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Purification, Properties, and Application of a Novel Acid Urease from *Enterobacter* sp.

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Abstract It has been demonstrated that acid urease is capable of decomposing urea in fermented beverage and foods. As urea is a precursor of ethylcarbamate, a potential carcinogenic compound, measures must be taken to control the level of urea. We herein describe the purification and characterization of a novel acid urease from *Enterobacter* sp. R-SYB082 and its application to the removal of urea in Chinese rice wine. The enzyme was purified to electrophoretic homogeneity using ethanol precipitation, Superdex 200 and Mono Q with a fold purification of 21.1 and a recovery of 49%. The molecular weight of the enzyme was 430,000 Da by gel filtration and 72,000 Da by sodium dodecyl sulfate polyacrylamide gel electrophoresis, suggesting that it was a hexamer. The activity of this purified enzyme was optimal at pH 4.5 and 35 °C. The temperature stability was under 55 °C, and the pH stability was 4.0~5.0. The enzyme exhibited an apparent K_m of 19.5 μmol/l and a V_{max} of 109 μmol urea/mg·min at 35 °C and pH 4.5. When incubating two different kinds of Chinese rice wine with the enzyme (0.08 U/ml) at 35 °C for 7 days, over 85% of urea was decomposed, and at 20 °C, above 78% was removed. The result showed that the enzyme is applicable to elimination of urea in Chinese rice wine.

Keywords *Enterobacter* sp. · Acid urease · Ethylcarbamate · Urea · Chinese rice wine

Introduction

Ethylcarbamate, which is known to be carcinogenic, is thought to be formed by the reaction of urea with ethanol in fermented beverages and foods [1]. Several factors influencing EC formation was reviewed, and the urea concentration was proportional to the concentrations of EC during storage [2]. Therefore, controlling the urea concentration may be important in limiting ethyl carbamate levels [3]. Reductions in the level of urea formation can be

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achieved by minimal fertilization, utilizing urease and isolating strains that release less urea. Among them, the decomposition of urea by urease is the most specific and efficient [4].

Urease (urea amido hydrolase, EC3.5.1.5) catalyzes the hydrolysis of urea into ammonia and carbamate. The latter spontaneously decomposes to yield another molecule of ammonia and carbonic acid. To eliminate the urea in wines, the urease should have catalytic activity in low pH range (pH 4.0–6.0) and a tolerance to ethanol (<20%) [5, 7]. But the commonly found types of this enzyme functions optimally at neutral or slightly alkaline pH and is hardly active under acidic conditions [8]. To remove urea from the fermented products, such as the Chinese rice wine, the urease used should be active under acidic conditions (pH 3.0–5.0) in the presence of significant concentrations of ethanol (<20%). Concerns about high ethyl carbamate levels in sake resulted in the development of bacterially produced urease enzymes [9, 10]. The effectiveness of acid urease in wine depends strongly on the wine type in which it is used, and to some extent, conditions in wines such as fluoride, malate, ethanol, and phenolic compounds may inhibit its activity [11, 12].

We have isolated a new strain producing acid urease, classified as *Enterobacte*r sp. R-SYB082, from the dejection of rat. The crude enzyme showed high efficiency on the decomposition of the urea in Chinese rice wine. The objects of this study were to purify and characterize the acid urease from *Enterobacte*r sp.

Materials and Methods

Materials

All the chemicals were of analytical grade and mainly purchased from Sionpharm Chemical Reagent Co., Ltd, China, unless otherwise mentioned. Protein molecule weight marker and bovine serum albumin were purchased from Sigma Chemical Co., Ltd, USA.

Microorganism and Crude Enzyme Preparation

Enterobacter sp. R-SYB082 was used throughout this study. The seed culture medium (pH 7.0) was composed of 2% D-glucose, 1% peptone, 0.5% yeast extract, 0.2% KH_2PO_4, 0.5% NaCl, 0.2% NaAc, and 0.5% urea. The fermentation medium (pH 5.5) components are as follows: 2% D-glucose, 1% peptone, 0.5% yeast extract, 0.2% KH_2PO_4, 0.5% NaCl, 0.2% NaAc, and 0.5% urea, 0.005% $MnSO_4$, and 0.005% $NiSO_4$.

For seed preparation, the microorganism from a fresh slant tube was inoculated into 100 ml fresh seed medium/500 ml flasks and cultivated at 35 °C for 24 h. Seed culture (5%, *v*/*v*) was then inoculated into the fermentation medium (initial pH value of 5.5) for production of acid urease in fermenter. The cultivation was carried out at 35 °C. After 36 h, the culture broth was centrifuged at 8,000×*g* for 20 min at 4 °C to collect microorganism cells. The harvested cells were washed with 0.9% NaCl and 0.05 mmol/l citric acid buffer (pH 5.5).

About 50 g (wet weight) of cells was suspended in 500 ml 0.05 mmol/l citric acid buffer (pH 5.5) and disrupted with Vcx750 ultrasonic processor(Sonics & Materials, Inc., USA) at 40% intensity for 15 min. Samples were kept in ice bath during cell disruption to prevent overheating. The temperature inside the samples was checked during treatment, and it was always below 10 °C. The suspension was centrifuged for 20 min at 15,000×*g* to remove the cell debris, and the resulting suspension was used as the crude enzyme preparation.

Analytical Methods

Enzyme Assay and Definition of Units

The hydrolysis of urea was routinely followed by the determination of the ammonia formed. A 0.8-ml enzyme solution (1 mg/ml) was added to 0.2 ml of 3% (*w*/*v*) substrate solution (3 g urea in 100 ml 50 mmol/l sodium citric buffer, pH 4.5), and the mixture was incubated at 35 °C for 15 min, and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. Ammonia formed in the reaction mixture was spectrophotometrically determined by the method of Berthelot reaction [13]. One unit (U) of enzyme was defined as the amount that catalyzed the hydrolysis of 1 μmol of urea per min at 35 °C.

Protein Determination

The amount of protein was determined by Bradford method [14], with bovine serum albumin as the standard.

Enzyme Purification

Except for ethanol precipitation done at 4 °C, the enzyme was purified at room temperature (20 °C) using AKTA explorer 100 (Amersham Biosciences). During the chromatographic purification steps, proteins were monitored by the measuring of absorbance at 280 nm.

Ethanol Precipitation

Chilled ethanol was added dropwise to the crude enzyme solution with a constant stirring, and the precipitate at a final ethanol concentration of 10–30% (*v*/*v*) was collected by centrifugation at 10,000×*g* for 20 min, dissolved in 100 ml 50 mmol/l citric acid buffer, pH 5.5.

Superdex 200 Chromatography

The 10–30% ethanol fraction was loaded on Superdex™ 200 10/300 GL column, pre-equilibrated with 50 mmol/l citric acid buffer, pH 5.5, and eluted with the same buffer at a flow rate of 0.5 ml/min, and fractions with acid urease activity was collected for determination of enzyme activity and protein content. The fraction with acid urease activity were pooled and dialyzed at 4 °C overnight against 50 mmol/l sodium phosphate buffer, pH 6.8.

Mono Q Chromatography

The active pool from the previous step was loaded on Mono Q™ 4.6/100 PE column, pre-equilibrated with 50 mmol/l sodium phosphate buffer, pH 6.8. The column was washed extensively with the same buffer and eluted with a linear gradient of 0.0–0.6 M NaCl with the same buffer at a flow rate of 1.5 ml/min. Fractions with acid urease activity were pooled and dialyzed overnight against 50 mmol/l citric acid buffer, pH 5.5, to determine the enzyme activity and protein content.

Electrophoretic Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) was carried out according to Laemmli [15] on a discontinuous gel containing 5% stacking gel (pH 6.8) and 12% resolving gel (pH 8.8), respectively. Electrophoresis was performed with 45 V fixed voltage. The proteins were stained with solution of Coomassie Brilliant Blue (CBB) R-250.

Relative Molecular Weight Estimation

The apparent molecular weight was determined by SDS-PAGE as described above, using low-range SDS-PAGE molecular weight standards purchased from Bio-Rad, USA: phosphorylase b (94,000 Da), serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da). Proteins were stained with CBB R-250, and the mobility was plotted against the molecular weight of proteins on a semilogarithmic scale.

To estimate native molecular weight, gel filtration in native conditions was carried out by using Superdex™ 200 10/300 GL column equilibrated with 50 mmol/l sodium phosphate buffer at pH 7.2, containing 0.15 mmol/l NaCl and calibrated with the Bio-Rad protein standards: thyroglobulin (669,000 Da), ferrtin (440,000 Da), fibrinogen (341,000 Da), and aldolase (158,000 Da). Equilibration, loading, and elution of the column were performed at a constant flow rate of 0.5 ml/min at room temperature. The elution volume (Ve) of each marker and the enzyme was determined by measurement of the absorbance at 280 nm. The native molecular weight of acid urease was estimated according to calibration curves obtained by plotting the log molecular weight of each standard protein against the corresponding Ve.

N-Terminal Sequencing and Analysis

Based on Edman method, polypeptide chain is degraded and sequenced one-by-one through the cycles of three chemical reactions: PITC coupling with N-terminal amino acid, cyclization cracking, and transformation to phenylthiohydantoin (PTH)-amino acid. Then one new free amino acid residue is exposed. By identifying PTH-amino acid, the sequence is determined. All the procedure is operated by ABI Procise™ 492cLC.

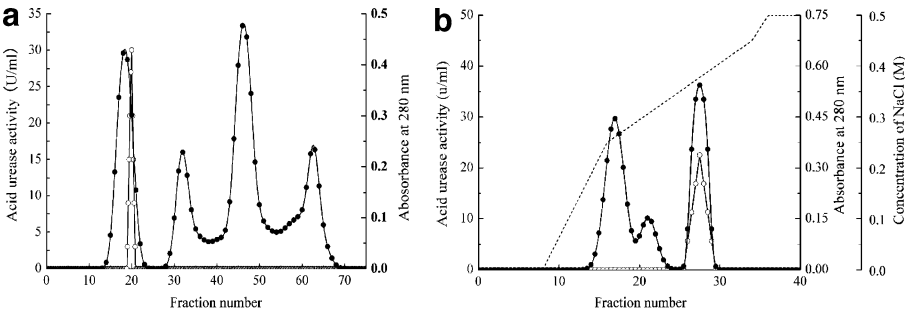

Fig. 1 Chromatography of crude acid urease from *Enterobacter* sp. R-SYB082 on Superdex 200 (**a**) and Mono (**b**)

Table 1 Summary of purification of acid urease from *Enterobacter* sp. R-SYB082.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme	1,453	300	4.8	100	1
10–30% ethanol precipitation	1,089	131	8.3	75	1.7
Superdex 200	938	17	58.1	68	12.1
Mono Q	610	6	101.7	42	21.1

Biochemical Characteristics of Acid Urease

The effect of pH on the enzyme activity was determined using the reaction mixtures as described previously except for with various buffers: pH 3.0–6.0, using 50 mmol/l citric acid buffer; pH 6.0–8.0, using 50 mmol/l sodium phosphate buffers. To check the pH stability, enzyme was pre-incubated with the above respective buffer at 35 °C for 30 min, and the assay of the remaining activity was carried out as described above. The effect of temperature on the enzyme activity was tested by assaying the activity at different temperatures in the range from 25 °C to 80 °C. Thermal stability was determined by pre-incubating the enzyme at various temperatures for 30 min, and the samples were placed in ice immediately. The remaining activities were then determined using the standard method described above.

Application in Chinese Rice Wine

The acid urease of *Enterobacter* sp. was added to two kinds of Chinese rice wine at a concentration of 0.08 U/ml and incubated at 20 °C and 37 °C. At appropriate time intervals, partial urea were decomposed, and the remaining urea content was determined by diacetylmonoxime reaction [16].

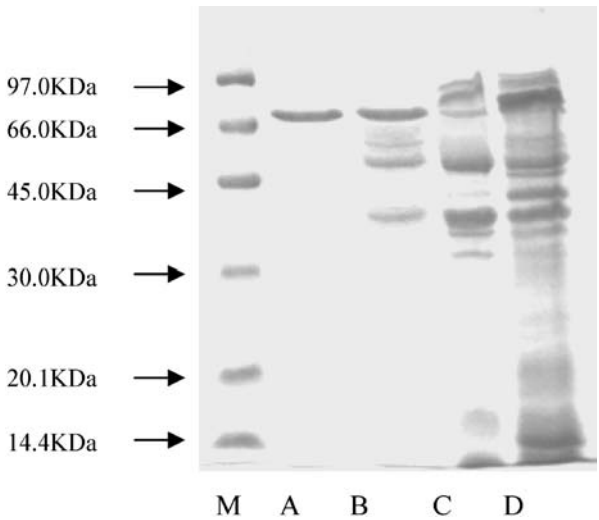

Fig. 2 SDS-PAGE of acid urease; *lane M* standard marker, *lane A* urease after Mono Q, *lane B* urease after Superdex 200, *lane C* urease after ethanol precipitation, *lane D* crude enzyme

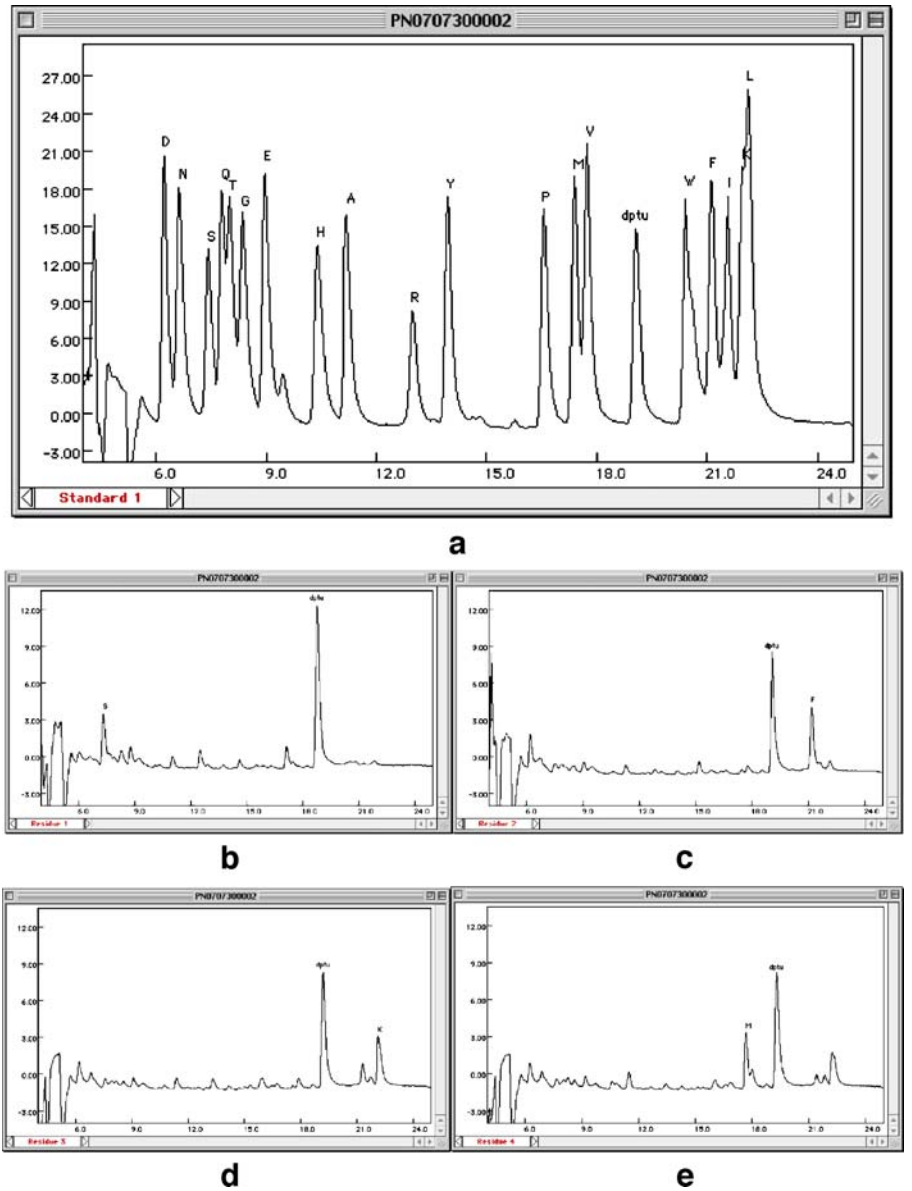

Fig. 3 **a** Amino acid standard, **b** residue 1-S, **c** residue 2-F, **d** residue 3-K, **e** residue 4-M, **f** residue 5-D, **g** residue 6-R, **h** residue 7-K, and **i** residue 8-Q

Result and Discussion

Purification, Molecular Weight, and Subunit Structure Determination

The acid urease was purified by the three-step procedure described in "Materials and Methods." In the first step, the crude enzyme was precipitated with ethanol. The 10–30%

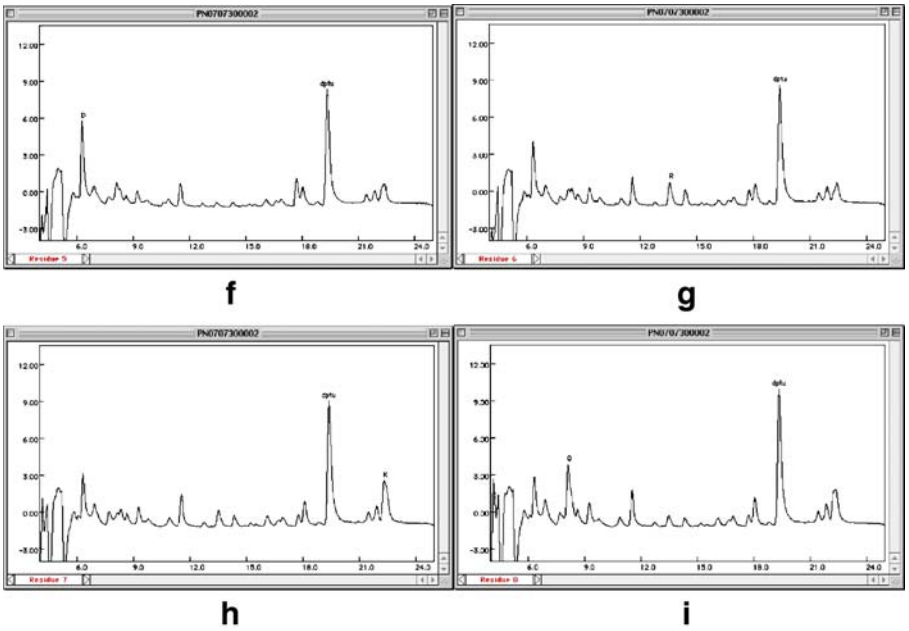

Fig. 3 (continued)

(*v*/*v*) ethanol fraction showed higher specific activity (8.3 U/mg). The 10–30% (*v*/*v*) ethanol fraction was then successively subjected to gel filtration on a Sephadex 200 column and to ion exchange chromatography on Mono Q column. The elution profiles of proteins and protease activities are shown in Fig. 1. The results of the purification procedure are summarized in Table 1. After the final purification step, the enzyme was purified 21.1-fold with a recovery of 42% and a specific activity of 101.7 U/mg. The purified acid urease was homogenous on SDS-PAGE (Fig. 2).

The relative molecular weight of native acid urease was estimated by gel filtration on a Superdex 200 column using AKTA explorer 100 system. The apparent molecular weight value obtained was 430,000 Da. A single protein band corresponding to a molecular weight

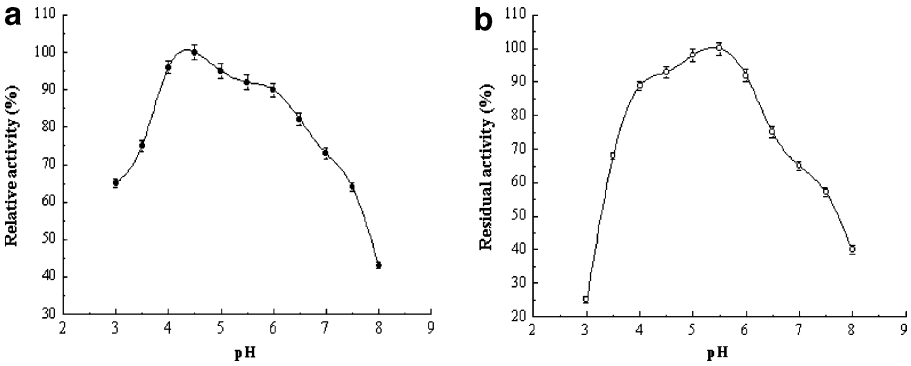

Fig. 4 Effect of pH on the activity (**a**) and stability (**b**) of purified acid urease from *Enterbacter* sp.

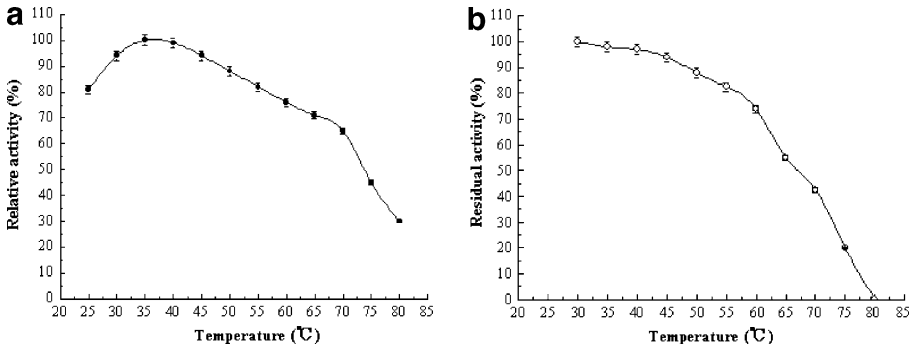

Fig. 5 Effect of temperature on the activity (**a**) and stability (**b**) of purified acid urease from *Enterbacter* sp.

of 72,000 Da was obtained by PAGE under denaturing conditions (Fig. 2). The results of gel filtration and SDS-PAGE demonstrate that the acid urease from *Enterobacter* sp. R-SYB082 is a homooligomeric enzyme of six identical subunits.

The quaternary structure of the enzyme is analogous to the well-studied plant enzyme, such as urease from jack bean (*Canavalia ensiformis*) and eucaryotic microorganisms, composing of six identical subunits [17, 18]. However, it is different from those of neutral and acid urease from known Gram-negative bacteria that possess two or three distinct subunits.

Transfer membrane is applied before N-terminal sequencing. Figure 3a–i shows the sequence of N-terminal initial amino acid residues.

The result clearly proves that the single band obtained by SDS-PAGE has reached electrophoretic purity, and N-terminal initial amino acid residues of this subunit are S-F-K-M-D-R-K-Q (Ser-Phe-Lys-Met-Asp-Arg-Lys-Gln).

Effect of pH on Enzyme Activity

The effect of pH on acid urease activity was determined using the reaction mixtures as described previously at pH 3.0–8.0 and at 35 °C. The enzyme exhibited optimum activity for the catalytic reaction of urea at pH 4.5 (Fig. 4a).The optimum pH of the enzyme from *Enterobacter* sp. was identical to that (pH 4.5) from *Streptococcus mitior* [19] and very close to that (pH 4.2) from *Arthobacter mobilis* [20] but higher than those (pH 2.0) reported for *Lactobacillus* enzymes. To test the stability of acid urease in buffers of different pH, the

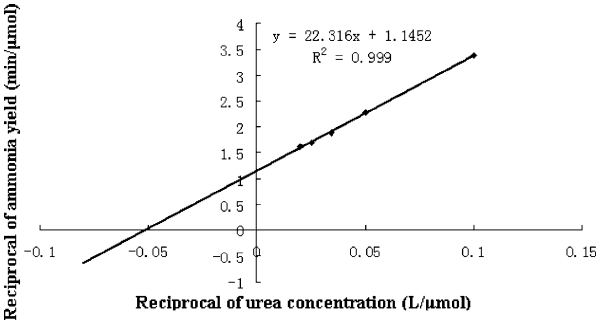

Fig. 6 Lineweaver–Burk plot of the purified acid urease

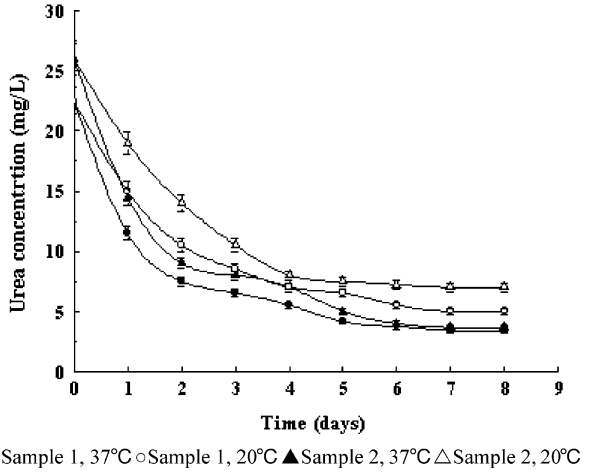

Fig. 7 Course of elimination of urea in Chinese rice wine

enzyme was incubated with buffers at 35 °C for 30 min, and the remaining activity was detected. Figure 4b shows that the enzyme is stable at pH 4.0–6.0. This feature makes it suitable for removing the urea in Chinese rice wine (pH 4.2).

Effect of Temperature on Enzyme Activity

The effect of temperature on the enzyme activity from 25 °C to 80 °C in a 50 mmol/l sodium citric buffer (pH 4.5) was shown in Fig. 5a. It can be seen that the enzyme showed the highest activity at 35 °C and retained more than 90% of its activity when temperature was below 50 °C.

Figure 5b represented the effect of temperature on enzyme stability of the enzyme. The results indicated that the enzyme was very stable below 50 °C. It was completely inactivated after incubation at 80 °C for 30 min.

Kinetics Parameters

The enzyme exhibited an apparent K_m for urea of 19.5 μmol/l and a V_{max} of 109 μmol urea/mg·min at 35 °C and pH 4.5, Fig. 6. Compared with other known acid ureases, such as *A. mobilis*, *Lactobacillus reuteri*, *L. fermentum*, and *S. mitior*, both of the values are considerably lower [6, 7, 19–21]. The lower K_m indicates that the acid urease has higher affinity to urea than those reported. Besides, in the study, we found that the novel acid urease has a tolerance at a certain extent to some compounds that existed in wine, including ethanol.

Application of the Enzyme to Elimination of Urea in Chinese Rice Wine

We applied the enzyme to two kinds of Chinese rice wine from different rice wine-making regions (Fig. 7). The concentration of the enzyme was 0.08 U/ml according to the study on model wine solution, which had the same pH, and ethanol and urea concentration with real rice wine. We found that 85% of urea in sample 1 and 92% of urea in sample 2 were decomposed after incubation at 35 °C for 7 days in comparison to those of 78% and 86% after incubation at 20 °C, respectively. The elimination effect was satisfying in Chinese rice

wine. Most of urea in the two samples could be effectively removed even at room temperature (20 °C). These results would generally be accepted by traditional Chinese rice wine makers for economical and practical reasons.

It was very interesting to note that the velocity of elimination of urea was very high in the first 2 days, and it became slow between the third and the seventh day, and the hydrolysis extent of urea could not be increased when the incubation time was increased from 7 to 8 days. It is partly because of the inactivation of the enzyme in such conditions, and the complex components in Chinese rice wine may have inhibited the enzyme activity.

Conclusions

In this report, an acid urease was obtained from *Enterobacter* sp. The gel filtration and SDS-PAGE revealed that the enzyme is a hexamer composed of six identical subunits. Urease is a high-molecular-weight, multimeric, nickel-containing enzyme. Eucaryotic microorganisms possess a homopolymeric urease, analogous to the well-studied plant enzyme composed of six identical subunits, such as urease from jack bean (*C. ensiformis*). Gram-positive bacteria may also possess homopolymeric ureases, but the evidence for this is not conclusive. In contrast, ureases from Gram-negative bacteria possess three distinct subunits with MW of 65,000 to 73,000 (alpha), 10,000 to 12,000 (beta), and 8,000 to 10,000 (gamma) [18]. The results above clearly demonstrate that the acid urease produced by *Enterobacter* sp. was different from that by *K. aerogenes* [22, 23]. Our studies indicated that the urease from *Enterobacter* sp. R-SYB082 was a novel acid urease.

Therefore, the conclusion can be drawn that acid urease derived from *Enterobacter* sp. exhibits its high rate of urea decomposition at acidic conditions and can be utilized for reduction of urea in Chinese rice wine.

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