Note

Characteristics of Acid Urease from *Streptococcus mitior*

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Ethylcarbamate, which is known to be carcinogenic, is thought to be formed from urea and ethyl alcohol in alcoholic beverages.¹⁾ Acid urease is effective in preventing the formation of ethylcarbamate, but only the enzyme from *Lactobacillus fermentum* has previously been partially purified and characterized.²⁾ We started our research by examining the distribution of acid urease-producing microorganisms and isolated 700 producers. These isolates belonged to 6 genera, strains of the genera *Streptococcus* and *Lactobacillus* being dominant.³⁾ The acid ureases from 37 representative isolates from these 700 producers were classified into 6 different types of the enzyme according to their electrophoretic mobility.⁴⁾ Two different types of acid urease, one from *L. reuteri*⁵⁾ and one from *L. fermentum*,⁶⁾ have been highly purified and characterized, but little is known of the properties of the acid ureases produced by *Streptococcus* strains. This report describes the purification

and characterization of the acid urease from a new isolate of *S. mitior*. This acid urease differs from the *Lactobacillus* acid ureases in electrophoretic mobility and molecular weight of the subunits.

S. mitior PG-118, which was isolated from rat feces,³⁾ was statically grown at 37°C for 48 hr in a medium containing 3.0% glucose (separately sterilized), 0.2% sodium acetate, 1.5% Polypepton (Nihon Seiyaku, Tokyo), 1.0% meat extract, 0.8% yeast extract, 0.5% NaCl, 0.5% urea, 0.005% MnSO₄·4-6H₂O, and 0.005% NiSO₄·6-H₂O. Cells were harvested by centrifugation, washed twice with 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0), and then stored at –80°C until use.

Acid urease activity 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 started by adding 2 ml of an enzyme solution to 2 ml of the standard reaction mixture. The reaction was stopped by adding 4 ml of 10% trichloroacetic acid. After the precipitate had been removed by centrifugation, the amount of ammonia in the supernatant was measured spectrophotometrically at 625 nm by the method of Weatherburn.⁷⁾ 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.

Ethylcarbamate was extracted from sake by the method of Joe *et al.*⁸⁾ Ethylcarbamate was measured with a GC-9A gas chromatography system (Shimadzu Corporation, Tokyo) on a Chromosorb W (AW) column (0.3 × 200 cm; Gasukuro Kogyo, Inc., Tokyo) coated with 10% ethylene glycol adipate.

Other materials and methods were the same as those described in our previous paper.⁵⁾

The acid urease from *S. mitior* was purified about 27-fold, with a recovery of 14.9% (Table I), by a multi-step procedure involving ethanol extraction, Toyopearl HW-55, DEAE-Sepharose CL-6B and affinity gel column chromatographic fractionations, and isoelectric focusing as described previously.⁵⁾ As shown in Fig. 1A, poly-

Table I. PURIFICATION OF ACID UREASE FROM *S. mitior*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Extraction	1150	19,500	16.9	1.0	100
Ethanol precipitation	436	17,700	40.6	2.4	90.8
Toyopearl HW-55F	105	11,900	113	6.7	61.0
DEAE-Sepharose CL-6B	56.0	9,850	176	10.4	50.5
First affinity gel	11.7	5,480	469	27.8	28.1
Chromatofocusing	NT ^a	3,490	NT	NT	17.9
Second affinity gel	6.3	2,900	458	27.1	14.9

^a NT, not tested.

Fig. 1. Polyacrylamide Gel Electrophoresis of Acid Urease from *S. mitior*.

(A) 6 μ g of each of the purified enzymes was electrophoresed on a 6% polyacrylamide gel. (B) 3 μ g of each of the purified enzymes was electrophoresed on a 15% SDS-polyacrylamide gel. Lanes: 1, *L. fermentum* IFO 14511⁶⁾; 2, *L. reuteri* Rt-5⁵⁾; 3, *S. mitior* PG-118.

acrylamide gel electrophoresis of the purified acid urease from *S. mitior* gave a single band. The mobility of the acid urease from *S. mitior* was different from that of *L. reuteri*⁵⁾ and from that of *L. fermentum*.⁶⁾

The molecular weight of the acid urease from *S. mitior* was about 200,000 by Sepharose CL-6B gel filtration. *S. mitior* acid urease was found to contain 3 kinds of subunits on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as shown in Fig. 1B. The molecular weights of the 3 subunits, α , β , and γ , of the acid urease from *S. mitior* were 66,000, 15,600, and 8,600, respectively, using a 15% acrylamide gel. The mobilities of the subunits of the acid urease from *S. mitior* were different from those of the acid urease from *L. reuteri* and from *L. fermentum* (Fig. 1B). The molecular weights of the 3 subunits, α , β , and γ , of the urease from *L. reuteri*⁵⁾ were 68,000, 16,100, and 8,800, respectively, and those of that from *L. fermentum*⁶⁾ were 67,000, 16,800, and 8,600, respectively. The approximate subunit ratio of the acid urease from *S. mitior* was 1:2:1, which was calculated from the integrated intensities of the gel scan profile and from calculations based on the molecular weight of 200,000 obtained on gel filtration chromatography and the molecular weights of the 3 subunits estimated by SDS-PAGE. The isoelectric point of *S. mitior* acid urease

Table II. AMINO ACID COMPOSITION OF ACID UREASE FROM *S. mitior*^a

Amino acid	Residues ^b per molecule	Mole percent (%)
Asp ^c	222.7	12.1
Thr ^d	137.3	7.4
Ser ^d	98.6	5.4
Glu ^c	156.8	8.5
Pro	76.9	4.2
Gly	187.8	10.2
Ala	150.8	8.2
H-cys ^e	23.0	1.2
Val	139.2	7.5
Met	63.4	3.4
Ile	112.1	6.1
Leu	114.9	6.2
Tyr	77.4	4.2
Phe	44.5	2.4
His	46.6	2.5
Lys	114.7	6.2
Arg	72.8	4.0
Trp	6.0	0.3

^a The values are the averages for 24-, 48- and 72-hr hydrolyses in 6N HCl containing 4% (v/v) thioglycolic acid at 110°C.

^b Calculations were based on a molecular weight of 200,000.

^c Free amino acid and corresponding amide.

^d Obtained by extrapolation to zero time of hydrolysis.

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

was pH 4.6 by isoelectric focusing.

The *S. mitior* acid urease also contained nickel, and the content was calculated to be 1.9 mol of nickel per mol of the $\alpha_1\beta_2\gamma_1$ structural unit. This indicates that this acid urease contained 2 mol of nickel per mol of the $\alpha_1\beta_2\gamma_1$ structural unit.

The amino acid composition of the acid urease from *S. mitior* is shown in Table II. This amino acid composition resembles those of the acid ureases from *L. reuteri*⁵⁾ and *L. fermentum*.⁶⁾

The optimum pH for the acid urease from *S. mitior* was around pH 4.5. The enzyme activity was maximum at 60 to 70°C, and was stable from pH 4 to 8 at 37°C for 30 min. When incubated for 30 min at pH 4, the enzyme activity was stable below 40°C.

The effects of metal ions (5.0 mM) on the enzyme activity of the acid urease from *S. mitior* were examined by adding metal ions, Mg²⁺, Ca²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Sr²⁺, Ag⁺, Sn²⁺, Ba²⁺, and Hg²⁺, to the standard reaction mixture. The activity was completely inhibited by Hg²⁺ and Ag⁺ and was reduced to 27% in the presence of Cu²⁺. The enzyme activity was strongly inhibited by sulphhydryl reagents such as *p*-chloromercuribenzoic acid

Table III. PREVENTION OF ETHYLCARBAMATE FORMATION IN SAKE^a

Urea (ppm)	Temperature (°C)	Ethylcarbamate (ppb)			
		Incubation time (months)			
		0	1	2	3
0 ^b	10	ND ^c	ND	ND	ND
	30	ND	ND	ND	ND
30	10	ND	ND	ND	ND
	30	ND	40	60	110

^a pH of the sake was 4.4 and the ethyl alcohol content was 19%.

^b Urea was eliminated by the action of 100 units/ml of *S. mitior* acid urease.

^c ND, not detected.

and by chelating reagents such as acetohydroxamic acid.

The acid urease was active against urea, but not against thiourea, methylurea, or ethylurea. This substrate specificity profile is very similar to those of the ureases from jack bean,⁹⁾ *L. reuteri*,⁵⁾ and *L. fermentum*.⁶⁾ The rate of hydrolysis of urea by the acid urease from *S. mitior* was measured at 37°C in 0.1 M citrate buffer, pH 4.5. Plots of the reaction velocity against urea concentration gave a normal Michaelis-Menten curve. The *K_m* for urea was 2.0 mM and the *V_{max}* was 460 units/mg.

The effect of temperature on the elimination of the urea in sake was examined using purified *S. mitior* acid urease. The urea was eliminated faster with increasing temperature up to 35°C, but the urea was not eliminated at 40°C. The

acid urease seems to be inactivated in sake at this temperature. The urea in sake was completely eliminated at 35°C in 4 days.

We examined the formation of ethylcarbamate using urea-free and urea-containing sake (Table III). When ethylcarbamate-free sake, which contained 30 ppm urea, was incubated at 30°C for 3 months, 110 ppb ethylcarbamate was formed. But, when the sake from which urea had been eliminated almost completely by the action of *S. mitior* acid urease was incubated at 30°C for 3 months, no ethylcarbamate was formed.

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