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ENZYMATIC PROPERTIES OF MICROCAPSULES CONTAINING ASPARAGINASE*

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SUMMARY

Nylon and polyurea microcapsules containing asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) were prepared by the interfacial polymerization method. The activities of nylon and polyurea microcapsules containing asparaginase were 37% and 42%, respectively, compared with that of native asparaginase.

The pH optimum of the microencapsulated enzymes was the same as that of native enzyme, although the pH–activity curves of native asparaginase were somewhat restricted by microencapsulation. The optimum temperature was lowered by microencapsulation. The apparent Michaelis constants of both microencapsulated enzymes were about 100 times higher than that of the native one. No marked difference of stability in prolonged storage was observed, whereas both microencapsulated asparaginases were found to be resistant to repeated uses and attack of proteolytic enzymes.

INTRODUCTION

Recently, microencapsulation of enzymes has been noted as one of the potential methods to solve some troubles in enzyme therapy such as production of antibody and loss of effectiveness of enzyme administered. In the case of microencapsulated enzymes, they do not leak out but still act efficiently on external permeable substrates, since the enzymes are encapsulated by the semipermeable polymer membrane. Thus, there is the possibility that the above troubles in enzyme therapy might be overcome by use of microcapsules containing enzymes. From these points of view, a number of microencapsulated enzymes have been prepared and studied as therapeutic agents.

Chang and his coworkers successfully prepared the microcapsules containing enzymes[1,2], and developed the interesting application of microcapsules in physi-

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ology[3,4]. Kitajima *et al.*[5] and Boguslaski and Janik[6] also reported on the preparation and some properties of microcapsules containing enzymes.

However, little is known about the enzymatic properties of these microencapsulated enzymes. In the foregoing paper[7], we reported on the detailed conditions for the preparation of nylon microcapsules containing asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) which has been used for the suppression of lymphosarcoma. In this paper we describe the preparation of more enzymatically active microcapsules containing asparaginase, with polyurea or nylon membranes, and the enzymatic properties of both microencapsulated asparaginases were investigated and compared with those of native asparaginase.

MATERIALS AND METHODS

Materials

Asparaginase was prepared from *Proteus vulgaris* according to the method of Tosa *et al.*[8], and its activity was 70 μmoles/min per mg of the preparation by standard enzyme assay.

Chymotrypsin (bovine pancreas) and trypsin (bovine pancreas) were obtained from Sigma Chemical Company, U.S.A., and Pronase-P (bacteria) was obtained from Kaken Kagaku Co., Tokyo, Japan.

Sebacoylchloride, 1,6-hexanediamine and 2,4-toluenediisocyanate were obtained from Tokyo Kasei Kogyo Co., Tokyo, Japan. Span 85 (detergent) was obtained from Kao-Atlas Co., Tokyo, Japan.

Preparation of nylon microcapsules containing asparaginase

The preparation of nylon microcapsules containing asparaginase was carried out under the optimal conditions described in the previous paper[7]. The method is as follows. To the mixed solution (pH 8.4) containing 1 mg of asparaginase, 10 mg of casein, 50 μmoles of L-aspartic acid and 800 μmoles of 1,6-hexanediamine, in a total volume of 3.5 ml, was added 20 ml of mixed solvent (cyclohexane–chloroform; 5:1, v/v) containing Span 85. The mixed solution was then mechanically emulsified at 4 °C. With stirring, 15 ml of the mixed solvent containing 450 μmoles of sebacoylchloride was added and stirred for 3 min. The resulting microcapsules were collected by filtration, then washed with ethanol and water. The activity of nylon microcapsules obtained was 37% compared with that of native asparaginase.

Preparation of polyurea microcapsules containing asparaginase

Polyurea microcapsules containing asparaginase were prepared by the same method as in the case of nylon microcapsules except for using 1400 μmoles of 2,4-toluenediisocyanate in place of sebacoylchloride. The activity of the polyurea microcapsules obtained was 42% relative to that of native asparaginase. The particle size of microcapsules could be controlled, and they became smaller at the higher rate of stirring and concentration of Span 85 in a first emulsification step as reported in the previous paper[7]. A photomicrograph of polyurea microcapsules containing asparaginase is shown in Fig. 1.

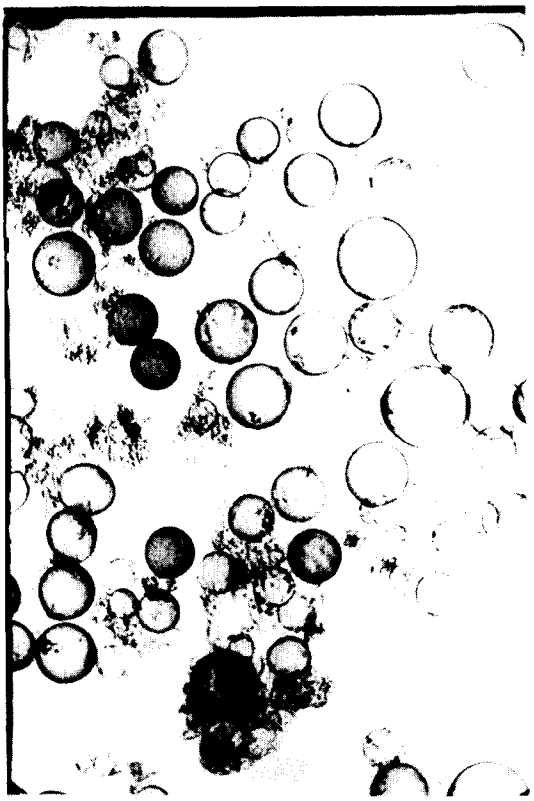

Fig. 1. Photomicrograph of polyurea microcapsules containing asparaginase.

Standard enzyme assay of native asparaginase

Except for varying the pH of the enzyme reaction from 8.4 to 8.0 and the amount of L-asparaginase from 50 μmoles to 100 μmoles, the enzyme reaction of native asparaginase was carried out by the method previously described[7]. The enzyme activity was expressed in μmoles of ammonia liberated per min.

Standard enzyme assay of microcapsules containing asparaginase

Unless otherwise noted, the enzyme reaction of microcapsules was carried out by incubating a mixture of 100 μmoles of borate buffer (pH 8.0), 100 μmoles of L-asparagine and microencapsulated asparaginase, in a total volume of 3 ml, for 10 min at 37 °C with shaking (100 cycles/min, 5-cm stroke). The reaction mixture was filtered, and the liberated ammonia in the filtrate was measured by the colorimetric method using Nessler's reagent. The effects of amount of microencapsulated enzyme, *i.e.* enzyme concentration, and incubation period on the reaction rate are shown in Figs 2 and 3, respectively.

In the case of microencapsulated enzymes, since the enzyme reaction is carried out through the microcapsule membrane, diffusion will become the limiting step of the enzyme reaction. Thus, the effect of shaking on the reaction rate was studied. As a result, it was confirmed that the reaction rate was increased with increase in

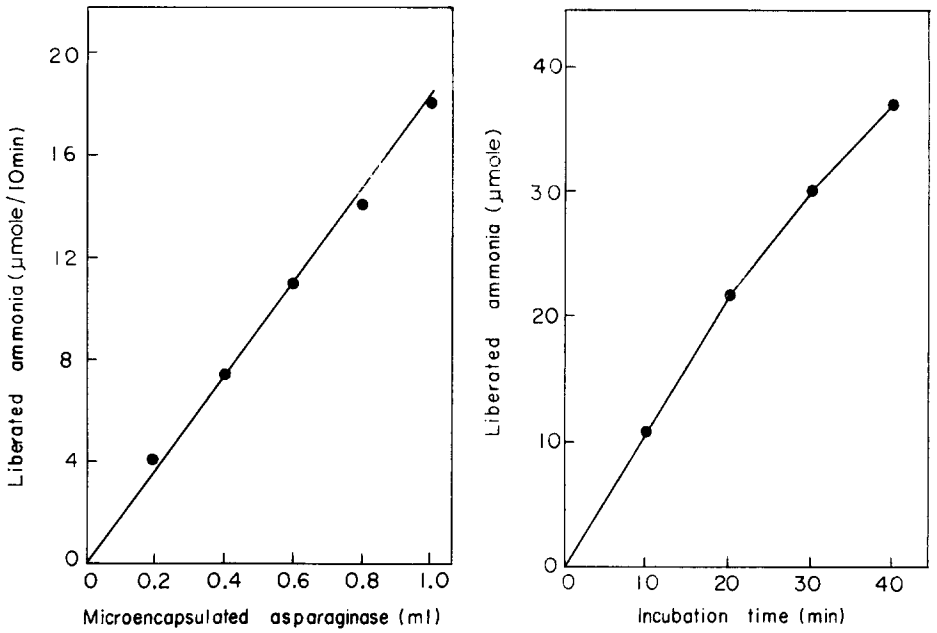

Fig. 2. Effect of concentration of microcapsules on the reaction rate. Polyurea microcapsules containing asparaginase were employed for the test. The enzyme assay was carried out under standard conditions with varied amounts of microcapsules.

Fig. 3. Effect of incubation period on the reaction rate. Polyurea microcapsules containing asparaginase were employed for the test. The enzyme assay was carried out under standard conditions with varied incubation time.

shaking but it had already reached a plateau level at the shaking speed of the standard enzyme assay, and the effect of diffusion on the reaction rate was negligible.

RESULTS

Effect of pH

The pH dependence of the initial rate of hydrolysis of L-asparagine with nylon and polyurea microcapsules containing asparaginase and native asparaginase is shown in Fig. 4. Both the microencapsulated enzymes show a more restricted range of activity than that of the native enzyme, although no difference in the optimum pH values of these three enzyme preparations are observed.

Effect of temperature

The effect of temperature on the reaction rate was investigated, and the results are shown in Fig. 5. The figure indicates that the optimum temperature for both microencapsulated enzymes is lower by about 10 °C than that for the native asparaginase. For the estimation of the apparent activation energy of these three enzyme preparations, the results given in Fig. 5 were plotted by the method of Arrhenius. The apparent activation energies below 37 °C were calculated to be 7440 cal/mole for the three enzyme preparations.

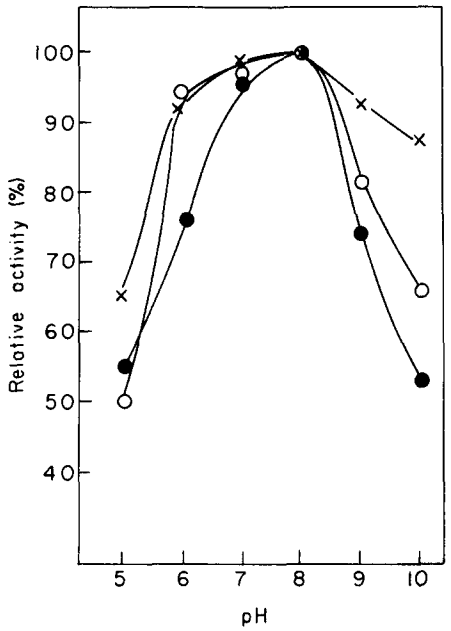

Fig. 4. Effect of pH on the reaction rate. The enzyme assay was carried out under standard conditions except for buffers employed. At pH 5.0–6.0, pH 6.0–8.0, and pH 8.0–10.0, 0.1 M acetate buffer, 0.1 M phosphate buffer, and 0.1 M borate buffer were employed, respectively. ×—×, native asparaginase; ○—○, nylon microcapsules containing asparaginase; ●—●, polyurea microcapsules containing asparaginase.

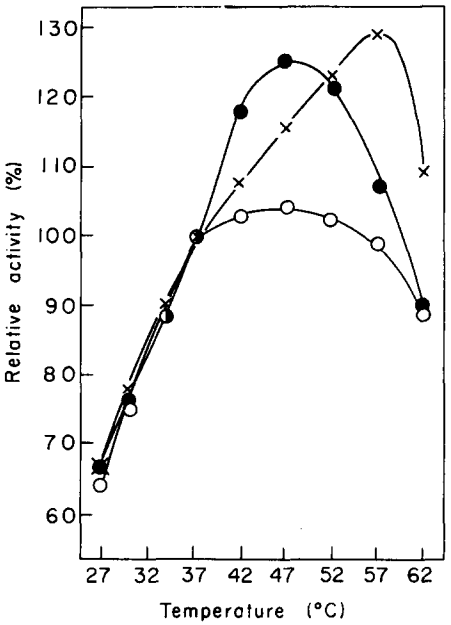

Fig. 5. Effect of temperature on the reaction rate. The enzyme assay was carried out under standard conditions modified by varying the incubation temperature. The reaction rates obtained at 37 °C were taken as 100%. ×—×, native asparaginase; ○—○, nylon microcapsules containing asparaginase; ●—●, polyurea microcapsules containing asparaginase.

Comparison of Michaelis constant

The effect of substrate concentration on the enzyme activity was investigated, and the results obtained were plotted by the method of Lineweaver and Burk for the estimation of the Michaelis constant (K_m). The results are shown in Fig. 6. From these figures, K_m for native asparaginase, nylon and polyurea microcapsules was calculated to be $1.8 \cdot 10^{-5}$ M, $1.3 \cdot 10^{-3}$ M and $2.0 \cdot 10^{-3}$ M, respectively.

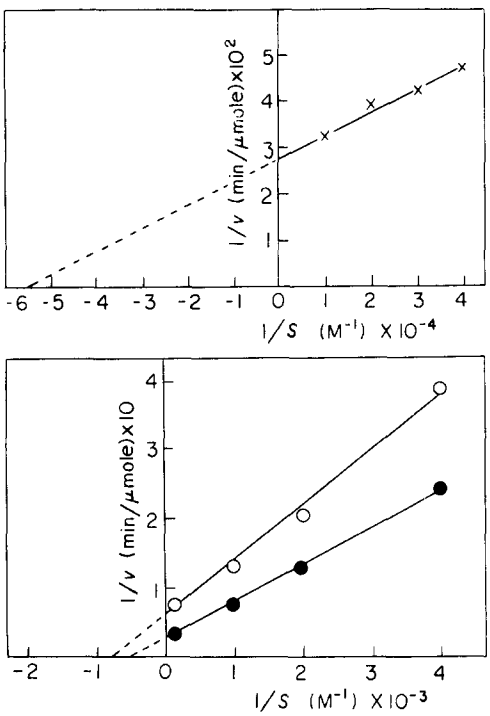

Fig. 6. Lineweaver–Burk plots for the native and microencapsulated asparaginase. In the case of native asparaginase, the enzyme reaction was started by the addition of 1 ml of enzyme solution into 4 ml of substrate solution (pH 8.0). The mixture was incubated for 10 min at 37 °C, and the reaction was stopped by the addition of 1 ml of Nessler's reagent. In the case of microencapsulated asparaginase, the enzyme reaction was started by the addition of 2 ml of microcapsules into 8 ml of substrate solution (pH 8.0). The mixture was incubated for 10 min at 37 °C with shaking, and the reaction was stopped by the filtration. To 5 ml of filtrate was added 1 ml of Nessler's reagent. The absorbance was measured at 410 nm. ×—×, native asparaginase; ○—○, nylon microcapsules containing asparaginase; ●—●, polyurea microcapsules containing asparaginase.

Stability of microencapsulated asparaginase after repeated use

Stability of nylon and polyurea microcapsules containing asparaginase after repeated use was investigated. As shown in Table I, no loss of activity in both microcapsules was observed after being used five times.

Stability in storage

The stability of microencapsulated and native asparaginases in prolonged storage at 4 °C and 30 °C was investigated, and the results are shown in Fig. 7. The

TABLE I

STABILITY OF MICROCAPSULES AFTER REPEATED USE

A mixture of 1 mmole of borate buffer (pH 8.0), 1 mmole of L-asparagine and microcapsules containing asparaginase, in a total volume of 30 ml, was incubated for 10 min at 37 °C with shaking, and then filtered. The residual microcapsules were washed with water, and again incubated under the same conditions as described above. This procedure was carried out repeatedly, and the time interval between each use was 30 min.

Number of times used	*Remaining activity (%)*	
	Nylon microcapsules	*Polyurea microcapsules*
1	100	100
2	91	103
3	98	94
4	103	93
5	96	97

figure shows that after storage at 30 °C the activity of both microencapsulated enzymes decreases with the same ratio as in the case of native asparaginase, but at 4 °C both microcapsules are more stable than native asparaginase.

Effect of proteases on the enzyme activity

To obtain further information on the stability of microencapsulated enzymes the effect of proteases on the microcapsules containing asparaginase was investigated, and compared with that on the native asparaginase. As shown in Table II, native asparaginase was almost inactivated by incubation with proteases for 10 min, but both microencapsulated enzymes were not affected by the same treatment.

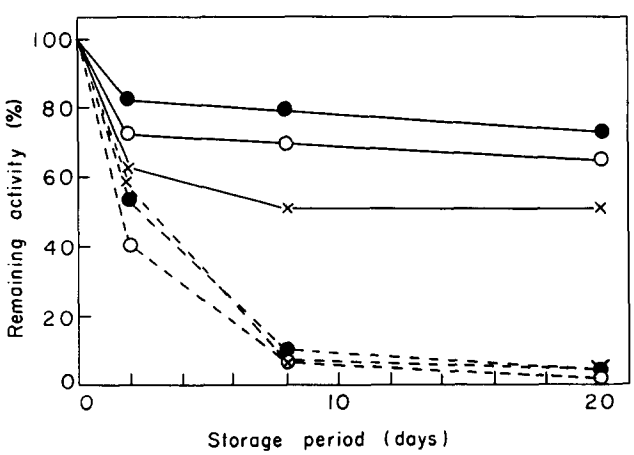

Fig. 7. Stability in storage. Microencapsulated and native asparaginases were stored for 20 days in physiological saline. After storage, the enzyme activity was measured under standard conditions. ×—×, native asparaginase; ○—○, nylon microcapsules containing asparaginase; ●—●, polyurea microcapsules containing asparaginase. ———, at 4 °C; — — —, at 30 °C.

TABLE II

EFFECT OF PROTEASES ON THE ENZYME ACTIVITY

In the case of native asparaginase, a mixture of 100 μmoles of borate buffer (pH 8.0), 1 mg of asparaginase and a specified amount of protease, in a total volume of 2 ml, was incubated at 37 °C for 10 min. In the case of microcapsules containing asparaginase, a mixture of 1 mmole of borate buffer (pH 8.0), microcapsules (containing 1 mg of native asparaginase) and specified amount of protease ,in a total volume of 11 ml, was incubated at 37 °C for 10 min with shaking. The amount of trypsin, chymotrypsin and Pronase-P used was 500, 10 and 20 μg, respectively. After these treatments, the remaining enzyme activity was immediately determined by the standard assay system.

Proteases	*Remaining activity (%)*		
	Native asparaginase	*Nylon microcapsules*	*Polyurea microcapsules*
Trypsin	28	104	100
Chymotrypsin	4	111	96
Pronase-P	12	106	107

DISCUSSION

In order to prepare a more active microencapsulated asparaginase, we have attempted to prepare the polyurea microcapsule as well as the nylon microcapsule. Enzyme activity of polyurea microcapsules containing asparaginase was higher than that of nylon microcapsules, and also considerably higher than that of the nylon microcapsules prepared by Chang[9].

The enzymatic properties of nylon and polyurea microcapsules containing asparaginase were compared with those of native enzyme. At the outset, the effect of enzyme concentration and incubation time on the reaction rate were investigated in order to clarify whether it is possible to assay the activity of the microencapsulated enzyme by the same method employed for the native enzyme. As shown in Figs 2 and 3, it was found that if the microencapsulated enzymes were uniformly suspended in the reaction medium with shaking, the activity could be assayed by the same method as in the case of the native enzyme. Accordingly, the Michaelis constant (K_m) could be obtained by the method of Lineweaver and Burk. The apparent K_m values of both microencapsulated asparaginases are about 100 times higher than that of native enzyme. The same phenomenon was observed by Allison *et al.*[10] in asparaginase covalently bound to the nylon tube. They consider that this loss of affinity for substrate is probably a result of a change in conformation during the coupling process and/or a result of charge–charge interactions between substrate and support. On the other hand, in the case of microencapsulated enzymes, this increase of K_m value may be explained by the change of permeation rate of substrate or product through the microcapsule membrane. The permeation rate will be logarithmically related to the concentration of substrate or product in the reaction medium. That is, transfer of substrate or product through the microcapsule membrane will be much limited when the concentration of substrate or product is very low, and the apparent K_m value becomes higher.

Other properties of the enzyme were also changed by microencapsulation. Although maximum activity was obtained at pH 8.0 with native enzyme and both

microencapsulated enzymes, the microencapsulated enzymes restricted the pH range on the activity more. Significant difference between the microencapsulated and native asparaginases was also observed in optimum temperature. The reason for changes in the pH profiles and optimum temperature by microencapsulation is not clear.

By conversion into a microencapsulated form the resistance to attack of proteolytic enzymes is very much enhanced. Furthermore, the microencapsulated enzymes were found to be very stable after repeated use. These results suggest that asparaginase is completely encapsulated in the semipermeable nylon or polyurea membrane and does not leak out from the microcapsule. These properties indicate that microencapsulated asparaginase is promising for future practical use in enzyme therapy.

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