urease could be used to reduce the levels of urea in non-
Table 2. Activity of immobilized urease

Activity of urease (20 mg) purified from *Aspergillus niger* and immobilized on 10 ml Phenyl Sepharose. Two solutions (pH 4.0 and 7.0) containing 2 mM-urea and 15% (v/v) ethanol were circulated over the column at a flow rate of 10 ml min\(^{-1}\) at 25 °C for 3 d. The pH of the solutions was maintained with 50 mM-sodium acetate (pH 4.0) and 50 mM-Tris/HCl (pH 7.0).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Urea concn (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.0</td>
<td>2.0 2.0</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>2.0 0.3</td>
</tr>
</tbody>
</table>

Table 3. Influence of Na\(_2\)EDTA, DTT, cations, anions, acetate, citrate and nitrate on urease purified from *Aspergillus niger*

The activity of urease in the standard assay mixture was used as a control (100%). Cations were chloride salts, while anions, acetate, citrate and nitrate were sodium salts. Most values have less than 1% variability, based on the mean of six trials from two separate purified enzyme preparations.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (%) at 2 mm</th>
<th>5 mm</th>
<th>20 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(_2)EDTA</td>
<td>130.3 127.5   108.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>129.2 123.0  129.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K(^+)</td>
<td>98.1  91.9   100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li(^+)</td>
<td>88.6  86.5   70.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(^+)</td>
<td>101.2 98.9   98.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br(^-)</td>
<td>126.2 129.4  126.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(^2+)</td>
<td>116.7 117.1  102.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni(^2+)</td>
<td>154  15.0   3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(^2+)</td>
<td>79.5  70.7   70.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn(^2+)</td>
<td>94.1  92.4   82.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br(^-)</td>
<td>100.5 101.8  96.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>101.2 98.9   101.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(^-)</td>
<td>66.5  43.4   59.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>95.9  93.1   103.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>120.6 135.8  132.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
<td>102.3 101.0  100.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

acidic solutions. For example, people experiencing renal distress often have elevated levels of urea in their blood, which is decreased by dialysis. If blood was circulated over this immobilized urease, only urea levels should be reduced because of the high specificity for the primary substrate urea (see above).

**Influence of Na\(_2\)EDTA and DTT on urease activity.** Urease activity was enhanced by, but not dependent on, low levels of Na\(_2\)EDTA and DTT (Table 3). Na\(_2\)EDTA and DTT concentrations of \(\leq 0.5\) and 100 \(\mu\)M, respectively, enhanced urease activity by 31.2 and 30.1%, while increasing levels of Na\(_2\)EDTA (> 20 mM) and DTT (> 5 mM) were inhibitory (data not shown). The literature, the role of these two compounds is ambiguous, suggesting that the influence of Na\(_2\)EDTA and DTT is dependent on the specific urease analysed. Urease activity can be enhanced by EDTA, DTT and \(\beta\)-mercaptoethanol (Christians *et al.*, 1991; Creaser & Porter, 1985; Evans *et al.*, 1991; Mahadevan *et al.*, 1977; Mobley & Hausinger, 1989; Rai & Singh, 1987). However, another possible explanation may lie in the purity of the enzyme extract. When initially optimizing the urease assay with a crude enzyme extract, all levels (1 to 20 mM) of Na\(_2\)EDTA and DTT were inhibitory (data not shown). This suggests that measurements of urease activity in crude enzyme preparations could be spurious because of interfering compounds or proteases affecting the apparent urease activity.

**Influence of cations, anions, acetate, citrate and nitrate on urease activity.** The urease assay mixture was supplemented with varying levels (0, 2, 5 and 20 mM) of cations (chloride salts) or anions, acetate, citrate and nitrate (sodium salts). Urease activity was not affected by Na\(^+\), K\(^+\), Cl\(^-\), Br\(^-\), acetate and nitrate, but decreased significantly with Li\(^+\), Mg\(^2+\), Ni\(^2+\), Zn\(^2+\) and I\(^-\) (Table 3). Surprisingly, urease activity was increased by a mean of 16.9, 27.8 and 28.2% by Ca\(^2+\), Ba\(^{2+}\) and citrate, respectively. The literature regarding the influence of metabolites on urease activity is highly contradictory (Mahadevan *et al.*, 1977; Rai & Singh, 1987). For example, our results suggest that Ba\(^{2+}\) enhances urease activity, while Mahadevan *et al.* (1977) found that Ba\(^2+\) was inhibitory. However, general trends in the literature suggest that Na\(^+\), K\(^+\) and nitrates have no effect on urease activity, while Mg\(^2+\), Ni\(^2+\) and Zn\(^2+\) are inhibitory (Table 3).

**Thermostability and storage of urease.** Purified urease was treated at 65, 80 and 100 °C through 30 min, then cooled to 4 °C and assayed for activity (Fig. 3). Urease activity decreased by 26.0% after 30 min at 65 °C, 86.5%
after 5 min at 80 °C and 100-0% after 1 min at 100 °C. These results are in agreement with previous studies of purified ureases. Ureases tend to be thermally stable up to 60 or 65 °C (Malthotra & Rani, 1969; Mobley & Hausinger, 1989).

The purified enzyme was transferred to 1.5 ml microcentrifuge tubes (100 μl per tube) and stored at 4, -20 and -80 °C. A 30-5% reduction in total activity was observed after 90 d at 4 °C. Freezing the samples at -20 and -80 °C for 2 h did not affect the enzyme activity. Storing the samples for 28 d at -20 and -80 °C reduced the activity by 20.5 and 22.2%, respectively. This data tends to agree with that of Mobley & Hausinger (1989), who demonstrated that storing ureases (without glycerol) at low temperatures can result in a significant loss of activity over time.

Conclusions

The data presented in this manuscript details the first isolation and characterization of urease from A. niger. The basic information (activation, inhibition, pH optimum and temperature stability) obtained from this study could be used in future applications of the enzyme. Further, the technology developed with the DEAE anion exchange column chromatography could be applied to the industrial purification of the enzyme. Our data indicates that when the purified A. niger urease was immobilized on a matrix, the immobilized enzyme reduced the levels of urea in non-acidic solutions (Table 2).

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References


