Purification and Characterization of Acid Urease from *Lactobacillus reuteri*

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Acid urease was purified from *Lactobacillus reuteri* and characterized. The enzyme preparation was electrophoretically homogeneous and the molecular weight of the enzyme was estimated to be 220,000. The enzyme consisted of three kinds of polypeptides, designated as α, β and γ, with molecular weights of 68,000, 16,100 and 8,800, respectively, in an (αβγ)3 structure. The isoelectric point of the enzyme was 4.7. The nickel content was found to be 1.8 atoms of nickel per αβγ unit. The amino acid profile was different from those of known bacterial neutral ureases. The enzyme was most active at pH 2 and at around 65°C. It was stable between pH 3 and 8, and below 50°C. The *Km* for urea was 2.8 mM at pH 2. The enzyme activity was inhibited by Ag+, Hg2+, Cu2+, p-chloromercuribenzoate and acetohydroxamate. The acid urease eliminated urea in alcoholic beverages that are acidic in general.

**Materials and Methods**

*Materials.* DEAE-Sepharose CL-6B, Sepharose CL-6B, Polybuffer 74, PBE 94, molecular weight marker proteins for sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Ampholine PAG plates (pH 4.0 ~ 6.5) were obtained from Pharmacia Biotechnology Products, Uppsala, Sweden. Toyopearl HW-55F was a product of Toyo Soda Mfg. Co., Ltd., Tokyo, Japan. Urease from jack bean (*Canavalia ensiformis*) and standard proteins for molecular weight measurement by gel filtration were purchased from Sigma Chemical Co., St. Louis, Mo. Affi-gel 202 was obtained from Bio-Rad Laboratories, Richmond, Calif. The food-analysis kit for urea determination was a product of Boehringer and Mannheim GmbH, Mannheim, W. Germany.

*Bacterium and cultivation.* *Lactobacillus reuteri* Rt-5, which was isolated from rat feces, was statically grown at 37°C for 48 hr in a medium containing 3.0% glucose (separately sterilized), 0.2% sodium acetate, 1.5% peptone, 1.0% meat extract, 0.8% yeast extract, 0.5% NaCl, 0.5% urea, 0.005% MnSO4·4H2O and 0.005% NiSO4·6H2O. Cells were harvested by centrifugation, washed twice with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0) and then stored at −20°C until used.

*Enzyme assay.* Acid urease was assayed at 37°C by measuring the rate of release of ammonia from urea. The standard reaction mixture contained 167 mM urea and 0.2 M citrate buffer (pH 4.0). The reaction was initiated by adding 2 ml of an enzyme solution to 2 ml of the standard reaction mixture. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid. After the precipitate had
been removed by centrifugation, the supernatant was used to measure ammonia. The released ammonia was determined spectrophotometrically at 625 nm by the method of Weatherburn. One unit of enzyme activity was defined as the amount of enzyme required for the formation of 1 μmol of ammonia from urea per min under the standard conditions.

**Protein measurement.** Protein was measured by the method of Lowry et al. with bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor the elution of proteins on column chromatography.

**Preparation of the affinity gel.** Affi-Gel 202 was coupled with hydroxyurea in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide by the method of Wong and Shobe.

**Determination of the molecular weight by gel filtration.** The molecular weight of the enzyme was determined by gel filtration on a Sepharose CL-6B column (2.6 x 60 cm). Samples were eluted at a flow rate of 15 ml/hr in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl. Apoferritin (molecular weight, 443,000), β-amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000) and carbonic anhydrase (29,000) were used as standards.

**Gel electrophoresis.** Gel electrophoresis was carried out by using the buffers described by Davis. A stacking gel containing 4% acrylamide and a running gel containing 6% acrylamide were used. The proteins were stained with Coomassie brilliant blue R 250.

**SDS-PAGE.** The molecular weight of the acid urease was estimated by SDS-PAGE, as described by Laemmli. The samples were denatured by heating for 5 min at 100°C in 0.0625 M Tris–hydrochloride buffer (pH 6.8) containing 1% SDS, 10% glycerol and 5% 2-mercaptoethanol. A stacking gel containing 4% acrylamide and a running gel containing 15% acrylamide or a gradient of 10% to 20% acrylamide were used. Phosphorylase b (molecular weight, 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400) and myoglobin fragments (14,600, 8,240, 6,380 and 2,560) were used as standards. Proteins were stained with Coomassie brilliant blue R 250.

**Estimation of the isoelectric point.** The isoelectric point of the purified enzyme was estimated by isoelectric focusing using commercially available Ampholine PAG plates, pH 4.0–6.5. Proteins were stained with Coomassie brilliant blue R 250.

**Determination of nickel.** Nickel was determined with a Hitachi Z-8000 polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi Ltd., Tokyo, Japan). Samples were hydrolyzed in 1 N nitric acid, dried at 120°C, charred at 800°C and then atomized at 2850°C.

**Amino acid analysis.** The enzyme protein was hydrolyzed in vacuo at 110°C for 24, 48 and 72 hr in 6 N HCl containing 4% (v/v) thioglycolic acid. Amino acid analysis was performed with a Hitachi 835 amino acid analyzer (Hitachi Ltd.).

**Determination of urea.** Urea in alcoholic beverages was determined with a food-analysis kit, according to the manufacturer’s instructions.

**Results and Discussion**

**Preparation of the acid urease**

Washed cells (42 g as wet weight) were suspended in 200 ml of 50 mM Tris–hydrochloride buffer (pH 7.0) containing 1 mM 2-mercaptoethanol (buffer T) and then disrupted by two passages of the suspension through a French pressure cell (Ohtake Works Co., Ltd., Tokyo, Japan) at 12,000 psi. The suspension of disrupted cells was centrifuged at 76,000 × g for 20 min. The supernatant was used as the cell-free extract. Cold ethanol (−20°C) was added to the supernatant at 0 to 5°C to a final concentration of 30%. After 30 min, the precipitate was collected by centrifugation and resuspended in a minimal volume of buffer T.

The suspension was applied to a column (2.6 x 60 cm) of Toyopearl HW-55F previously equilibrated with buffer T containing 0.1 M NaCl. Proteins were eluted with the same buffer at the flow rate of 15 ml/hr. Fractions of 13 ml were collected. The active fractions were concentrated by ultrafiltration and then dialyzed against buffer T. The dialyze was applied to a column (2.6 x 60 cm) of Toyopearl HW-55F previously equilibrated with buffer T containing 0.1 M NaCl. Proteins were eluted with the same buffer at the flow rate of 15 ml/hr. Fractions of 13 ml were collected. The active fractions were concentrated by ultrafiltration and then dialyzed against 5 mM potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol (buffer P). The dialyze was applied to the first column (4 x 40 cm) of the affinity gel previously
Acid Urease from *Lactobacillus reuteri*

The pooled fractions obtained on affinity chromatography were applied to a column of PBE 94 and then proteins were separated using a pH gradient generated with Polybuffer 74. The eluate was monitored for absorbance at 280 nm (—), and fractions (4 ml each) were assayed for acid urease activity (○) and pH (△). The fractions pooled are indicated by the bar.

**Table I. Purification of Acid Urease from *L. reuteri***

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>1410</td>
<td>18,300</td>
<td>13.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>521</td>
<td>15,800</td>
<td>30.3</td>
<td>2.3</td>
<td>86.3</td>
</tr>
<tr>
<td>Toyopearl HW-55F</td>
<td>140</td>
<td>13,440</td>
<td>96.0</td>
<td>7.4</td>
<td>73.4</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>78.2</td>
<td>10,248</td>
<td>131</td>
<td>10.1</td>
<td>56.0</td>
</tr>
<tr>
<td>First affinity gel</td>
<td>18.0</td>
<td>6,426</td>
<td>357</td>
<td>27.5</td>
<td>35.1</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>NT*</td>
<td>3,274</td>
<td>NT</td>
<td>NT</td>
<td>17.9</td>
</tr>
<tr>
<td>Second affinity gel</td>
<td>7.2</td>
<td>2,520</td>
<td>350</td>
<td>26.9</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* NT, not tested.

equilibrated with buffer P. The column was washed with buffer P and then proteins were eluted with 100 mM buffer P at the flow rate of 140 ml/hr. Fractions of 15 ml were collected. The active fractions were concentrated and then dialyzed against 25 mM imidazole-hydrochloride buffer (pH 7.4). The dialyze was applied to a column (1 x 20 cm) of PBE 94 previously equilibrated with 25 mM imidazole-hydrochloride (pH 7.4) for chromatofocusing. The proteins were eluted with 9-fold diluted Polybuffer 74 (pH 4.0) at the flow rate of 20 ml/hr. Fractions of 4 ml were collected. As shown in Fig. 1, acid urease was eluted as a single peak at about pH 4.2. The active fractions were pooled, concentrated and then dialyzed against buffer P. The dialyze was applied to the second column (1 x 20 cm) of the affinity gel previously equilibrated with buffer P to remove Polybuffer. The column was washed with buffer P and then the proteins were eluted with 100 mM buffer P at the flow rate of 100 ml/hr. Fractions of 5.2 ml were collected. The active fractions were pooled and then stored in small aliquots at —70°C.

Purification of the *L. reuteri* acid urease is summarized in Table I. The acid urease was purified about 27-fold, with a recovery of 13.8%. Polyacrylamide gel electrophoresis of the purified acid urease gave a single band.
Fig. 2. Polyacrylamide Gel Electrophoresis of Acid Urease from *L. reuteri*.

(A) 10 μg of the purified enzyme was electrophoresed on a 6% polyacrylamide gel. (B) 5 μg of the purified enzyme (right lane) and 3 μg each of the standard proteins (left lane) were electrophoresed on a 10–20% SDS-polyacrylamide gradient gel. The details of the methods are described under Materials and Methods.

(Molecular weight and isoelectric point)

The molecular weight of the acid urease was estimated to be about 220,000 by Sepharose CL-6B gel filtration. *L. reuteri* acid urease was found to contain three kinds of polypeptides on SDS-PAGE, as shown in Fig. 2B. The molecular weights of the three polypeptides α, β, and γ, were estimated to be 68,000, 16,100, and 8,800, respectively, using 15% acrylamide gel. Recently, bacterial neutral ureases were found to consist of three kinds of subunit in a α2β2γ4 structure,11,12 whereas jack bean urease is a hexamer of the unit protein.13 The approximate subunit ratio of the acid urease was 1:2:1, which was determined from the integrated intensities of the gel scan profile, and calculation based on the molecular weight of 220,000 obtained on gel filtration chromatography and the molecular weights of the three polypeptides estimated by SDS-PAGE. The result suggests that the acid urease consists of 2 mol of the 68,000-dalton peptide, 4 mol of the 16,100-dalton peptide and 2 mol of the 8,800-dalton peptide.

The isoelectric point of *L. reuteri* acid urease was found to be pH 4.7 on isoelectric focusing.

### Table II. Amino Acid Composition of Acid Urease from *L. reuteri*^a^

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues^b^ per molecule</th>
<th>Mole percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp^c^</td>
<td>230</td>
<td>11.5</td>
</tr>
<tr>
<td>Thr</td>
<td>127^d^</td>
<td>6.3</td>
</tr>
<tr>
<td>Ser</td>
<td>76^d^</td>
<td>3.8</td>
</tr>
<tr>
<td>Glu^c^</td>
<td>187</td>
<td>9.3</td>
</tr>
<tr>
<td>Pro</td>
<td>86</td>
<td>4.3</td>
</tr>
<tr>
<td>Gly</td>
<td>207</td>
<td>10.3</td>
</tr>
<tr>
<td>Ala</td>
<td>178</td>
<td>8.9</td>
</tr>
<tr>
<td>H-cys^e^</td>
<td>28</td>
<td>1.4</td>
</tr>
<tr>
<td>Val</td>
<td>146</td>
<td>7.3</td>
</tr>
<tr>
<td>Met</td>
<td>72</td>
<td>3.6</td>
</tr>
<tr>
<td>Ile</td>
<td>119</td>
<td>5.9</td>
</tr>
<tr>
<td>Leu</td>
<td>143</td>
<td>7.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>84</td>
<td>4.2</td>
</tr>
<tr>
<td>Phe</td>
<td>59</td>
<td>2.9</td>
</tr>
<tr>
<td>His</td>
<td>41</td>
<td>2.0</td>
</tr>
<tr>
<td>Lys</td>
<td>115</td>
<td>5.7</td>
</tr>
<tr>
<td>Arg</td>
<td>82</td>
<td>4.1</td>
</tr>
<tr>
<td>Trp</td>
<td>27</td>
<td>1.3</td>
</tr>
</tbody>
</table>

^a^ The values are the averages for 24-, 48-, and 72-hr hydrolysates in 6 n HCl at 110°C.

^b^ Calculation were based on a molecular weight of 220,000.

^c^ Free amino acid and corresponding amide.

^d^ Obtained by extrapolation to zero time of hydrolysis.

^e^ H-cys: Half-cystine determined as cysteic acid in a 24-hr hydrolysate after performic acid oxidation.

Analysis of nickel

Ureases from jack bean14) and soybean15) were reported to be nickel metallo-proteins containing 2 mol of nickel per mol of subunit, whereas 116,17) or 211,18) mol of nickel per mol of subunit were found for bacterial neutral ureases. The *L. reuteri* acid urease was found to contain 2.2 μg nickel per 2.4 mg protein on atomic absorption spectrometry; the content was calculated to be 1.8 mol of nickel per mol of the αβ2γ4 structural unit.

No other metal ions were detected in the acid urease on atomic absorption analysis.

Amino acid composition

The amino acid composition of the acid urease is shown in Table II. It is noticeable that the methionine and tyrosine contents were
Acid Urease from \textit{Lactobacillus reuteri}

almost twice those of the \textit{B. ammoniagenes}^{16}) and \textit{K. aerogenes}^{11}) neutral ureases.

\textbf{Substrate specificity}

The acid urease was active toward urea. Hydroxyurea was hydrolyzed 120 times slower than urea, while thiourea, methylurea and ethylurea were not attacked at all. This substrate specificity profile is about the same as that of the jack bean urease.\textsuperscript{19})

\textbf{Effects of pH and temperature on the activity and stability}

The pH optimum of the acid urease was around pH 2 (Fig. 3A). The enzyme activity was maximum at 60 to 70°C (Fig. 3B), and was stable in the pH range of 3 to 8 at 37°C for 30 min in various buffers (Fig. 3C). When incubated for 30 min at pH 4, the enzyme activity was stable below 50°C, and about 80\% of the original activity was retained at 60°C (Fig. 3D).

\textbf{Effects of metal ions and various chemicals on the activity}

The effects of metal ions (5.0 mm) on the enzyme activity were examined by adding metal ions, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Sr\textsuperscript{2+}, Ag\textsuperscript{+}, Sn\textsuperscript{2+}, Ba\textsuperscript{2+}, and Hg\textsuperscript{2+}, to the standard reaction mixture. The activity was completely inhibited by Hg\textsuperscript{2+} and Ag\textsuperscript{+}, and reduced to 59\% in the presence of Cu\textsuperscript{2+}.

\begin{table}[h]
\centering
\caption{Effects of Various Chemicals on Acid Urease Activity in \textit{L. reuteri}}
\begin{tabular}{lll}
\hline
Reagent & Concentration (mm) & Relative activity (\%) \\
\hline
None & — & 100 \\
PCMB\textsuperscript{a} & 0.1 & 3 \\
NEM\textsuperscript{b} & 10 & 101 \\
CH\textsubscript{4}ICOOH & 1 & 99 \\
Acetohydroxamic acid & 10 & 10 \\
Nitritotriacetic acid & 10 & 104 \\
EDTA\textsuperscript{c} & 10 & 100 \\
NaCN & 10 & 97 \\
Na\textsubscript{2}S & 10 & 100 \\
\hline
\end{tabular}
\label{table:chemicals}
\end{table}

\textsuperscript{a} \textit{p}-Chloromercuribenzoic acid.
\textsuperscript{b} \textit{N}-Ethylmaleimide.
\textsuperscript{c} Ethylenediaminetetraacetic acid.

The effects of chemical reagents on the acid
urease are shown in Table III. The enzyme activity was inhibited by sulfhydryl reagents, such as p-chloromercuribenzoic acid, and chelating reagents, such as acetohydroxamic acid.

**Kinetic parameters**

The rate of hydrolysis of urea was measured at 37°C in 0.1 m sodium acetate–hydrochloride buffer, pH 2, and 0.1 m citrate buffer, pH 4. Plots of the reaction velocity against urea concentration gave a normal Michaelis-Menten curve. The $K_m$ and $V_{max}$ were calculated from Lineweaver-Burk plots. At pH 2, $K_m$ for urea was 2.8 mM and $V_{max}$ was 580 units/mg. At pH 4, $K_m$ for urea was 1.7 mM and $V_{max}$ was 360 units/mg.

**Elimination of urea in Japanese sake by the acid urease**

Yamamoto et al. reported that Japanese sake on the market contained 10 to 38 ppm urea. We tried to eliminate urea in sake which contained 25 ppm urea. As shown in Fig. 4, urea in the sake was completely eliminated at 20°C in 6 days on adding 0.01 units/ml of the purified *L. reuteri* acid urease, whereas 1 unit/ml of jack bean neutral urease failed to eliminate urea in the sake, because this urease was inactive under acidic conditions (about pH 4) and a high ethanol concentration (about 16%).

The purified *L. reuteri* acid urease effectively eliminated urea in not only sake but also in wine and shaohsing wine (data not shown).

The crude enzymes from newly isolated acid urease producers belonging to the genera *Lactobacillus* and *Streptococcus* were also added to the sake under the same conditions as in Fig. 4. Urea was completely eliminated at almost the same rate as mentioned above. Thus we confirmed that acid ureases from a wide variety of strains can effectively eliminate urea in alcoholic beverages.

Further work is in progress to determine the diversity of the acid ureases from various newly isolated strains.

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**References**

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