Urethane-Hydrolyzing Enzyme from *Citrobacter* sp.

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Received September 4, 1989

Urethane, a cancer-causing chemical, was reported to contaminate alcoholic beverages such as whisky, liquor, wine and sake. Enzymatic removal of urethane would be a possible approach to remove this potentially hazardous chemical from alcoholic beverages. We found that *Citrobacter* sp. isolated from mouse feces stoichiometrically decomposed urethane to ethanol and ammonia. We named this enzyme "urethanase." Partially purified urethanase could hydrolyze several carbamates and some amides. However, urea, *N*-alkyl ureas and ethyl esters of organic acids were not hydrolyzed at all. These results suggest that urethanase belongs to the category of amidase. The enzyme was inactive in high concentrations of alcohol and at acidic pH and was practically ineffective for the elimination of urethane from alcoholic beverages.

Keywords ethylcarbamate; carcinogenicity; alcoholic beverage; amidase; *Citrobacter* sp.; mouse feces; urethanase

Ethylcarbamate (urethane), which is known to be potentially carcinogenic[1] and teratogenic[2] to humans, was reported to contaminate various kinds of alcoholic beverages (several scores of ppb to several ppm).[3] Urethane[4] was chemically produced from carbamyl compounds, in particular urea in sake and wine, by heating with ethanol under the acidic conditions of fermentation. In experimental models, urethane contamination levels were dependent upon acidity, temperature, time and the concentrations of urea and of ethanol. We[5] have already succeeded in preventing the production of urethane by removal of urea from sake with bacterial acid urease. However, urethane once formed by distillation or during long-term storage could not be decomposed by urease. ^{14}C-Labelled urethane orally administered to mice was reported in the 1960s to be decomposed to carbon dioxide, which was exhaled.[1] Recently, Yamamoto *et al.*[6] reported trace activity for urethane hydrolysis in a homogenate of mouse liver. However, they did not confirm the hydrolysis of urethane to be an enzymic reaction owing to the low activity. So we searched for intestinal bacteria producing urethane-hydrolyzing enzyme(s) in animals.

Materials and Methods

Isolation and Identification of Urethane-Splitting Bacteria A suspension of fresh animal feces (0.1 mg of wet feces of mouse, rat or rabbit) was inoculated into 10 ml of a screening medium, pH 5.0, which contained 5.0 g/l urethane, 1.0 g/l ammonium chloride, 1 g/l mineral mixture (Oriental Yeast Co., Tokyo, Japan), 2.4 g/l *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES), 30 mg/ml choline HCl, 10 mg/l nicotinamide, 2.5 mg/l each of 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, and was cultured at 37 °C for 5 to 21 d, aerobically or anaerobically. Though bacterial growth was detectable in all cases only under the aerobic condition, bacteria from rat and rabbit feces died after several successive cultivations. Only bacteria from mouse feces retained the ability to grow in the screening medium. After seven successive cultivations, 20 species of bacteria were isolated on the brain heart infusion (2-fold dilution, Difco Co., U.S.A.) agar plate, pH 5.0, containing 5.0 g/l urethane, and 19 species of them hydrolyzed urethane. The bacterium showing the highest activity among them was characterized according to the method of Cowan.[7] The bacterium grew aerobically and anaerobically. The cell grown on a nutrient agar plate was negative in Gram-staining, and rod-shaped, 0.60—0.75 μm in width and 1.2—2.3 μm in length. Perilrichous flagella were observed in 5.0 g/l phosphotungstic acid-stained cells with a JEM-100 U electron microscope operating at 80 kV. Some biochemical characteristics are listed in Table I. It differed from *Klebsiella* in the mobility test, *Serratia* and *Enterobacter* in the growth test on KCN-medium, *Proteus* in the acid production test with mannitol and rhamnose and *Salmonella* in the indole-production test and acid production from sucrose. Overall, the data suggested that the isolated bacterium belongs to *Citrobacter* sp.

Partial Purification of Urethane-Hydrolyzing Enzyme *Citrobacter* sp. cells were grown aerobically in 10 l of the screening medium for 1 week. Total urethane-hydrolyzing activity varied from 0.4 to 2.0 μmol of urethane decomposed per min in different cultures. Cells harvested from the screening medium (10 l) were suspended in 9 ml of 20 mM phosphate buffer, pH 7.3, containing 1 mM each of ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol, and disrupted by sonication for 2.5 min. Eighty percent of the original activity was recovered in the supernatant fluid (crude extract) obtained after centrifugation at 100000 × *g* for 1 h. The crude extract was applied to a diethylaminoethyl (DEAE)-cellulose column (1.5 × 6 cm) equilibrated with 20 mM phosphate buffer, pH 7.0, with 1 mM each of EDTA and 2-mercaptoethanol. Proteins were eluted stepwise with 0.1, 0.25 and 0.5 M sodium chloride solutions. Hydrolytic activities toward acylcarbamate (containing urethane) and acylamide derivatives were eluted with 0.1 M sodium chloride in the same buffer. Urease activity was detected in fractions eluted with 0.25 M sodium chloride in the same buffer. Recovery of urethane-hydrolyzing activity was 50%. After desalting on an Econo-Pac 10DG column (BIO-RAD, U.S.A.), the active fraction was concentrated with an Ultracent-30 (Toyo Soda, Japan), and then applied on a Sephacryl S-300 column (1.7 × 46 cm)

TABLE I. Characteristics of the Isolated Bacterium

	The isolated bacterium	*Citrobacter*[7] *diversus*	*freundii*
Growth in KCN-medium	w	−	+
Utilization of citrate	+	+	−
Catalase	+	+	+
Oxidase	−	−	−
OF test	F	F	F
Reduction of nitrate	+	+	+
VP test	+	+	+
Indole production	w	+	−
H_2S production	w	−	+
Gas production from glucose	+	+	+
Acid production of carbohydrate			
Adonitol	−	+	−
Arabinose	w	+	+
Lactose	−	d	d
Mannitol	+	+	+
Rhamnose	+	+	+
Salicin	+	+	d
Sucrose	+	d	d
Xylose	+	+	+
Esculin	−	−	−

w: the reactions are slow and weak. d: 11—89% of strains are positive.

equilibrated with 50 mM phosphate buffer, pH 7.0, containing 1 mM each of EDTA and 2-mercaptoethanol. Urethane-hydrolyzing activity was detected in fractions from 41 to 44 ml of the elution buffer, which corresponded to a molecular mass of 500 to 600 kDa. These active fractions (recovery, 12%; specific activity, 0.254 unit/mg·protein) were used for the measurement of hydrolysis of test compounds.

Hydrolysis of Carbamyl, Urea and Amide Derivatives For the measurement of urethane-hydrolyzing activity, an enzyme preparation (100 μl) was incubated with 300 μl of 13.3 mM urethane in 100 mM phosphate buffer, pH 7.0, at 37 °C for the indicated periods of time. After the incubation, 100 μl of 1 N sulfuric acid was added to terminate the reaction. The concentrations of ammonia and ethanol were determined with phenol–alkali reagents[8] and by using the alcohol dehydrogenase–aldehyde dehydrogenase system,[9] respectively. One unit represents 1 μmol of ammonia or ethanol produced from urethane in 1 min under the reaction conditions described above. For other carbamyl-, amide-, or N-substituted urea derivatives and urea, the concentrations of ammonia produced after the incubation were determined by the same method as described above or by the glutamate dehydrogenase method[10] when test compounds interfered with the former method. For measurement of the inhibitory activity of test compounds, a mixture of inhibitor (50 μl), 10 mM urethane in 10 mM phosphate buffer, pH 7.0 (250 μl) and the enzyme (100 μl) was incubated at 37 °C for 2 h. After the incubation, the ammonia produced was measured according to the method[8] described above. The inhibition percentage was calculated based on the control experiment, where 50 μl of distilled water was added in place of the inhibitor. The concentration which caused 50% inhibition (I_{50}) was determined graphically.

Results and Discussion

Fresh animal feces were used as sources of bacteria, because the fecal flora[11] consisted of a large number of bacteria of many kinds. If some bacteria utilize urethane as a nitrogen or carbon source, it is very probable that they produce an enzyme(s) for urethane hydrolysis. Therefore we prepared a special screening medium containing urethane as a sole carbon source. *Citrobacter* sp. isolated from mouse feces decomposed urethane to ethanol and ammonia. All the activity of urethane hydrolysis was detected in the cells and 80% of this activity was recovered in a cytosol fraction (crude extract) after sonication. When the crude extract was incubated with 10 mM urethane in 100 mM phosphate buffer, pH 7.0, both ammonia and ethanol were produced stoichiometrically and progressively with time (Fig. 1). The specific activity of the crude extract was 0.023 unit/mg·protein in the same buffer as described above. Urethane-hydrolyzing activities were observed from pH 6 to 8 and half of the maximum activity was found even at pH 5.0. The K_m value for urethane was 1.6 mM. The activity was completely lost after heating at 65 °C for 15 min, and completely inhibited by the addition of 1 mM Ag^+, Hg^{2+} or F^- and also by the addition of 35 mM sodium laurylsulfate. The enzyme did not require any cofactor such as a metal ion and was not affected by the addition of EDTA and 2-mercaptoethanol. The urethane hydrolysis described in this study is enzymatic, and has not been reported hitherto. We named the enzyme "urethanase."

Urethanase was partially purified from a crude extract by diethylaminoethyl (DEAE)-cellulose and Sephacryl S-300 column chromatographies with a recovery of 12%. The purified enzyme (specific activity, 0.254 unit/mg·protein) hydrolyzed six carbamyl derivatives and three amide derivatives except glycinamide (Table II). However, the enzyme did not hydrolyze urea, five urea derivatives (methyl-, ethyl-, *n*-butyl-, allyl- and phenylurea), or three esters (ethyl acetate, ethyl benzoate and diethyl carbonate). Therefore, it seems likely that urethanase belongs to the class of amidases. Urethanase reported in this study is quite different from aminoacylase (Sigma Chemical Co., St. Louis, MO, U.S.A.) and from ureido compound-hydrolyzing enzymes, which include ureidopropionase,[12] *N*-benzyloxycarbonyl-amino acid hydrolase,[13] phenylcarbamate hydrolase[14] and some acyl amidases[15] as reported previously. Urethanase was inhibited by phenylphosphorodiamide, phenylthiophosphorodiamide and *N*-benzoylphosphoric triamide, which are known to be potent and specific inhibitors[16] and also suicide substrates of urease[17]; the concentrations giving 50% inhibition (I_{50} value) were 27, 175 and 720 nM,

TABLE II. Substrate Specificity of Urethanase from *Citrobacter* sp.

Substrate	Activity (unit/mg·protein)
Methylcarbamate	0.16
Ethylcarbamate	0.25
n-Butylcarbamate	0.56
tert-Butylcarbamate	0.061
Phenylcarbamate	0.24
Benzylcarbamate	0.37
Acetamide	2.2
n-Butylamide	2.5
Glycinamide	0
Benzamide	1.7

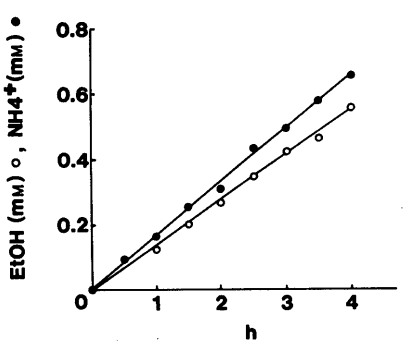

Fig. 1. Production of Ammonia and Ethanol from Urethane with Crude Extract from *Citrobacter* sp.

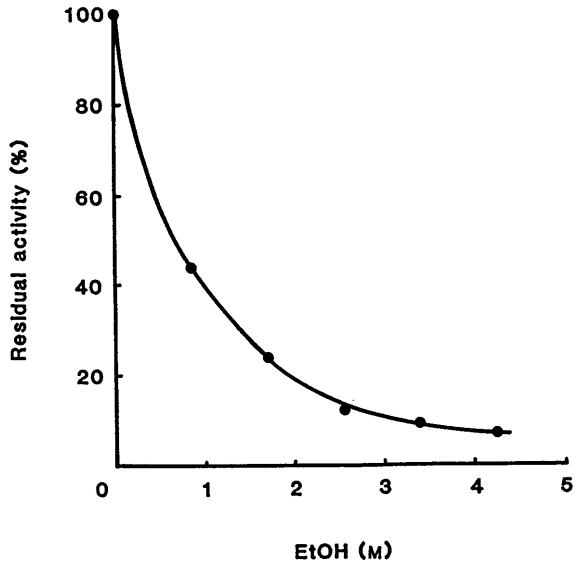

Fig. 2. Inactivation of Urethanase by Ethanol

Urethanase was incubated with 10 mM urethane at various concentrations of ethanol at 37 °C for 60 min.

respectively. However, these organophosphorus compounds were not hydrolyzed by urethanase at all. *O,O*-Dimethyl-*O*-(2,2-dichlorovinyl) phosphate and *O,O*-dimethyl-*O*-(3-methyl-4-nitrophenyl) phosphate also inhibited urethanase and their I_{50} values were 0.1 and 8.2 μM, respectively. But diethylpyrocarbonate, *N*-ethylmaleimide and iodoacetamide did not affect the enzyme activity at 100 μM.

Urethane, a cancer-causing chemical, has been reported to contaminate alcoholic beverages such as whisky, liquor, wine and sake. Thus, removal of urethane from alcoholic beverages is an urgent world-wide problem. Enzymatic decomposition of urethane might be one possible solution. In the present report, a new enzyme, urethanase, was discovered in *Citrobacter* sp., but the enzyme was inhibited by high concentrations of ethanol, with an I_{50} value of 0.7 M (Fig. 2). Therefore the enzyme is not applicable practically for the elimination of urethane from alcoholic beverages, because Japanese sake contains 3.4 M ethanol and liquors generally contain more than 8 M ethanol. However, we intend to search for a more ethanol-resistant urethanase.

Acknowledgement We thank Dr. K. Mitsui of our faculty for his help in electron microscopy, Ms. K. Tadaka and Mr. Y. Tsukamoto of our laboratory for their technical assistance and Ms. S. Takayanagi for her help in the preparation of the manuscript.

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