[Chem. Pharm. Bull. 36(2) 693—699 (1988)]

Acid Urease from *Lactobacillus* of Rat Intestine

SACHIKO TAKEBE and KYOICHI KOBASHI*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama-shi, Toyama 930-01, Japan

(Received July 25, 1987)

The effect of pH on the urease activity of the extract from ileum-cecum contents of rats revealed the presence of an enzyme having high activity in the acid region (pH 2—3). This type of enzyme was designated as acid urease to distinguish it from the enzyme having high activity in the neutral region. The bacterium producing acid urease was isolated from the cecum contents of rats and identified as *Lactobacillus fermentum*. Acid urease was partially purified from the extract of *L. fermentum* TK1214. The enzyme had the optimal pH at 2.4, the isoelectric point at 4.2, and a K_m of 17 mM, and was inhibited by caprylohydroxamic acid, *N*-ethylmaleimide and *p*-chloromercuribenzoic acid, though these compounds were considerably less inhibitory to acid urease than to neutral urease.

Keywords——acid urease; *Lactobacillus fermentum*; rat intestine; alcholic beverage

Introduction

It has been established that conventional animals have high urease activity in their intestinal mucosa and contents, but germ-free animals have no activity.[1,2] We reported previously[3,4] that ureolysis *in vivo* was depressed by the oral administration of hydroxamate, which is known to be a potent and specific inhibitor of urease activity.[5] Furthermore, we found that intestinal bacteria such as *Eubacterium*, *Fusobacterium*, *Peptococcus* and *Lactobacillus*[6] produced urease, and concluded that ureolysis *in vivo* was attributable to the bacterial enzyme.

An examination of the pH–activity profile of ileum-cecum contents of rats revealed urease activity not only in the neutral region, but also in the acidic region. All the data previously reported on ureases of various origins showed their optimal pHs to be around 7, including ruminal enzymes.[7-10] We named the enzyme having a low optimal pH "acid urease" and attempted the isolation of the acid urease-producing bacterium from the cecum contents of rats.

Experimental

Animals——Rats (Wistar strain, female, body weight 200 to 250 g), and mice (ddYS strain, male, body weight 25 to 30 g) were maintained on commercial feed (Nihon Clea CE-II) and rabbits (male, body weight 4 kg) on feed from Funabashi Co.

Preparation of Crude Ureases of Various Origins——Intestinal Contents of Experimental Animals: Fresh ileum-cecum contents (total wet weight was 55.9 g) of nine rats were suspended in 10 ml of cold isotonic buffer, pH 7.0, composed of 0.864% $NaHCO_3$ and 0.932% KH_2PO_4, and centrifuged at 10000 × *g* at 4 °C for 1 h. The sediment was washed twice with two volumes of the same buffer and resuspended in 40 ml of the same buffer, followed by repeated freezing and thawing in a dry ice–acetone bath. After centrifugation, the supernatant fluid was dialyzed against 2 l of 0.1 M phosphate buffer, pH 7.0, containing 0.4 mM ethylenediaminetetraacetic acid (EDTA). The dialysate was applied to a column (2.5 × 64 cm) of Sephadex G-100 previously equilibrated with the buffer used for dialysis and eluted with the same buffer, fractionating 6 ml per tube. Urease activities were eluted in fractions 24—30, which were

pooled and concentrated to a small volume by ultrafiltration (PM10 membrane, Amicon Co.).

Fresh cecum contents (16.2 g) of two rabbits were suspended in 30 ml of 0.2 M phosphate buffer, pH 7.0, and centrifuged at 10000 × *g* at 4 °C for 30 min, followed by freezing and thawing five times. The supernatant fluid was applied to a column (3.0 × 122 cm) of Sephadex G-100 in the same way as in the case of rats. Active fractions were pooled and concentrated by ultrafiltration.

Fresh ileum-cecum contents (9.3 g) of twelve mice were suspended in 10 ml of 10 mM phosphate buffer, pH 7.7, containing 1 mM EDTA. The crude urease preparation was obtained by the same procedure as in the cases of rats and rabbits.

Human Feces: Acetone powder (1 g) of human feces (female, healthy) was suspended in 15 ml of 10 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 mM β-mercaptoethanol (β-ME), and extracted at 25 °C for 1 h. The soluble fraction was dialyzed against 10 mM phosphate buffer, pH 7.0, at 4 °C for 4 h.

Proteus mirabilis: *P. mirabilis* cells as a starting material were grown aerobically at 37 °C for 18 h in 100 ml of the nutrient broth, pH 7.0, containing 1% beef extract (BBL), 1% peptone (Eiken Co.), 1% glucose and 0.5% NaCl. Harvested cells were suspended in 5 ml of 5 mM phosphate buffer, pH 6.8, and disrupted by ultrasonic vibration with a Kontes Sonifier at 8 A (Kontes Inc., Vineland, N. J.) four times for 3 min at intervals of 2 min in an ice bath and centrifuged at 10000 × *g* for 15 min. The supernatant fluid was dialyzed twice against 1 l of the same buffer overnight at 4 °C, and the dialysate was used as a crude extract (specific activity was 15.3 IU/mg protein).

Lactobacillus fermentum TK1214: *L. fermentum* cells as a starting material were grown anaerobically at 37 °C for 16 h in 6 l of LBS medium prepared according to the method of Mitsuoka.[11] Harvested cells were disrupted by repeated freezing and thawing, and extracted at room temperature for 40 min with 60 ml of 10 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 10 mM β-ME. After removal of cell debris by centrifugation, the supernatant fluid was concentrated to a small volume by ultrafiltration, applied to a column (2.5 × 41 cm) of Sepharose 6B and eluted with the same buffer as described above. The urease was eluted as a single peak between two major peaks of absorbance at 280 nm. The active fractions were concentrated by ultrafiltration and again applied to a column (2.5 × 63.5 cm) of Sepharose 4B because the specific activity was not constant in each fraction. In the second gel filtration, urease was eluted from 214 to 301 ml, where two peaks and two shoulders of activity were observed. This elution pattern of activity did not coincide with that of absorbance at 280 nm. All the active fractions were pooled and concentrated by ultrafiltration, followed by isoelectrofocusing using carrier Ampholyte (pH 4—6) with a sucrose gradient. Urease was found as a single peak at pH 4.2. The specific activity of the enzyme preparation obtained after removal of Ampholyte by Sephadex G-50 gel filtration was 47 IU/mg protein, which represented a 17-fold purification from the crude extract of *L. fermentum*. However, the enzyme was not homogeneous, because two protein bands having no urease activity were detected by disc electrophoresis using 5% acrylamide.

Isolation of Acid Urease-Producing Bacterium——Media: Acidic beef extract medium was composed of BBL 1% glucose and 0.05% cysteine·HCl·H_2O, adjusted to pH 4.0 with 3 N HCl. General anaerobic medium (GAM, Nissui Co.) was acidified to pH 4.0 with 3 N HCl. Selective media for *Bacteroidaceae* (NBGT), *Veillonella* (VS), *Bifidobacterium* (BS) and *Lactobacillus* (LBS) were prepared by the method of Mitsuoka.[11]

Isolation of a Bacterium: Cecum was taken into a sterile dish from narcotized rats with ethyl ether, and about 47 mg of its contents was suspended in 1 ml of 70 mM phosphate buffer, pH 7.2, containing 0.05% cysteine·HCl·H_2O and 0.1% agar. Then 0.1 ml of this suspension was inoculated on an acidic GAM agar plate and cultivated anaerobically at 37 °C for one week. Subsequently, the bacterial colonies obtained were cultivated anaerobically in LBS, NBGT, BS and VS media for one week. The growth of bacteria was observed in all media except in VS medium, but urease activity was detected only in LBS and BS media. Ten colonies grown in LBS medium were taken out and cultivated in GAM broth. Each bacterium harvested from GAM was suspended in 10 mM phosphate buffer, pH 7.0, containing 0.85% NaCl and analyzed for acid urease activity at pH 2.2.

Identification of the Isolated Bacterium: The isolated bacterium was identified on the criteria of acid production from carbohydrates, gas production, growth temperature and Gram staining by the method of Mitsuoka.[12]

Measurement of Urease Activity——Activity of Intestinal Contents: A mixture of 0.05 ml of enzyme solution, 0.65 ml of a buffer, as described in the legend to Fig. 1, and 0.05 ml of 5 M urea was incubated at 37 °C for 30 min, followed by the addition of 0.2 ml of 1 N H_2SO_4. To 0.5 ml of the reaction mixture, 2.5 ml each of phenol reagent (1% phenol and 0.005% sodium nitroprusside) and alkali reagent (0.54% $Na_2HPO_4 \cdot 12H_2O$, 0.5% NaOH and 0.1% NaOCl) were added. After incubation at 65 °C for 20 min, the absorbance at 630 nm was measured according to the method of ammonia determination of Okuda and Fujii[13] with a minor modification.

Activity of Bacteria: The cells collected by centrifugation at 10000 × *g*, at 4 °C for 15 min were washed twice with physiological saline and suspended in the same solution. A mixture of 0.1 ml of this suspension, 0.6 ml of a buffer, and 0.05 ml of 5 M urea was incubated at 37 °C for 30 min, followed by the addition of 0.2 ml of 1 N H_2SO_4. The activity of acid urease partially purified from *L. fermentum* TK1214 was measured at 6.6 mM HCl-10 mM KCl, pH 2.5. The amounts of liberated ammonia were determined by the method described above.

Activity was expressed as international unit (IU), one unit being defined as the amount of enzyme that hydrolyzed 1 μmol of urea per minute at 37 °C.

Measurement of Apparent 50% Inhibition (I_{50}) Values——A mixture of 0.05 ml each of the enzyme solution and

test compound dissolved in 10 mM phosphate buffer, pH 7.0, was preincubated at 37 °C for 30 min. After the preincubation, the urease activity was measured according to the method described above. The inhibition percentage was calculated, based on the control experiment where 0.05 ml of the buffer was added in place of the test compound. The final concentration which caused I_{50} was determined graphically.

Measurement of Protein——The protein of the soluble fraction was measured by the Folin–Lowry method.[14] The protein of bacterial cells was measured in the same way after heating a mixture of 0.5 ml each of the cell suspension and 1 N NaOH at 65 °C for 10 min.

Results

Distribution of Urease Activity in Gut of Rats

Most of the bacterial urease activity was distributed in the contents of the ileum-cecum and colon. A low activity was detected in the stomach, probably derived not only from microorganisms, but also from commercial feed which has a considerable activity of plant origin (Table I). Eighty percent of the total activity of ileum-cecum contents was present in the insoluble fraction, which suggests most of the enzyme to be intracellular.

Effect of pH on Urease Activity from Ileum-Cecum Contents of Rats, Mice and Rabbits

As shown in Fig. 1A, the crude soluble preparation from ileum-cecum contents of rats showed the optimal activity at pH 4.5 and exhibited a broad shoulder in the neutral region. The profiles of the preparations from ileum-cecum contents of mice and rabbits, and also from human feces are shown in Fig. 1B. In the case of rabbits, the lower the pH of incubation, the higher the activity; the activity at pH 4.0 was 5-fold higher than that at pH 7.0. However in mice, the pH–activity profile was found to be bell-shaped having a peak at pH 7.0, though the activity at pH 5.0 in acetate buffer was 2.5-fold higher than that at the same pH in phosphate buffer. In human feces, the optimal pH was 7.4. In jack bean and *P. mirabilis*, the optimal pHs were 6.7 and 7.2, respectively. These results revealed that ureases having high activity in the acid region of pH 3—5 were present in the gut of rats and rabbits. We designated this type of enzyme as acid urease to distinguish it from the ureases having high activity in the neutral region (named neutral urease). The distribution of acid urease in the gastrointestinal contents of rats is shown in Table I. The activity of acid urease was high in the ileum-cecum and colon contents. However, the ratio of acid activity to neutral activity was the highest in the jejunum contents (5.2) and stomach-duodenum contents (3.4). This observation showed that the acid urease was dominant in the upper parts of the alimentary tract.

Effect of pH on the Stability of Neutral and Acid Urease

When the enzyme solution from ileum-cecum contents of rats was incubated at an acidic

TABLE I. Urease Activities of Gastrointestinal Contents of Rat

Contents[a]	pH	Urease activity[b] (IU/g dry weight)	
		Neutral	Acid
Stomach-duodenum	4.1	0.166	0.572
Jejunum	7.2	0.048	0.252
Ileum-cecum	7.3	14.9	13.3
Colon	7.0	9.43	13.0
Feces		(19.7)[c]	(11.4)[c]
Feed		2.40	1.09

a) Contents of gastrointestinal parts, fresh feces and feed (Nihon Clea) were suspended in cold physiological saline, and the pH of the suspension was measured by a Hitachi pH meter at 4 °C. *b*) Neutral and acid urease activities of these suspensions were measured at pH 7.0 and 5.0, respectively, according to Experimental. *c*) IU/g wet weight.

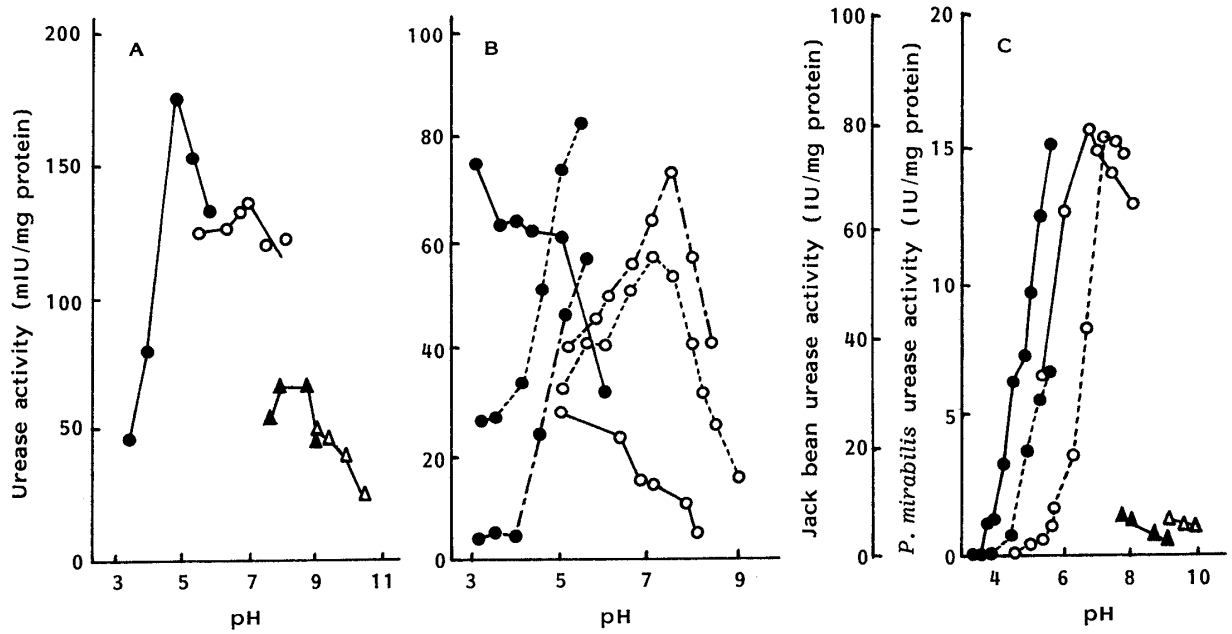

Fig. 1. Effect of pH on Urease Activity from Ileum-Cecum Contents of Rats, Mice and Rabbits, and from Human Feces

The following buffers were used: 0.2 M acetate buffer (●), 0.2 M phosphate buffer (○), 0.1 M phosphate–0.05 M borate buffer (▲), 0.05 M borate–0.05 M carbonate buffer (△).
A shows the pH-activity profile of the enzyme solution obtained from ileum-cecum contents of rats. B shows that from ileum-cecum contents of mice (----) and rabbits (——) and from human feces (— · —). C shows that from *P. mirabilis* (----) and jack bean (——).

pH, the neutral activity disappeared rapidly, while the acid activity was considerably resistant to acid treatment. For example, at pH 3.5 for 17 h, the neutral activity was completely lost, but the acid activity decreased to 25% of the original activity. This inactivation of both ureases was not prevented by the addition of boiled enzyme solution, 0.5 M NaCl,6.6 mM β-ME or 0.1 mM cysteine, or by incubation under anaerobic conditions. However, the addition of 25% glycerol prevented this inactivation of both enzymes.

pH–Activity Profile of Urease after Acid Treatment

The optimal pH of urease dialyzed against acidic buffer was around 3.0, as shown in Fig. 2. Dialysis under acidic conditions led to almost complete loss of neutral activity. On the other hand, a slight increase of specific activity of acid urease was observed. Attempts to separate acid and neutral ureases were unsuccessful by the following methods: 1, fractionation with acetone; 2, chromatography on Sepharose 6B or DEAE cellulose; 3, disc electrophoresis. These results suggest that the two enzymes are closely similar in their physicochemical properties.

Identification of the Acid Urease-Producing Bacterium from Cecum Contents of Rats

The bacterium producing acid urease was gram-positive, rod-shaped, and non-spore-forming. For the identification of the bacterium, we examined acid production from carbohydrates, growth temperature and gas production (Table II); the results showed this bacterium to be *L. fermentum* sp. In the pH–activity profile of this cell suspension, the lower the pH of incubation, the higher the activity.

L. fermentum TK1214 isolated from cecum contents, produced acid urease in the primary cultivation in neutral medium (GAM, pH 7.3) and acid medium (LBS, pH 5.5) but it did not produce acid urease any longer in a neutral medium upon successive cultivation. However, when the bacterium grown in a neutral medium was inoculated in an acid medium,

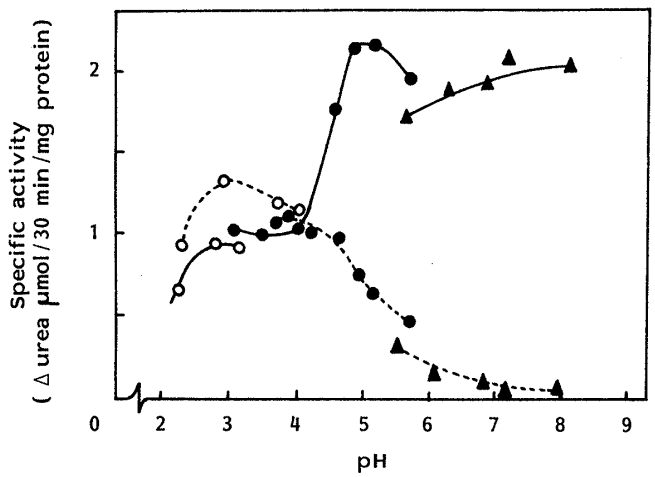

Fig. 2. pH–Activity Profile of Urease after Acid Treatment

Enzyme solution (2 ml) was dialyzed overnight against 1 l of 10 mM acetate buffer, pH 4.6, containing 1 mM EDTA at 4 °C.

——, before the acid treatment; – – – –, after the acid treatment; ○, 0.2 M HCl–KCl buffer; ●, 0.2 M acetate buffer; ▲, 0.2 M phosphate buffer.

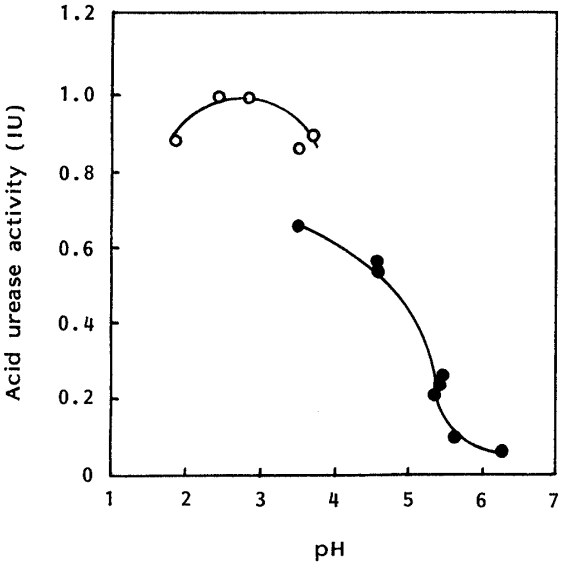

Fig. 3. Effect of pH on Partially Purified Acid Urease

Symbols are the same as in Fig. 2.

TABLE II. Acid Production from Carbohydrates by *L. fermentum* TK 1214

Carbohydrates	TK 1214	*L. fermentum*[a)]	Carbohydrates	TK 1214	*L. fermentum*[a)]
Arabinose	−	±	Melibiose	+	±
Xylose	−	±	Raffinose	+	+
Rhamnose	−	−	Melezitose	−	−
Ribose	+	±	Starch	−	−
Glucose	+	+	Mannit	−	−
Mannose	−	±	Sorbit	−	−
Fructose	−	±	Esculin	−	−
Galactose	+	+	Salicin	−	−
Sucrose	+	±	Sorbose	−	−
Maltose	+	+			
Cellobiose	−	−	Gas production	+	+
Lactose	+	+	Growth at 15 °C	−	−
Trehalose	−	±	Growth at 45 °C	+	+

a) Data from Mitsuoka.[12)]

the production of acid urease started again. From this observation it seems that the ability to produce acid urease was controlled by the pH of the environment.

Properties of the Partially Purified Acid Urease

Acid urease was partially purified from the extract of *L. fermentum* TK1214 according to the procedure described in Experimental. The enzyme obtained by isoelectric focusing showed classical Michaelis–Menten kinetics for urea with a K_m of 17 mM, which is similar to the values reported for other bacterial enzymes. The optimal pH in KCl–HCl buffer was 2.4 (Fig. 3) and the isoelectric point using carrier Ampholytes (pH 4—6) with a sucrose gradient was 4.2. After incubation in 10 mM phosphate buffer, pH 7.0, at 65 °C for 15 min, 50% of the original activity remained. The effects of several sulfhydryl reagents and hydroxamic acid on the activity were examined. The mixture of enzyme and inhibitor was incubated under neutral conditions,

followed by measurement of the activity in acidic conditions. Acid urease was inhibited by caprylohydroxamic acid, *p*-chloromercuribenzoic acid and N-ethylmaleimide: the concentrations giving 50% inhibition were 41, 50 and 700 μM, respectively, which are about ten times higher than those in the case of neutral urease.

Discussion

Many investigations have been undertaken on the enzymatic properties of ureases in the lumen from ruminants in order to clarify the process of utilization of non-protein nitrogen *in vivo*. These ureases have been reported to show optimal pHs in the neutral region, pH 8.0 or 6.8 in bovine,[7,8] pH 7.6 in sheep[9] and pH 8.5 in cattle.[10] We[6] previously reported that the strains originally isolated from human feces, such as *Eubacterium aerofaciens*, *Eubacterium lentum*, *Bacteroides multiacidus*, *Fusobacterium varium* and *Peptostreptococcus productus*[15] produced ureases having optimal pHs in the neutral region (pH 7—8). However, only two strains, *Lactobacillus salivarius*[16] and *L. fermentum*,[6] in the cell suspension were reported to show ureolytic activity at pH 3.0 and 4.5, respectively. Recently, several bacterial ureases have been purified from *Brevibacterium ammoniagenes*,[17] *Arthrobacter oxydans*,[18] *Selenomonas ruminantium*,[19] *Klebsiella aerogenes*[20] and *Ureaplasma urealyticum* (unpublished data). All of their optimal pHs also lie in the neutral region. In the present paper we demonstrated the presence of urease having acidic optimal pH (acid urease) in the intestinal flora of rats and rabbits. Acid urease partially purified from the extract of *L. fermentum* TK1214 had the optimal pH of 2.4, which was markedly lower than those of ureases from the bacteria described above. Acid urease was inhibited much less than neutral urease by hydroxamic acid, which suggests that an area adjacent to the binding site of hydroxamic acid in acid urease differs from that in neutral urease. It still remains to be explained why bacteria growing in the alimentary tract, whose pH is neutral, produce acid urease. On the other hand, the acid urease-producing bacteria were present in the stomach contents, whose pH is acidic, and therefore the bacteria may flow into the lower parts of the alimentary tract. Some investigators reported that *Aerobacter aerogens*[21] was the dominant bacterium causing ureolysis in the stomach and it produced neutral urease, whose activity is highest at pH 7 to 7.5.[22] Others[23] reported that marked activity in the stomach was also observed at pH 7.9. Their results suggest that the optimal pH of urease is not directly related to the pH of the alimentary tract. However, in this study, it is suggested that ureolysis in the stomach may be caused chiefly by acid urease, because the activity ratios of acid urease to neutral urease in the stomach and the upper parts of the small intestine were considerably higher than those of the lower parts. We examined the enzymatic properties of acid urease using a preparation partially purified from *L. fermentum* TK1214, but its physiological role in the host animals is not clear. Moreau *et al.*[24] showed that the intact cells of *Lactobacillus* sp. (producing acid urease) isolated from conventional rats had higher antigenic acitivity in gnotobiotic rats (contaminated with the same bacterium) than *Actinobacillus* (producing neutral urease). The relevance of this to the physiological role of acid urease *in vivo* remains to be examined.

Acid urease was found to be useful for the removal of urea produced in alcoholic brewing,[25] and is expected to be applicable for the prevention of formation of ethylcarbamate, a cancer-causing chemical, because ethylcarbamate is formed by heating urea with ethyl alcohol and alcoholic beverages in general are acidic (pH 4—5). We are examining the effect of acid urease treatment on ethylcarbamate concentrations in various kinds of alcoholic beverages with a view to application of the enzyme in the fermentation industry.

Acknowledgement We wish to thank Dr. N. Nakai of Fukui Medical University and Dr. K. Mitsui of our university for useful discussions, and Miss S. Takayanagi for her help in the preparation of the manuscript.

References

1) S. M. Levenson, L. H. Crowley, R. E. Horowity and O. J. Malon, *J. Biol. Chem.*, **234**, 2061 (1959).
2) A. M. Delluva, K. Markley and R. E. Davies, *Biochim. Biophys. Acta*, **151**, 646 (1968).
3) K. Kobashi, S. Takebe, N. Harada and J. Hase, *J. Germfree*, **4**, 11 (1974).
4) S. Takebe, K. Kobashi, J. Hase and T. Koizumi, *Chem. Pharm. Bull.*, **31**, 600 (1983).
5) K. Kobashi, J. Hase and K. Uehara, *Biochim. Biophys. Acta*, **65**, 380 (1962).
6) K. Suzuki, Y. Benno, T. Mitsuoka, S. Takebe, K. Kobashi and J. Hase, *Appl. Environ. Microbiol.*, **37**, 379 (1979).
7) S. Mahadevan, F. D. Saller and J. D. Erfle, *Biochem. J.*, **163**, 495 (1977).
8) B. E. Brent, A. Adepoju and F. Portella, *J. Anim. Sci.*, **32**, 794 (1971).
9) K. Baintner, Jr., *Allatenyestez*, **13**, 17 (1964).
10) S. A. Rahman and P. Decker, *Nature* (London), **209**, 618 (1966).
11) T. Mitsuoka, *Kansensyo-gakkaishi*, **45**, 406 (1970).
12) T. Mitsuoka, *Rinshokensa*, **18**, 1163 (1974).
13) T. Okuda and S. Fujii, *Saishin Igaku*, **21**, 622 (1966).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) H. Teshima and Y. Tanaka, *Nippon Saikinkagaku Zasshi*, **31**, 175 (1975).
16) P. Raibaud, J. V. Galpin, R. Ducluzean, G. Mocquot and G. Oliver, *Ann. Microbiol.* (Paris), **124A**, 83 (1973).
17) H. Nakano, S. Takenishi and Y. Watanabe, *Agric. Biol. Chem.*, **48**, 1495 (1984).
18) J. Schneider and H. Kaltwasser, *Arch. Microbiol.*, **139**, 355 (1984).
19) R. P. Hausinger, *J. Biol. Chem.*, **261**, 7866 (1986).
20) M. J. Todd and R. P. Hausinger, *J. Biol. Chem.*, **262**, 5963 (1986).
21) H. L. Kornberg and R. E. Davies, *Physiol. Rev.*, **35**, 169 (1955).
22) M. Y. Kamel and R. R. Hamed, *Acta Biol. Med. Germ.*, **34**, 169 (1975).
23) S. Maramna, *Gastroenterology*, **50**, 657 (1966).
24) M. C. Moreau, R. Ducluzeaum and D. Raiband, *Infect. Immu.*, **13**, 9 (1976).
25) K. Kobashi, S. Takebe and T. Sakai, *J. Appl. Toxicol.*, **8**, in press (1988).