Purification and Characterization of Iron-Containing Urethanase from *Bacillus licheniformis*

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Urethane is potentially carcinogenic and teratogenic to human and has been reported to be a contaminant of various kinds of alcoholic beverages. Enzymatic removal of urethane is one possible approach to remove this cancer-causing chemical from alcoholic beverages. Among *Bacillus licheniformis* strains, IFO 12107 showed the highest urethane hydrolyzing activity when cultivated in a urethane-containing white medium. The enzyme was purified about 300-fold by means of several chromatographic steps to homogeneity. The enzyme activity was strongly inhibited by batho- and *o*-phenanthroline. The complete loss of enzyme activity following treatment with bathophenanthroline was fully restored by the addition of Fe^{3+} at a ratio of 4 iron atoms to 1 mol apoenzyme. This result was obtained by the incorporation of $^{59}\text{Fe}^{3+}$ into apourethanase. ESR spectroscopy showed that the enzyme contained a typical high-spin Fe^{3+} . The urethanase hydrolyzed carbamyl ester derivatives more rapidly than amide derivatives. The molecular weight of the native enzyme was about 160 kDa (gel-filtration), and that of the subunit was 42 kDa (sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS–PAGE). This shows the enzyme to be a homotetramer. The pI and K_m values were 5.5 and 0.17 mM, respectively. The enzyme was considerably resistant to high concentrations of ethanol, which is a great advantage for the industrial removal of urethane from alcoholic beverages.

Keywords ethylcarbamate (urethane); *Bacillus licheniformis*; carcinogenic; urethanase; ferric ion-containing hydrolase; alcoholic beverage

Urethane (ethylcarbamate) had been used to promote the dissolution of drugs and as an anaesthetic in humans; it is still used as an anaesthetic for laboratory animals.¹⁾ Urethane was discovered to be effective for the treatment of leukaemia in humans in 1946 and has been used as a therapeutic agent. Unfortunately, urethane was found to be carcinogenic in mice in 1943²⁾ and to be mutagenic in *Drosophila melanogaster*. Finally, urethane was banned as a drug in the 1970s in many countries. However, humans have been exposed to urethane naturally-occurring in alcoholic beverages and some kinds of food.^{3,4)} The mechanism of formation of urethane in alcoholic beverages has been investigated^{5–7)} and it has been shown that urethane is produced by the chemical reaction of urea with ethanol in wine^{8–10)} and sake,¹¹⁾ by heating or long periods of storage. Removal of urea, the major precursor, by an acid urease from wine^{12,13)} and sake¹⁴⁾ has been successful. However, once formed urethane is chemically stable and cannot be easily decomposed. Recently, we reported that *Citrobacter* sp.¹⁵⁾ and *Bacillus licheniformis*¹⁶⁾ isolated from intestinal flora produced urethanase under neutral and acidic conditions, respectively. We also reported that urethanase activity was present in rat tissues and investigated the enzymatic properties, which are probably due to a type of Zn enzyme.¹⁷⁾

In this report, we measured the urethanase activity in eight strains of *B. licheniformis* and found that the IFO 12107 strain showed the highest activity under acidic conditions. The enzyme was purified to homogeneity and its enzymatic properties studied.

MATERIALS AND METHODS

Chemicals Urethane used as a substrate for the enzyme assay was purchased from Tokyo Chemical Industry Co.

Diethylaminoethyl (DEAE)-cellulose, Sephacryl S-300, Hydroxyapatite and Polybuffer-exchanger-94 (PBE) for chromatofocusing were purchased from Pharmacia Fine Chemicals. Reagents for electrophoretic calibration (including myosin, β -galactosidase, phosphorylase, bovine serum albumin, ovalbumin and carbonic anhydrase) were purchased from Sigma Chemical Co., St. Louis, Missouri. Iron-59 (NEZ-037, half life: 44.5 d, FeCl_3 in ca. 0.5 M HCl solution, 604 MBq/mg ^{59}Fe , 291 MBq/ml, total 3.7 MBq) was from Dupont.

Microorganisms Eight strains of *Bacillus licheniformis* were obtained from Nagase Biochemicals, Ltd., Fukuchiyama. The strains were cultivated in nutrient medium and urethane-containing white medium.¹⁵⁾

Culture The urethane-containing white medium was composed of 0.24% HEPES: 2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.1% vitamin mixture (30 g/l choline HCl, 10 mg/l nicotinamide, 2.5 mg/l for both Ca pantothenate and thiamine HCl, 1.25 mg/l riboflavin, 0.75 mg/l pyridoxine HCl, 0.6 mg/l *p*-aminobenzoate, 0.5 mg/l folic acid and 0.1 mg/l biotin), 0.02% mineral mixture (Oriental Yeast Co., Tokyo), containing 5% urethane. The culture experiments were carried out aerobically at 37 °C for 6 d, and bacteria were collected by centrifugation (5000 $\times g$, 20 min) and disrupted using a sonicator (70 W, Heat System-Ultrasonics, U.S.A.). Urethanase activity was measured at pH 4.5. The bacterium showing the highest urethanase activity of the eight strains was investigated further.

Preparation of Crude Enzyme from *B. licheniformis* IFO 12107 The bacterium showing the highest urethanase activity was aerobically grown in 10 l of urethane-white medium for 6 d. Cells harvested from the medium were suspended in 10 ml phosphate buffered saline (PBS), pH 7.0, and disrupted 5 times by sonication at intervals of

1 min in an ice bath. After centrifugation ($20000 \times g$, 15 min) the supernatant was used as the crude enzyme solution.

Enzyme Assay Methods Urethanase was assayed at 37°C by measuring the amount of ammonia or ethanol produced from urethane. The standard reaction mixture contained 10 mM urethane and 0.1 M 2-(*N*-morpholino)-ethane sulfonic acid (MES) buffer, pH 4.5. The reaction was initiated by adding 100 μl of the enzyme to 300 μl of the standard reaction mixture. The reaction was terminated by adding 100 μl 1 N sulfuric acid. After removing the precipitate by centrifugation, the supernatant was analyzed for ammonia or ethanol. The ammonia was determined spectrophotometrically at 630 nm by the indophenol method,¹⁸⁾ and ethanol was measured enzymatically using alcohol dehydrogenase.¹⁹⁾ The glutamate dehydrogenase method²⁰⁾ was used to measure the ammonia produced from some substrates.

Protein Measurement Protein was measured by the Folin-Lowry method²¹⁾ using bovine serum albumin as a standard.

Determination of the Molecular Weight by Gel Filtration The molecular weight of the enzyme was estimated by gel filtration as described by Andrews.²²⁾ A column of Sephacryl S-300 was equilibrated with 10 mM phosphate buffer containing 200 mM saline, pH 7.0 (PBS). Elution was carried out at a flow rate of 10 ml/h and 5 ml fractions were collected.

Catalase (MW 232000), alcohol dehydrogenase (MW 150000), bovine serum albumin (MW 66000), ovalbumin (MW 43000), and carbonic anhydrase (MW 29000) were used as the standards.

Electrophoresis Polyacrylamide gel electrophoresis (PAGE) was carried out as described by Davis²³⁾ on a slab of 10% polyacrylamide gel with a pH 8.8 buffer system. Electrophoresis was performed at 4°C at a current of 20 mA. The gel was cut and half was stained with silver nitrate, while other half was stained for urethanase activity by the following method: The staining solution contained 1.0 μmol urethane, 15 units ALDH, 100 units ADH, 1.0 μmol NAD^+ , 4.0 mg nitroblue tetrazolium, 0.4 mg phenazine methosulfate, 150 units diaphorase and 0.1 M MES buffer to give a final volume of 20 ml. The reaction was carried out by incubating the gels in the solution for 1 h for 37°C and then stopped by immersing the gels in 10% acetic acid. Sodium dodecyl sulfate (SDS)-PAGE was carried as described by Weber and Osborn,²⁴⁾ and the gel was stained with Quick-CBB. β -Galactosidase (MW 116000), phosphorylase (MW 97400), bovine serum albumin, ovalbumin, and carbonic anhydrase were used as molecular weight standards.

Radioactivity Measurement ^{59}Fe solution was diluted with an ^{56}Fe solution (32 $\mu\text{g}/\text{ml}$ in 10 mM PBS, pH 7.0). A mixture of 1 ml radioactive iron solution and 1 ml apoenzyme solution (1 mg/ml) was incubated at 37°C for 1 h. Then the sample was applied to a column (Bio-Gel P-6 gel) and eluted with 10 mM PBS, pH 7.0, containing 1 mM EDTA. The radioactivity in the eluted fractions was measured in a γ -ray counter (Auto Well Gamma System, Aloka ARC-500) and the absorbance of the fractions was monitored at 280 nm.

ESR Measurement ESR spectra were obtained using a JES-FE3X type spectrometer²⁵⁾ (X band, 100 kHz modulation) with the following instrumental settings: power, 20 mW; modulation amplitude, 0.63 mT; scan rate, 3.125 mT/min; time constant, 1 s; gain, 2×1000 .

RESULTS AND DISCUSSION

Urethanase Activity in *B. licheniformis* Cells The urethanase activities of the eight strains cultivated in a nutrient medium was generally low (data not shown). When they were cultivated in urethane-white medium, the activities of some strains increased significantly probably due to induction by the substrate. Strain IFO 12107 exhibited the highest activity under the experimental conditions used (Table I).

Purification of Urethanase The bacterium was cultivated aerobically in 10 l of urethane-white medium for 6 d and the urethanase from harvested cells was purified by the following procedures.

1. Ammonium Sulfate Fractionation: About 80% urethanase activity was present in the supernatant of the sonicated suspension. The supernatant (10 ml) was brought to 40% saturation by slow addition of solid ammonium sulfate, followed by stirring for 2 h in the cold room and then centrifugation at 13000 rpm for 20 min at 4°C . The pellet was discarded and the supernatant was brought to 70% saturation with more ammonium sulfate then stirred overnight in the cold room. Next day the preparation was centrifuged at 13000 rpm for 20 min as described above. The supernatant was discarded and the pellet was dissolved in a minimum amount of 10 mM PBS, pH 7.0, and dialyzed against the same buffer.

2. DEAE-Cellulose Chromatograph: The dialyzed sample was applied to a DEAE-cellulose column and eluted with a linear gradient of sodium chloride (0 to 0.5 M) at a flow rate of 60 ml/h. The enzyme was eluted at around 0.2 M NaCl as shown in Fig. 1. The fractions exhibiting urethanase activity were pooled and concentrated by ultrafiltration.

3. Sephacryl S-300 Chromatography: Further purification was achieved by gel filtration. The concentrated enzyme solution from the previous step was directly applied to a Sephacryl S-300 column (1.8×90 cm) at 4°C and eluted with 10 mM PBS, pH 7.0, at a flow rate of 20 ml/h. The absorbance of the fractions (5 ml each) was monitored at 280 nm and urethanase activity determined by the standard assay method. The active fractions were pooled and concentrated by ultrafiltration.

4. Hydroxyapatite Chromatography: The concentrated enzyme solution obtained from Sephacryl S-3000 chromatography was dialyzed against 10 mM PBS, pH 7.0, and applied to a hydroxyapatite column (1.8×30 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of phosphate buffer (0.01 to 0.2 M) at a flow rate of 24 ml/h and 3 ml fractions collected. The active fractions were pooled.

5. Isoelectric Chromatofocusing: The active fractions were concentrated and then dialyzed against 25 mM imidazole buffer (pH 7.4). The dialyzate was applied to a PBE-94 column to measure the isoelectric point of

Fig. 1. DEAE-Cellulose Chromatography

The enzyme solution from the dialyate was applied to the column (4.0×50 cm), which was thermostated at 4°C , and eluted at a flow rate of 60 ml/h with a gradient buffer of sodium chloride (0 to 0.5 M) in 10 mM PBS, pH 7.0. Fractions (5 ml/tube) were collected and their absorbance monitored at 280 nm (○). Urethanase activity was determined by the indophenol method (▲).

TABLE I. Urethanase Activity of *Bacillus licheniformis* Strains Cultivated in Urethane-White Medium

Organism	Cultivation time (d)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
<i>Bacillus licheniformis</i> :				
IFO 12200	6	20.0	252	12.6
IFO 12197	6	10.0	11.3	1.13
IFO 12107	6	19.5	804	41.2
IFO 12195	8	9.0	12.7	1.41
IFO 12198	8	7.0	4.8	0.69
IFO 12202	6	13.0	112	8.62
IFO 12201	6	18.5	142	7.68
IAM 11054	8	6.0	5.7	0.95

The eight strains of the bacteria in the table was cultivated in 100 ml of white medium containing 5% urethane at 37°C for the indicated number of days. The urethanase activity of each strain was measured as described in Materials and Methods. White medium was sterilized through a Millipore filter ($0.4 \mu\text{m}$).

TABLE II. Summary of the Purification of Urethanase from *B. licheniformis* IFO 12107

Steps	Total protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity (nmol/min/mg)	Yield (%)
Crude extract	2200	92.4	0.042	100
40–70% $(\text{NH}_4)_2\text{SO}_4$	1320	88.4	0.067	96
DEAE-cellulose	245	66.4	0.271	72
Sephacryl-S 300	48.4	56.2	1.162	60.9
Hydroxyapatite	6.7	24.3	3.627	26.3
Isoelectric chromatofocusing	0.94	11.3	12.04	12.2

urethanase. As shown in Fig. 2, urethanase was eluted as a single peak at pH 5.5. The active fraction was applied to a Sephadex G-25 column to remove the Polybuffer 74 which had been used as the eluent.

Summary of Purification Procedures The results of the purification procedure are summarized step by step in Table II. Urethanase was purified about 300-fold from the crude extract of *B. licheniformis* IFO 12107 cells and the yield was about 12%.

Fig. 2. Polybuffer Exchanger-94 Chromatofocusing

The enzyme from the previous step was dialyzed against 25 mM imidazole buffer, pH 7.4. The dialyate was applied to a column (1.0×12 cm) of PBE-94 previously equilibrated with the same buffer for chromatofocusing. The protein was eluted with 10-fold diluted Polybuffer 74, pH 4.0, at the flow rate of 20 ml/h. Fractions (3 ml/tube) were collected. The active fractions were applied to a Sephadex G-75 column to remove the Polybuffer.

PAGE of Urethanase PAGE of the purified enzyme gave a single band in the presence and absence of SDS as shown in Figs. 3A and B, respectively. Following staining for urethanase activity, both crude and purified enzyme preparations showed only one band. These results suggest that there is no isozyme in the bacterium and the purified enzyme is electrophoretically homogeneous.

Effects of Chelating Agents on Urethanase Activity The enzyme was preincubated with various chelating agents at 37°C for 30 min in 10 mM PBS, pH 7.0, and the residual activities were then assayed under the standard conditions. As shown in Fig. 4, the enzyme was strongly inhibited in the following order bathophenanthroline > *o*-phenanthroline > phosphoramidon > phenylmethylsulfonyl fluoride (PMSF) and the 50% inhibition concentrations were found to be 42, 76 nM, 0.1, and $0.7 \mu\text{M}$, respectively.

The enzyme activity was not affected by 1 mM EDTA and EGTA. From these observations, it would seem that metal ions, which have a high affinity for these chelating agents and histidyl residues, are involved in the active site of urethanase.

Effect of Fe^{3+} Concentrations on the Apoenzyme Activity

The enzyme inhibited by 1 mM bathophenanthroline was applied to a Sephadex G-25 column. After gel filtration, the inactive protein fraction was preincubated with Fe^{3+} at various concentrations at 37 °C for 1 h in 10 mM PBS, pH 7.0. After preincubation, the enzyme activity was assayed. The degree of urethanase activity restored depended upon the Fe^{3+} concentrations as shown in Fig. 5. The activity was completely restored by the addition of Fe^{3+} in the ratio of 4 mol of Fe^{3+} to 1 mol inactive protein, the apoenzyme. However, no activation was detected when other metal ions such as Co^{2+} , Ni^{2+} , Mn^{2+} , Cu^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} and Ca^{2+} , were used at a final concentration of 1 mM.

Separation of ^{59}Fe Enzyme The apoenzyme isolated as described above was incubated with radioactive ^{59}Fe at room temperature for 1 h. The sample was applied to

Fig. 4. Inhibition of Urethanase by Chelating Agents

The enzyme solutions were preincubated at 37 °C for 1 h with bathophenanthroline (▲), *o*-phenanthroline (●), phosphoramidon (○) and PMSF (△) at the various concentrations. The enzyme activity in the samples was assayed as described in Materials and Methods. Controls, without chelating agents were run under the same conditions and taken as exhibiting 100% activity.

Fig. 3. Electrophoretic Analysis of the Purified Urethanase

(A): SDS-PAGE. Lane 1, molecular weight markers; lane 2, crude extract; lane 3, purified enzyme, stained with Quick-CBB. (B): Non-SDS-PAGE. Lane 1,3, crude extract; 2,4, purified enzyme; lane 1, 2, stained with silver nitrate; 3,4, stained for urethanase activity.

Fig. 5. Effects of Fe^{3+} Concentration on the Activity of Apourethanase Treated with Bathophenanthroline

After gel-filtration, apoenzyme was preincubated with different concentrations of Fe^{3+} for 1 h at 37 °C. Enzyme activities were determined as described in Materials and Methods. The original activity (12.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein) was taken as 100%.

Fig. 6. Incorporation of Iron (^{59}Fe) into Apourethanase

The apoenzyme (1.0 mg/ml) was incubated with ^{59}Fe solution for 1 h at 37 °C. After gel-filtration on a Bio-Gel P-6 column (1.6 × 5 cm), the radioactivity (●), urethanase activity (△) and absorbance at 280 nm for each fraction were measured.

Fig. 7. ESR Spectrum of Native Urethanase from *Bacillus licheniformis* IFO 12107

The ESR spectrum of the enzyme sample (0.05 mM) in 0.01 M PBS, pH 7.0 obtained using a JES-FE3X spectrometer (X band, 100 kHz modulation) equipped with a JES-VT-3A2 variable temperature controller and a JES-UCT-2AX variable temperature adapter. ESR measuremental conditions were described as in Materials and Methods.

TABLE III. Substrate Specificity

Substrate	Activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	(%)
Methylcarbamate ^{a)}	7.7	64
Ethylcarbamate ^{a)}	12.0	100
<i>n</i> -Butylcarbamate ^{a)}	23.0	192
<i>tert</i> -Butylcarbamate ^{a)}	21.6	118
Phenylcarbamate ^{b)}	23.6	197
Benzylcarbamate ^{b)}	15.8	132
Acetamide ^{a)}	9.4	78
<i>n</i> -Butyramide ^{a)}	11.2	93
Benzamide ^{b)}	3.6	30

a) Indophenol method.¹⁸⁾ b) Glutamate dehydrogenase method.²⁰⁾ The activity towards ethylcarbamate (12.0 $\mu\text{mol}/\text{min}/\text{mg}$ protein) was taken as 100. No activity was observed when urea, methylurea, ethylurea, *n*-butylurea, *tert*-butylurea, phenylurea, and *p*-nitrophenyl acetate were used as substrates.

TABLE IV. Properties of Urethanase in *B. licheniformis* IFO 12107 Strain

Molecular weight	160000
Subunit weight	4 $\times$ (42000)
pI	5.5
Optimum pH	4.5
Stable pH	4–8
Optimum temp	37 °C
Stable temp	< 55 °C
K_m for urethane	0.17 mM
V_{max}	13.3 $\mu\text{mol}/\text{min}/\text{mg}$ protein
Resistance to 20% EtOH	64% activity retained

a Bio-Gel P-6 column. After gel-filtration, the urethanase activity and radioactivity in the protein fractions were measured. The results showed that the apoenzyme clearly incorporated Fe^{3+} with a concomitant restoration of activity as shown in Fig. 6.

ERS Spectrum of the Urethanase An ESR spectrum of the enzyme is shown in Fig. 7. A signal at $g=4.3$ was observed, showing that the urethanase contained a typical high-spin Fe^{3+} . These results suggest that urethanase is probably an iron enzyme, but a novel type, different from the rat kidney enzyme reported previously,¹⁷⁾ whose activity was strongly inhibited by EDTA but was completely restored by the addition of Zn^{2+} . Further studies of the mechanism of the present urethanase are necessary to clarify its relationship to other Fe-enzymes,

such as endonuclease,²⁶⁾ nitrile hydratase^{27–29)} and aconitase.

Substrate Specificity of the Urethanase The purified enzyme hydrolyzed carbamyl ester derivatives more rapidly than amide derivatives, as shown in Table III. However, the enzyme did not hydrolyze urea and urea derivatives, such as methylurea, ethylurea, *n*-butylurea, *tert*-butylurea, and phenylurea. It was also unable to hydrolyze *p*-nitrophenyl acetate.

Some Properties of the Urethanase Other properties of the urethanase were investigated and these are summarized in Table IV. The optimum pH was 4.5, and the optimum temperature was 37 °C. The K_m value for urethane was calculated to be 0.17 mM, which is lower than that of other urethanases from *Citrobacter* (1.6 mM),¹⁵⁾ *B. licheniformis* 1013 (42 mM),¹⁶⁾ and rat kidney (2.5 mM).¹⁷⁾ The urethanase activity decreased with increasing ethanol concentration. About 64% activity remained in 20% ethanol solutions corresponding to the ethanol concentration of sake. Thus this enzyme was more resistant to ethanol than the other enzymes from *Citrobacter* (8.9%) or *B. licheniformis* 1013 (54%) under the same experimental conditions.

The urethanase of *Bacillus licheniformis* IFO 12107 reported in this study is quite different from the other enzymes reported previously.^{15–17)} The urethanase in this report exhibited higher activity under acidic conditions (pH 4.5) and was more resistant to ethanol. Furthermore, the enzyme had a lower K_m value and may be a practical means of removing urethane from alcoholic beverages.

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