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Characterization, stabilization and activity of uricase loaded in lipid vesicles

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ABSTRACT

Uricase-containing lipid vesicles (UOXLVs) were prepared by reverse-phase evaporation method with high efficiency and the characteristics of UOXLVs were described. The average size and zeta potential of UOXLVs obtained by the optimized formulation were 205.47 nm and −37.33 mV, respectively. Uricase was encapsulated in the alkaline aqueous phase of the lipid vesicle and the stability of its tetrameric structure was thus improved and its activity preserved. The storage stability of uricase in lipid vesicles was significantly increased compared to that of free uricase at 4 °C in borate buffer of pH 8.5. At 55 °C, free uricase was deactivated much more quickly especially at lower concentration predominantly due to enhanced dissociation of uricase into subunits. An intrinsic tryptophan of uricase recovered from the lipid vesicle thermally treated at 55 °C revealed that a partially denatured uricase molecule was stabilized through its hydrophobic interaction with lipid vesicle membrane. This interaction was depressed mainly by dissociation of uricase into subunits. At the physiological pH, significant increase of enzyme activity was found for the uricase entrapped in the lipid vesicles (1.8 times that of free uricase) at their respective optimum pH. The shift of optimum pH and increased uricolytic activity suggested the conformation change of the uricase during the entrapment process. The stability to proteolytic digestion was increased obviously by entrapping the uricase in the lipid vesicles. UOXLVs also showed relatively slower loss in activity compared with free uricase when treated with some chemical reagents. Lastly, *in vitro* study explicitly indicated that the uricase entrapped by UOXLVs possessed higher uricolytic activity than that of native uricase solution.

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1. Introduction

Uricase (urate oxidase, EC 1.7.3.3, UOX) could catalyze the oxidation of uric acid, a final product of purine catabolism, to allantoin which is more soluble and more easily excreted than the starting compound. Uricase has been administered intravenously for nearly 40 years, in patients to treat hyperuricemia caused by tumor lysis syndrome. Uricase was also used to treat gout, which is characterized by persistent hyperuricemia (Sherman et al., 2008; Paust et al., 2008).

The uricase molecule has a molecular weight of 120,000 and is composed of four identical subunits with a molecular weight of 30,000. The amino acid composition was determined and the N-terminal amino acid was identified as methionine (Nishimura et al., 1982). Under physiological conditions, however, the uricase application was limited due to its undesirable biochemical properties, such as low stability *in vitro* and *in vivo* (the uricase is readily deactivated) and relative low retention of enzyme activity (approximately 20–65% of the uricase activity remained in physiological pH 7.4 compared with the maximum activity at optimum pH of 8.5–9.2) (Bomalaski et al., 2002). Therefore, much attention has been devoted to developing the uricase preparations to overcome the shortcomings of the native form of uricase. The stability of uricase was improved by a variety of means: immobilized alginate microcapsules (Oloughlin et al., 2004), cross-linked uricase-albumin (Poznansky, 1979), immobilization of uricase in erythrocytes as carriers or coupling of uricase to the extracellular erythrocyte membrane (Ihler et al., 1975; Magnani et al., 1992), micromolecular conjugates of uricase with dextran and polyethylene glycol (Yasuda et al., 1990), and poly(ethylene glycol) modification of uricase (such as Puricase®). Nevertheless, disad-

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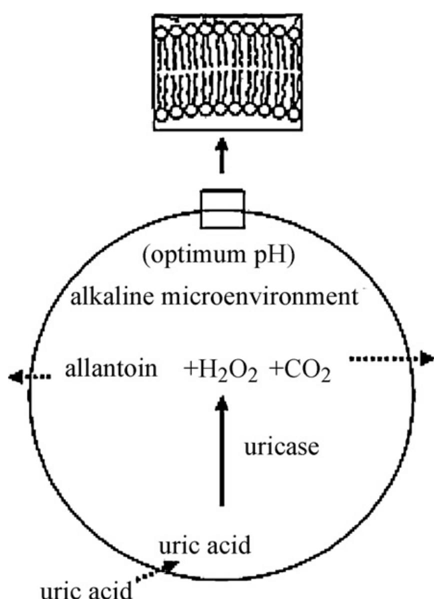

Fig. 1. Schematic representation of the principle of alkaline micro environmental lipid vesicles containing uricase.

vantages of the immobilized uricase are considered to be enzyme deactivation during immobilization reaction (including conformational change of the immobilized enzyme) or interaction with a hydrophobic interface. Other shortcomings, such as physical instability of the support to the oxygen bubbles produced (Sherman et al., 2004, 2008), low uricolytic activity at physiological pH, and readiness for uricase to fall off the vesicles, may also be involved.

The enzyme reactions occurring in lipid vesicles have received considerable attention because both stability and reactivity of the lipid vesicle enzyme are potentially regulated by keeping an intact molecular structure of the enzyme. The selective permeability of phospholipids bilayer membranes allows compartmentalization of biomacromolecules in lipid vesicles as well as modulation of apparent selectivity of the lipid vesicle enzyme to different substrates present in the liquid bulk (Yoshimoto et al., 2004). In addition, some interactions of lipid membranes with enzyme molecules were reported to stabilize the enzymatic activity (Kuboi et al., 1997). In immobilization of the UOXLVs through chemical bonding between lipid membranes and supports, no enzyme essentially underwent either deactivation or conformational change because the liposomal aqueous environment is rather independent of the environment outside the lipid vesicle (Yoshimoto et al., 2005). Oral administration to chickens of polyethylene glycol-modified uricase entrapped in liposomes produced a 50% fall in blood uric acid accompanied by a rise in plasma uricolytic activity (Nishida et al., 1984). The oxidation of glucose catalyzed by the liposomal glucose oxidase in the presence of liposomal catalase proceeded much more steadily than the reaction catalyzed by free enzyme systems (Yoshimoto et al., 2006). As far as we know, however, up to now there are few studies on the characteristics, stability and activity of the lipid vesicle loaded uricase considering its tetrameric structure as well as the lipid vesicle–uricase interaction.

In this work, alkaline micro environmental lipid vesicles containing uricase were designed (see Fig. 1) and prepared in an attempt that a general strategy to stabilize and deliver multimeric enzymes through entrapping them in the lipid vesicles might be affirmed. The UOXLVs were well characterized and studied in terms of the stability and activity at different temperatures and pH with an emphasis on the tetrameric structure of uricase and its interaction with lipid membrane, and the UOXLVs prepared under optimized conditions were expected to have higher stability and activity than that of free uricase at physiological condition (pH 7.4 and 37 °C).

2. Materials and methods

2.1. Materials

Uricase from Candida species (activity of 3.2 units/mg powder at 25 °C), fluorescein isothiocyanate (FITC) and bovine serum albumin (BSA) were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA). Hydrogenated soybean phosphatidylcholine was from Lucas Meyer GmbH (Hamburg, Germany). Cholesterol, an imported product, was obtained from Tianma Fine Chemical Plant (Guangzhou, China). Sephadex G-200 was obtained from Kaixiang Biotechnology Co. Ltd. (Shanghai, China). All other reagents and solvents used in the study were of analytical grade.

2.2. Preparation and characteristics of uricase-containing lipid vesicles

2.2.1. Basic protocol

Uricase-containing lipid vesicles (UOXLVs) were prepared by reverse-phase evaporation method. Briefly, hydrogenated soybean phosphatidylcholine (SPC) and cholesterol (CH) were dissolved in 30 mL chloroform solution with molar ration of 1:1. Chloroform was removed by rotary evaporation, and the lipid film obtained was redissolved in 30 mL diethyl ether. The organic phase was then added to 10 mL of 50 mmol/L borate buffer of pH 8.5, containing 16.7 nmol/L uricase and 14.7 nmol/L bovine serum albumin. The two-phase system was sonicated in a bath sonicator until the mixture became a stable and homogeneous opalescent dispersion. Then, the mixture was placed on the rotary evaporator and diethyl ether was removed under reduced pressure. As the majority of diethyl ether evaporated, the material formed a viscous gel, and then the gel collapsed into a lipid-in-water suspension. At this point, another 10 mL of the borate buffer (50 mmol/L, pH 8.5) was added, and the mixture was rotated under further decreased pressure for an additional 30 min to remove traces of the solvent. Size of uricase-containing lipid vesicles was homogenized by passing the sample through a 0.22 μm mixed cellulose filter.

2.2.2. Measurement of activity of UOXLVs and free uricase

The activity of uricase was measured with uric acid as substrate as follows. The uricase activity was assayed by a patented kinetic uricase method (Zhao et al., 2006). Briefly, 20 μL of uricase solution were added to the borate buffer solutions containing 75 μmol/L uric acid, and the overall uricase concentrations obtained were between 1.0 and 2.0 μg/mL. Then the time course of the decomposition of uric acid was determined based on the absorbance for uric acid at 293 nm with the molar extinction coefficient of $\varepsilon_{293\,\mathrm{nm}} = 11.5\,\mathrm{mol\,L^{-1}\,cm^{-1}}$ using a spectrophotometer. For lipid vesicle uricase, 1 mL of UOXLVs were added equal volume of chloroform, and then the mixture was swayed carefully and centrifuged at 3000 rpm for 15 min; the supernatant was taken out for further analysis in the same way as above.

2.2.3. Morphology, structure, size distribution, and zeta potential

Morphology and structure of lipid vesicles containing uricase–FITC conjugates were determined using Laser microdissection system (Leica AS LMD, Wetzlar, Germany) and photomicrographs were taken at suitable magnifications. The average diameter and polydispersity index of UOXLVs were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS90 (Malvern Instrument Ltd., Worcestershire,

UK). The zeta potential was performed with the same device (Koppel, 1972).

2.2.4. Determination of percent entrapment efficiency

Uricase-containing lipid vesicles were separated from free uricase by gel exclusion chromatography performed with a 15 mm × 400 mm Sephadex G-200 column. 100 μL of the entrapped uricase lipid vesicles was mixed with 200 μL of mixed solvents of isopropanol and ethanol (80:20), to form a clear solution. And then the protein content was determined by the Coomassie blue method initially reported by Bradford (1976) and modified by Georgiou et al. (2008).

Percentage entrapment was calculated using the following formula (Zhang et al., 2005):

$$\text{Percentage entrapment}\,(\%) = \frac{W_{\text{entrapped protein}}}{W_{\text{total protein}}} \times 100\%$$

where "$W_{\text{total protein}}$" and "$W_{\text{entrapped protein}}$" refer to the mass of the total protein and the entrapped protein detected in the lipid vesicle preparations respectively.

2.2.5. Measurement of FITC fluorescence

Free FITC molecules in an aqueous solution showed intense fluorescence. In contrast, the FITC molecules were found to show low fluorescence in lipid membranes in our experiment. The anionic FITC molecules are considered to be incorporated in the interfacial region of the membrane. The FITC fluorescence in lipid vesicle suspensions was measured in the presence and absence of uricase using a fluorescence Spectrophotometer (F-2500, HITACHI, Japan) to examine a lipid vesicle–uricase interaction. The lipid vesicles used contained no enzyme and had an average diameter of 200 nm. The interaction could be detected as an increase in the FITC fluorescence intensity of the lipid vesicle occurred due to a competitive interaction of the FITC and uricase molecules with lipid vesicle membranes. The measurements were performed by mixing 980 μL of a lipid vesicle suspension in the borate buffer and 20 μL of a borate buffer containing FITC to give the FITC concentration of 5.14 μmol/L. After incubating the lipid vesicle–FITC mixture for 5 min in the dark, the emission fluorescence intensity was recorded from 500 to 600 nm at the excitation wavelength of 480 nm. The excitation and emission slits were both 10 nm. The sample volume was 1.0 mL. All measurements were carried out in a cuvette with 1.0 cm optical length at 25 ± 0.5 °C. The effect of uricase was examined by incubating uricase (0.1 mg/mL) with lipid vesicles for 1 h at 25 °C before adding the FITC solution. The FITC-free lipid vesicles showed negligible fluorescence. To examine the uricase–FITC interaction, the FITC measurement was also performed in the absence of lipid vesicles at the uricase and FITC concentration of 0.1 mg/mL and 5.14 μmol/L.

2.3. Measurement of stability of UOXLVs and free uricase

2.3.1. Measurement of storage stability of UOXLVs and free uricase

The lipid vesicles and the free uricase (0.1 mg/mL) were stored in borate buffer (pH 8.5) at 4 °C. Then the activity of the lipid vesicle or free uricase was measured at designated time intervals with uric acid as substrate as described above.

2.3.2. Determination of optimum temperature of the UOXLVs and free uricase

The temperature effect on the activity of the free and lipid vesicles loaded uricase against uric acid was studied. The effect of temperature on UOXLVs and free uricase was investigated in the temperature range from 20 to 70 °C. The uricase activity was assayed with uric acid as substrate as described above.

The relative activity of the free uricase or the UOXLVs was defined as the percentage of its activity relative to the maximum activity of the free uricase.

2.3.3. Measurement of thermal stability of UOXLVs and free uricase

The lipid vesicles and the free uricase (5–100 μg/mL) were incubated at 55 ± 0.5 °C in tightly capped plastic tubes. The activity of each catalyst was successively measured at predetermined time intervals with uric acid as substrate as described above. For UOXLVs, the activity was measured after solubilization of lipid vesicles.

2.3.4. Measurement of steady-state fluorescence of uricase

The steady-state fluorescence was measured with the spectrofluorometer to evaluate the polarity of microenvironment of the tryptophan residues in uricase molecules. The excitation wavelength was set at 380 nm and the emission was scanned from 500 to 600 nm. The excitation and emission slits were both 10 nm. All the measurements were carried out in a cuvette with 1.0 cm optical length at 25 ± 0.5 °C. The lipid vesicles suspended in the borate buffer containing the overall uricase concentration of 0.3 mg/mL was incubated at 55 °C for 3 h. The steady-state fluorescence spectrum was measured for the sample of the above heat-treated lipid vesicles after solubilization of membrane with 5% Triton at 25 °C. The same measurement was also made for the sample of the intact UOXLVs before the heat treatment for comparison. For the free catalase in borate buffer of pH 8.5, its steady-state fluorescence was measured before and after the corresponding heat treatment in which the free uricase at the same concentration as the uricase concentration in UOXLVs, was incubated at 55 °C for 3 h.

2.3.5. Determination of optimum pH of UOXLVs and free uricase

The effect of pH on the free and the UOXLVs preparation was investigated in the pH range between 6.5 and 9.5 in borate buffers. The uricase activity was assayed by a patented kinetic uricase method described as above (Zhao et al., 2006).

The relative activity of the free uricase or the UOXLVs was defined as the percentage of its activity relative to maximum activity of the free uricase.

2.3.6. Measurement of pH stability of UOXLVs and free uricase

The lipid vesicles suspended in different borate buffer ranging from pH 5.0 to 9.5 containing the overall uricase concentration of 0.1 mg/mL was incubated at 40 °C for 3 h. For lipid vesicle uricase, equal volume of chloroform was added, and then the mixture was swayed carefully and centrifuged at 3000 rpm for 15 min; the supernatant was taken out for further analysis in the same way as describe above. The remaining activity of uricase was recorded.

2.3.7. Measurement of stability to proteolytic digestion

The UOXLVs and free uricase were treated with trypsin in 0.05 mmol/L borate buffer at 37 °C and pH 8.5 at the final uricase and trypsin concentration of 0.15 and 0.05 mg/mL, respectively. Aliquots were taken at various time intervals and the activity of uricase was measured.

2.3.8. Effect of various chemical agents on uricase activity

The UOXLVs and free uricase was treated with different chemical agents in 0.05 mmol/L borate buffer pH 8.5 and incubated for 1 h at 25 °C. The resulting uricase, D-sorbitol, sodium deoxycholate and mercaptoethanol were 0.15 mg/mL, 0.5 mmol/L, 0.5 mmol/L, and 0.05 mmol/L. Then the activity of uricase was measured followed by the method described above.

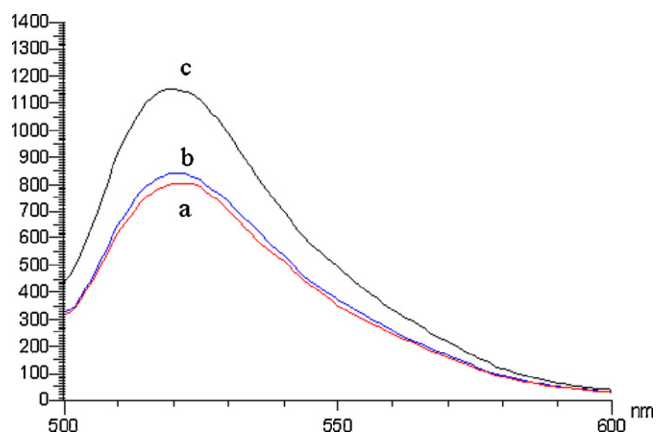

Fig. 2. FITC fluorescence spectra of (a) lipid vesicle, (b) lipid vesicles containing uricase and (c) uricase in the absence of lipid vesicles. Concentration of FITC was fixed at 5.14 μmol/L.

2.4. Uricolytic activity in vitro

300 μL of UOXLVs (containing 0.1 mg/mL uricase) prepared with borate buffer at pH 8.5, or 300 μL of native uricase solution (containing 0.1 mg/mL uricase dissolved in the borate buffer at pH 8.5) as controls, was added to 30 mL of pH 7.4 PBS containing 0.595 mmol/L of uric acid. Then the mixture was thermostatted at 37 ± 0.5 °C and shaken at 42 ± 3 revolutions/min. After 0, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 8, 16, and 24 h, 0.5 mL of medium was removed. Then the content of uric acid for each sample and the residual uric acid percent were calculated (Lu et al., 2003). The uric acid concentrations were determined according to the modified method (Stove et al., 2007; Zhao et al., 2006; Chuang et al., 2006): 0.5 mL of medium was mixed with 1 mL chloroform, then supplemented with 1 mL 8% perchloric acid (PCA) and 0.75 mL of 0.7 mol/L tripotassium phosphated. After centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant was analyzed. Uric acid analysis was performed by ultraviolet spectrophotometry at the maximum wavelength of 293 nm.

3. Results and discussion

3.1. Preparation and characteristics of UOXLVs

UOXLVs prepared by the reverse evaporation (REV) method were determined to have a high entrapment efficiency (64.27 ± 2.26%, $n = 3$), which might be resulted from a high aqueous space-to-lipid ratio in the vesicles. The entrapment efficiency of UOXLVs prepared here was much higher than those of other enzyme vesicles prepared by REV reported before (ranged from below 10% to more than 30%) (Walde and Ichikawa, 2001). The different entrapment efficiency might be due to the different type of enzyme entrapped, process parameter, and the size of resulting vesicles, etc.

Result of photomicrograph revealed that the UOXLVs were almost spherical in shape, having the appearance of a coated surface. The size of the optimized formulation was 205.47 ± 6.40 nm with the polydispersity index of 0.130 ± 0.02. The zeta potential of the optimized formulation was −37.33 ± 5.24 mV. Previous work has shown that negatively charged lipid vesicles (especially the charge value was about −30 mV) had good stability and then could be optimal for drug delivery (Zhang et al., 2007).

3.2. Interaction of uricase with lipid vesicle membranes

An interaction of uricase with lipid vesicle membrane was examined at 25 °C using enzyme-free lipid vesicles. Fig. 2 showed the FITC fluorescence spectra of the lipid vesicle suspensions with and without containing uricase. It was found that the FITC and uricase molecules competitively interacted with the interfacial region of zwitterionic lipid vesicle membranes through electrostatic and hydrophobic interactions (Domènech et al., 2007; Yoshimoto et al., 2007). As shown in Fig. 2, the smallest intensity of the FITC fluorescence was observed for an FITC–lipid vesicle in the absence of uricase (curve a in Fig. 2). This was because the lipid vesicles with the zeta potential −37.33 ± 5.24 mV were negative in charge and thus electrostatic repulsion occurred between the anionic FITC and the lipid vesicles. In the presence of uricase (curve b in Fig. 2), the fluorescence intensity of the lipid vesicle was slightly increased, indicating that the FITC–lipid vesicle interaction was inhibited by the presence of the uricase molecules interacting with the lipid vesicles. In Fig. 2 a much larger FITC fluorescence intensity was seen for the uricase solution in the absence of lipid vesicle (curve c in Fig. 2). The uricase–lipid vesicle interaction described above should occur in both outer and inner surface regions of the lipid vesicles. Considering that the UOXLVs were subjected to gel permeation chromatography for separating the lipid vesicle from free uricase, the amount of membrane-associated uricase in the lipid vesicle was considered to be much lower compared to that of the encapsulated one. In the lipid vesicle aqueous phase, the uricase molecules encapsulated are under a moderate interaction with the inner surface of the membranes.

3.3. Stabilities of UOXLVs and free uricase

3.3.1. Storage stabilities of UOXLVs and free uricase

As shown in Fig. 3, the activity of the free uricase and UOXLVs normalized by the initial activity was plotted as a function of the elapsed time of storage at 4 °C in the borate buffer (pH 8.5). Approximately 70% of the initial activity of free uricase was lost at the storage time of 28 days as shown in the figure. In our work, the stability of free uricase increased with increasing enzyme concentration up to 100 μg/mL (in the range of 5–100 μg/mL, data not shown). On the other hand, practically no deactivation of uricase was seen in Fig. 3 for UOXLVs during the storage period of 28 days. The UOXLVs showed a higher stability than free uricase. This comparison revealed that encapsulation of uricase in lipid vesicles was advantageous to maintain the activity of uricase at 4 °C. The interaction of the encapsulated uricase molecules with the lipid vesicle membrane was responsible for stabilizing the high concentrations of uricase.

3.3.2. Determination of optimum temperature of the UOXLVs and free uricase

Although significant increase of enzyme activity was found for the uricase entrapped in the lipid vesicles at every identical tem-

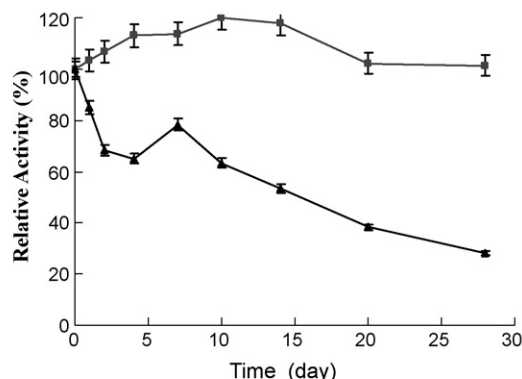

Fig. 3. Time courses of remaining activity of UOXLVs (closed squares) and free uricase (closed triangles) during storage at 4 °C.

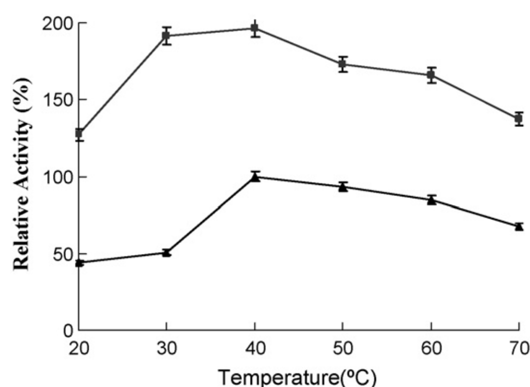

Fig. 4. Temperature profiles of free and lipid vesicle loaded uricase. The temperature effect on the activity of UOXLVs (closed squares) and free uricase (closed triangles).

perature, the optimum temperature of UOXLVs and free uricase were the same as 40 °C (Fig. 4). The temperature range at which UOXLVs remained high activity was larger than that of free uricase. The UOXLVs remained higher activity at the temperature from 30 to 60 °C (from 167% to 197%) than that at temperature at 20 °C (127%), while the free uricase remained higher activity at the high temperature from 40 to 60 °C (more than 85%) than that at low temperature from 20 to 30 °C (less than 50%).

3.3.3. Thermal stabilities of UOXLVs and free uricase

Fig. 5 showed the time courses of the remaining activity of free uricase incubated at 55 °C at the enzyme concentrations of 100, 50, 10, and 5 μg/mL. The remaining activity was calculated as percentage of the activity of free uricase solution at each elapsed time to the initial activity of free uricase solution. Regardless the concentration of uricase, the remaining enzyme activity decreased with the incubation time elapsed. This indicated that the enzymatically active tetrameric form of uricase was increasingly dissociated into inactive dimmers or subunits as observed for other oligomeric proteins (Attwood and Geeves, 2002; Reddy et al., 1999). This change in the quaternary structure was reported to be reversible (Poltorak et al., 1998). During the experiment time of 5 h, the appearance of the uricase solution kept clear and no aggregation was visually observed. In case of catalase, at 5 h incubation at 55 °C, an aggregation was visually observed in the enzyme solution containing 16 and 5 mg/mL catalase, and the conformational change in the tertiary structure of subunits was considered to be responsible for the formation of irreversible intermolecular aggregate observed

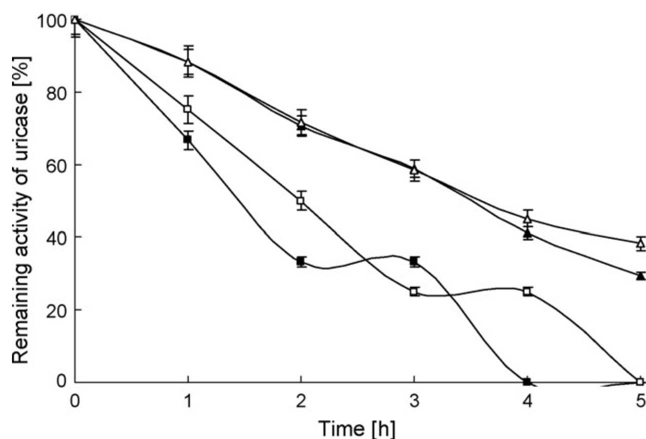

Fig. 5. Time courses of remaining activity of free uricase at enzyme concentrations of 100 μg/mL (open triangles), 50 μg/mL (closed triangles), 10 μg/mL (open squares), 5 μg/mL (closed squares) at 55 °C.

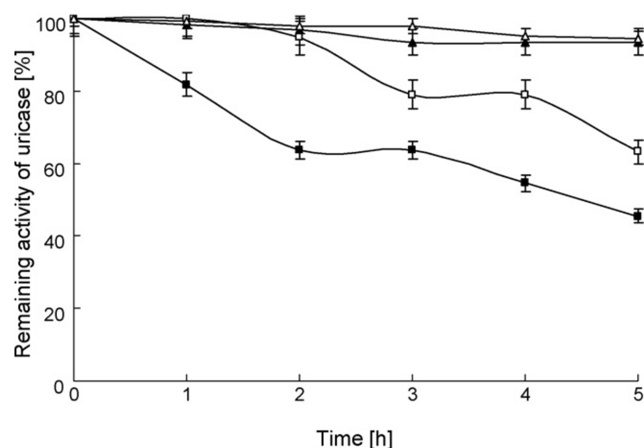

Fig. 6. Time courses of remaining activity of UOXLVs at enzyme concentrations of 100 μg/mL (open triangles), 50 μg/mL (closed triangles), 10 μg/mL (open squares), 5 μg/mL (closed squares) at 55 °C.

(Hashemnia et al., 2006). The difference of the uricase and catalase solutions' appearance in the thermal stability experiments was partly due to the different concentration of the enzyme (the concentration of catalase from 5 to 16 mg/mL was much higher than that of uricase from 5 to 100 μg/mL), the different type of the enzyme and the different deactivation mechanism (the changes in the quaternary and tertiary structures were responsible for the enzyme aggregates at high catalase concentration and for the enzyme deactivation at low uricase concentration here). An important feature seen in Fig. 5 was that the thermal deactivation rate of free uricase depended on the enzyme concentration. The lower the concentration of uricase, the larger the extent of the enzyme deactivation at 55 °C. The free uricase at 5 μg/mL was rapidly deactivated to about 30% of the initial activity within an incubation time of 2 h, while more than 70% of the initial enzyme activity remained for the uricase at 100 μg/mL in the identical incubation time. The rapid loss of activity observed for 5 μg/mL free uricase suggested that the dissociation of the tetrameric enzyme into its dimmers or subunits was pronounced at such a low enzyme concentration for the tetrameric enzyme urate oxidase. It was suggested that the predominant deactivation mechanism was uricase deactivation at the concentration from 5 to 100 μg/mL. Consequently, there should be an optimal catalase concentration for stabilizing the enzyme activity at 55 °C through maintaining its intact quaternary structure and avoiding the formation of the aggregates.

Fig. 6 showed the stability of UOXLVs incubated at 55 °C at various enzyme concentrations (100–5 μg/mL). In the figure, the UOXLVs at the lowest uricase concentration (5 μg/mL) exhibited the lowest stability among the UOXLVs suspensions examined. The stability of the lipid vesicle uricase was much less than that of free uricase shown in Fig. 6. The free uricase at the same concentration (in Fig. 5) showed much lower stability than the UOXLVs (in Fig. 6). This result was derived from a peculiar feature of the lipid vesicle system, i.e., in the lipid vesicle system, its aqueous microenvironment had local high enzyme concentration essentially irrelevant to the liquid bulk surrounding lipid bilayer membrane. The decreased stability observed for the lowest uricase concentration of UOXLVs were probably related to the distribution of uricase molecules in the lipid vesicle system. Namely, a part of uricase molecules were incorporated in the lipid bilayer membranes facing to the liquid bulk as indicated by the FITC fluorescence measurement (Fig. 2) for detecting the interaction of uricase with the interfacial region of the lipid membranes. This form of the lipid vesicle uricase molecules should behave like free uricase. At high uricase concentrations of UOXLVs, the uricase molecules dissociated from the lipid membranes could be stabilized through the interaction with the outer

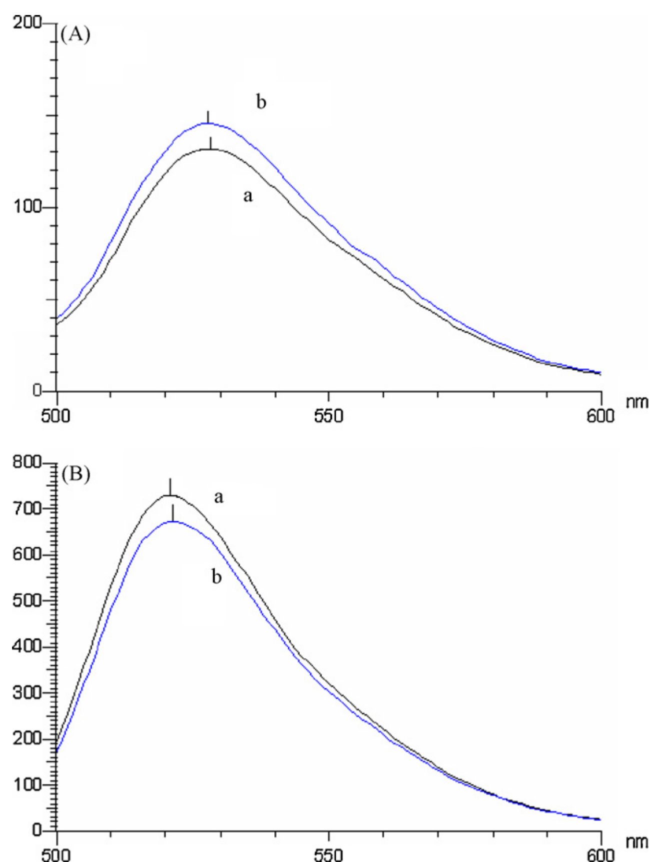

Fig. 7. Steady-state fluorescence spectra of (A) uricase released from lipid vesicle and (B) free uricase measured at fixed uricase concentration of 0.3 mg/mL. Curves a and b in each figure show spectra measured before and after heat treatment, respectively. For heat treatment, free uricase solution and UOXLVs were incubated at 55 °C for 3 h. Fluorescence measurements of uricase in UOXLVs were performed after solubilization of lipid vesicles with 5% Triton-100. Position of λ_{max} for each spectrum was indicated by vertical line.

surface of the suspended lipid vesicles. At low UOXLVs concentrations, on the other hand, the uricase molecules in the liquid bulk might be readily deactivated due to their insufficient interaction with the lipid membranes. Although the amount of the membrane-incorporated uricase was difficult to quantitatively determine, the uricase molecules entirely encapsulated in the lipid vesicle aqueous phase were suggested to possess a higher intrinsic thermal stability than the other lipid vesicle uricase molecules including the membrane-incorporated ones. The results in Fig. 6 implied that there was an optimal uricase content in a lipid vesicle to maintain the activity of uricase at 55 °C, as in the case of the thermal stability of free uricase (Fig. 5). Among the four concentrations of UOXLVs examined, the UOXLVs at the uricase concentration of 100 and 50 μg/mL provided the higher appropriate internal aqueous phase condition for the activity of uricase, and showed much higher stability than that of free uricase at the corresponding concentration (closed and open triangles in Fig. 5, respectively). The interaction between uricase and the inner surface region of lipid membranes contributed to stabilization the high concentration of uricase.

3.3.4. Structure of UOXLVs and free uricase

The steady-state fluorescence was examined for uricase to see if a change in its tertiary structure determining the quaternary one could be correlated with the thermal stability of the enzyme. Fig. 7 showed the steady-state fluorescence spectra measured for the free uricase and the uricase released from the lipid vesicle with 5% Triton-100. The measurements were performed before and after their incubation at 55 °C for 3 h. For free uricase (Fig. 7B), the fluorescence intensity was significantly decreased after the heat treatment compared to the intensity obtained before the treatment. In addition, a slight red shift in the maximum wavelength of the fluorescence λ_{max} was seen for the heat-treated free uricase. These results indicated that the microenvironment of the aromatic groups, typically the tryptophan residues, irreversibly became more hydrophobic at 55 °C due to intermolecular hydrophobic interactions. This was supported by the observation that the precipitated aggregates were formed in the heat-treated free uricase solution. On the other hand, the uricase released from the heat-treated UOXLVs showed a slight blue shift of the λ_{max} value accompanying an decrease in the fluorescence intensity, as compared to the enzyme from the intact UOXLVs (Fig. 7A). It should be noted that practically no increase in turbidity was observed in the uricase solution obtained by solubilizing the heat-treated UOXLVs membrane compared to the turbidity of the solution obtained by solubilizing the intact UOXLVs. These results indicated that a conformational changed uricase molecules induced by the heat treatment were stabilized in lipid vesicles so that the formation of intermolecular aggregates of uricase was prevented. It was reported that the partial conformational change of proteins induced the exposure of hydrophobic clusters to the aqueous phase (Prajapati et al., 1998; Mei et al., 1997). This intermediate state of protein was reported to interact with lipid bilayer membranes (Kuboi et al., 1997; Roberts et al., 2005). These previous observations support the assumption that the partially denatured uricase molecules at 55 °C form a complex with liposomal membranes. In this case, the hydrophobic interaction between uricase and the membranes might play a crucial role because the enzyme is conformationally changed as described above and the lipid membranes of the UOXLVs were electrically neutral as a whole. The thermal stability of the liposomal uricase was considered to be determined by a balance between the following three major interactions: intersubunit interaction keeping the tetrameric form of active uricase; interaction between conformationally changed uricase molecules triggering the formation of inactive aggregates and interactions of the conformationally changed uricase with lipid membranes. The intrinsic fluorescence and turbidity measurements reveal that the interaction of the conformationally changed uricase with lipid membranes had the most predominant influence on the thermal stability of the uricase in UOXLVs.

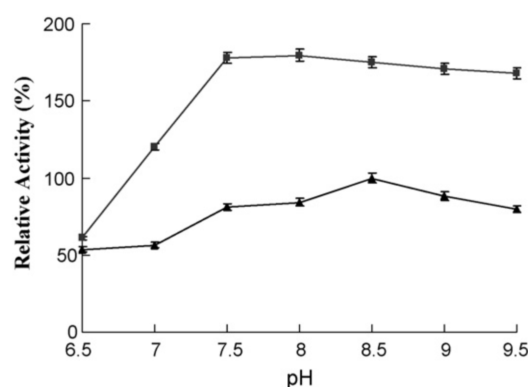

Fig. 8. pH profiles of free and lipid vesicles loaded uricase. The pH effect on the activity of UOXLVs and free uricase (closed squares and closed triangles, respectively).

3.3.5. Determination of optimum pH of the UOXLVs

Initial rate of absorbance change at 293 nm was measured at 37 ± 0.5 °C using 75 μmol/L uric acid, and one unit of uricase activity was the amount of uricase able to oxidize 1 μmol/L of uric acid/min using an absorptivity of 11.5 $\text{mmol}^{-1}\,\text{L}\,\text{cm}^{-1}$. As shown in Fig. 8, UOXLVs did shift the optimal pH of enzyme activity from 8.5 (for free uricase) to 8.0. The activity was almost the same from

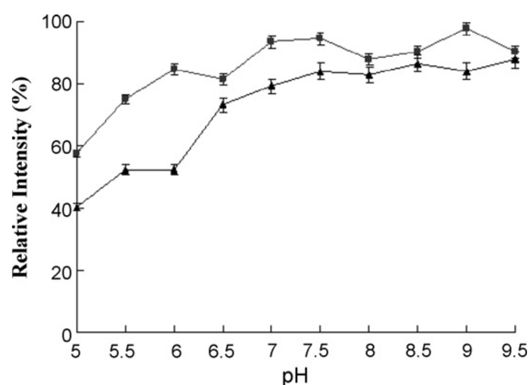

Fig. 9. pH stabilities of UOXLVs and free uricase (closed squares and closed triangles, respectively).

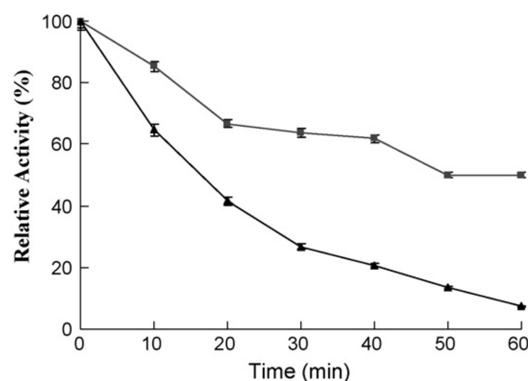

Fig. 10. Proteolytic stabilities of lipid vesicles contained uricase and free uricase (closed squares and closed triangles, respectively).

7.5 to 8.0 for the uricase loaded in the lipid vesicles. Very interestingly, significant increase of enzyme activity was found for the uricase entrapped in the lipid vesicles (1.8 times that of free uricase) at their respective optimum pH. The shift of optimum pH and increased uricolytic activity suggested the conformation change of the uricase during the entrapment process. At the physiological pH (around 7.5), free uricase remained 81.16% of its maximum activity, while the uricase loaded in the lipid vesicles remained almost the same high activity (178.26%) as its optimum activity (179.72%). In our experiment, the remaining enzyme activity of free uricase from *C. utilis* was higher than that reported before (approximately 65% of the uricase activity remained in physiological pH 7.4 compared with the maximum activity in optimum pH of 8.5) (Bomalaski et al., 2002). The difference of enzyme activity might be due to the different incubation temperature (24 °C was used before and 37 °C was used here).

3.3.6. Measurement of pH stabilities of UOXLVs and free uricase

The lipid vesicles suspended in different borate buffer with pH ranging from 5.0 to 9.5 containing the overall uricase concentration of 0.1 mg/mL were incubated at 40 °C for 40 min. The remaining activity of uricase was recorded at the optimum pH 8.5 and optimum temperature of 37 °C. As shown in Fig. 9, the UOXLVs remained higher activity than free uricase at every identified time. In case of UOXLVs, the remaining activity kept more than 90% during the pH of 7–9.5, and the maximum remaining activity was 98.04% at pH 8.0. While for the free uricase, though the remaining activity kept higher at alkaline condition (pH 7–9.5) than those at weak acidic conditions (pH of 5–6.5), the maximum remaining activity was 86.59% at pH 8.5. That was to say, both UOXLVs and free uricase kept good pH stability at their optimum pH, and these results also indicated that the conformation of UOXLVs and free uricase was stable at their optimum pH. The UOXLVs kept higher activity (94.57%) than free uricase (84.15%) at physiological pH.

3.3.7. Stability to proteolytic digestion

The UOXLVs and free uricase were treated with trypsin in 0.05 mmol/L borate buffer at 37 °C and pH 8.5. As shown in Fig. 10, free uricase showed rapid decrease in its enzymatic activity with a half-life of less than 20 min when incubated with trypsin. On the other hand, UOXLVs gradually lost their activity but still 50% of the original activity remained after 60 min (remaining activity was 7.32% in case of free uricase). Thus uricase was stabilized by entrapping into the lipid vesicles.

3.3.8. Effect of various chemical agents on uricase activity

The UOXLVs and free uricase were treated with several chemical agents in 0.05 mmol/L borate buffer pH 8.5 and incubated for 1 h at 25 °C. As shown in Fig. 11, D-sorbitol, sodium deoxycholate and mercaptoethanol had obvious effect on the free uricase, and among them, mercaptoethanol had the maximum effect (uricase showed only 60% of the original activity after incubation with mercaptoethanol). On the other hand, UOXLVS showed relatively slower activity loss compared with free uricase. The results showed that uricase was stabilized by incorporating into the lipid vesicles.

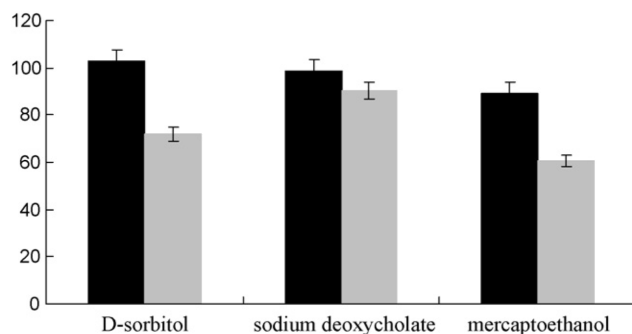

Fig. 11. Effect of several chemical agents on uricase activity of UOXLVs and free uricase (black columns and grey colomns, respectively).

3.4. Uricolytic activity in vitro

The enzymatic activities of UOXLVs were measured using the ability of uricase to decrease the concentration of uric acid at pH 7.4 (see Fig. 12). The same amount of UOXLVs or free uricase solution (both contained 0.1 mg/mL uricase) was placed into an

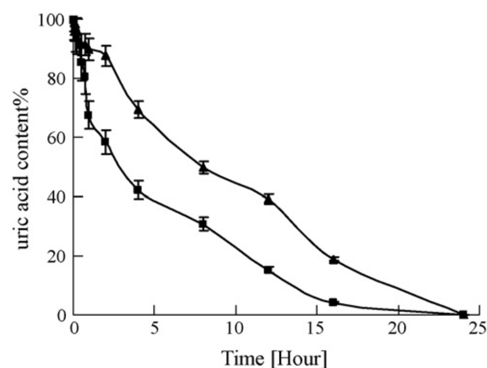

Fig. 12. Uricolytic activity of uricase preparations *in vitro*. The uricolytic activity of UOXLVs prepared with borate buffer of pH 8.5 in external medium phosphate buffer pH 7.4 containing of 0.595 mmol/L of uric acid at the beginning (closed squares). The uricolytic activity of native uricase in external medium of phosphate buffer pH 7.4 containing of 0.595 mmol/L of uric acid at the beginning (closed triangles).

external medium at the physiological pH 7.4. Compared with native uricase solution (from 11.56 h), it took about one half time (6.17 h) for uric acid decreasing from 0.595 mmol/L to normal levels (generally defined as $\leq$0.24 mmol/L) when the same dosage of UOXLVs were given. *In vitro* study explicitly indicated that the uricase entrapped by UOXLVs possessed higher uricolytic activity than native uricase solution. The reason might be that the micro environmental pH value of the vesicles was equal to the optimum pH of the uricase in UOXLVs determined as about pH 8.0. The UOXLVs were more favorable for efficiently utilizing enzyme from an economical viewpoint. Therefore, it was evident that the UOXLVs were more effective than free uricase. The lipid vesicles containing uricase were thus expected to be a more efficient enzyme delivery system.

4. Conclusion

The uricase-containing lipid vesicles with high entrapment efficiency were prepared and characterized in terms of their stability and activity. The storage stability of the UOXLVS at 25 °C was higher than that of free uricase. At 55 °C, the thermal stability of free uricase was significantly decreased with decreasing concentration mainly due to the dissociation of the tetrameric uricase into subunits. These results as well as the intrinsic fluorescence measurements of the uricase in the lipid vesicles indicated that the lipid membranes interacted with partially denatured uricase molecules, which prevented the formation of an intermolecular inactive aggregate of uricase. The storage stability of the UOXLVs was affected by the favorable uricase concentrating effect of lipid vesicles and uricase–lipid membrane. The proteolytic stabilities were increased obviously by entrapping the uricase in the lipid vesicles. UOXLVs showed relatively slower activity loss compared with free uricase when they were treated with some chemical reagents such as D-sorbitol, sodium deoxycholate and mercaptoethanol. All the results obtained showed that the UOXLVs were advantageous to maintain the tetrameric structure and activity of the encapsulated uricase not only when storing even at 55 °C but also when catalyzing the decomposition of uric acid at 37 °C. *In vitro* study explicitly indicated that the uricase entrapped by UOXLVs possessed higher uricolytic activity than native uricase solution. The UOXLVs are promising and need to be studied extensively which may ease the pressure of developing new uricolytic enzymes and enzyme preparations.

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