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Reactivity and Stability of Microencapsulated Placental Alkaline Phosphatase

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Alkaline phosphatase originating from placenta was microencapsulated with polystyrene by employing a drying-in-liquid method. The reaction rate of the resultant microcapsules was slower than that of the native enzyme. The kinetic data for both native enzyme and microencapsulated enzyme satisfied the Lineweaver–Burk relationship, but the kinetic parameters were different. When the concentration of substrate was much larger than the Michaelis constant, the reaction kinetics of the native enzyme were zero-order, while those of the microencapsulated enzyme were not. The reaction rate of the microcapcules was affected the amount of polystyrene used as a wall material and by the stirring rate of the reaction system. The reaction still continued, though slowly, after the microcapsules were removed from the system. These findings suggested that the internal diffusion of substrate in microcapsules and the capillary flow of enzyme molecules through a few pin holes on the surfaces of microcapsules were the rate-determining steps of the reaction in the case of the microcapsules. It was confirmed that the enzyme was fairly well immobilized, because sixty percent of the initial enzyme activity in the microcapsules remained even after the microcapsules had been used six times. When the native enzyme was stored at 60°C, the activity fell to a quarter of the initial activity after two hours, whereas microcapsules stored under the same conditions retained 50% of the initial activity.

Keywords——alkaline phosphatase; microcapsule; reactivity; stability to heat and repeated usage; immobilization internal diffusion; capillary flow

Introduction

Microencapsulation is a suitable method for producing pharmaceutical dosage form with better bioavailability, for compounding incompatible substances, and for stabilizing unstable drugs against oxygen, humidity, *etc.*[1] This technique is also one of the established ways to immobilize an enzyme.[2] Chang[3] firstly immobilized urease by means of microcapsules with nylon semipermeable film prepared by the interfacial polymerization method. The microencapsulated urease increased the concentration of ammonia in the blood when it was intravenously injected. Miyawaki *et al.*[4] improved the method of Chang by employing a liquid-air nozzle, which allowed good control of the diameter of microcapsules of urease. By a drying-in-liquid method, Kitajima *et al.*[5] produced microcapsules of catalase, urease and lipase with synthetic high polymers, such as polystyrene, ethylcellulose and silicone derivatives. These preparations were suitable for repeated usage.

In the present study, placental alkaline phosphatase, which is widely compounded in cosmetics[6] and is used for an immunoassay,[7] was immobilized by microencapsulation using a drying-in-liquid method. The aim of the present study was to elucidate the reaction kinetics of the microencapsulated enzyme and to test its stability to heat and repeated usage, as well as to clarify the parameters affecting the reactivity and the stability of the resultant microcapsules.

Experimental

Preparation of Microcapsule of Alkaline Phosphatase——A dichloromethane solution of 10 to 20% polystyrene (styrene polymer n=1700, Kishida Chemical Co., Japan) and an aqueous solution of placental

alkaline phosphatase amounting to 500 nitrophenol units (Placenand V-500 NPU, Ichimaru Co., Matsuhora, Takatomi, Gifu, Japan) and containing 4% gelatin were prepared separately. The aqueous enzyme solution (4 parts) was gradually added to the polystyrene solution (12 parts) with stirring, then the mixture was agitated strongly for 10 minutes in a jet-type homomixer (Homo-mixer Type M, Tokushukika Kogyo, Japan) to form a homogeneous W/O type emulsion. This emulsion was poured gradually into aqueous 10% gelatin solution, which was held at 15°C and gently stirred at 250 rpm. By this procedure, a stable complex emulsion (W/O/W) was prepared. The resultant complex emulsion was warmed to 40°C with stirring for several hours to remove dichloromethane from the system, and microcapsules with a polystyrene wall were formed. The microcapsules were separated from the system and washed with distilled water to remove enzyme and gelatin adsorbed on them. Then the microcapsules were dried for 3 hours at 30°C in a fluidizing dryer (Type TR 2, Haltingen-Bingen, West Germany). The diameter and the particle density of the dried microcapsules were determined using a standard test sieve as specified in J.P.X. and a helium comparison pycnometer (Model 1302, Micromeritics Instrument Co., U.S.A.), respectively. The surface topography of the microcapsules was examined with a scanning electron microscope (JMS-SI, Nihon Denshi, Japan). The resultant microcapsules were stored at 4°C in a freezer until required, unless they were used for a heat stability test.

Measurement of Alkaline Phosphatase Activity——The activity of alkaline phosphatase was defined as millimoles of *p*-nitrophenol produced from *p*-nitrophenyl sodium phosphate by the native enzyme or the microcapsules crushed in a mortar, in buffer solution (pH=10.5) containing glycine, sodium chloride and sodium hydroxide, at 37°C for 15 minutes. The activity of microcapsules was expressed in terms of the initial reaction rate with the same substrate. The concentration of *p*-nitrophenol was determined spectrophotometrically at 430 nm. The reaction with microencapsulated enzyme was carried out in a 500 ml beaker with 3 baffles. The reaction system was agitated by a turbine type stirrer with 6 blades, 4.8 cm in diameter, and was thermally controlled in a water bath.

Results and Discussion

Characteristics of Alkaline Phosphatase Microcapsules

The diameter of the microcapsules ranged from 100 to 500 μm. Scanning electron microscopic photographs of the surface and the cross-section of microcapsules are shown in Fig. 1. The surface of the microcapsule was smooth, but a few pin holes were observed. The diameter of these holes was less than 1μm. The cross-section of the microcapsule was like a bees'nest, so that the microcapsule was a complex capsule containing primary minute capsules. The weak agitation employed in the preparation of the secondary emulsion (W/O/W) compared to the primary one (O/W) might have resulted in such a characteristic. The microcapsules were fractionated into four sizes as listed in Table I and the activity of each fraction was determined. The activity and the density of microcapsules increased with increasing size, as can be seen from Table I.

Table I. Activity and Density of Microcapsules

Particle size (μm)	ρ (g/cm^3)	Activity (NPU/kg)
125—210	1.12	86.3
210—350	1.28	219.8
350—420	1.34	231.9
420—500	1.41	431.5

The temperature and pH dependencies of the activity of native and microencapsulated enzyme were investigated. The optimum temperature for the reaction was 37.5°C and 55°C for the native enzyme and the microencapsulated enzyme, respectively, as seen in Fig. 2. When the temperature rises above 37.5°C, the activity of intact enzyme should decrease, whereas the permeation of substrate and products through the microcapsule wall might be improved. The relative changes in the two effects might raise the optimum temperature for the reaction of microcapsules. By raising the temperature above 65°C, the reactivity could again be improved. This was because the microcapsule wall was destroyed by heat,

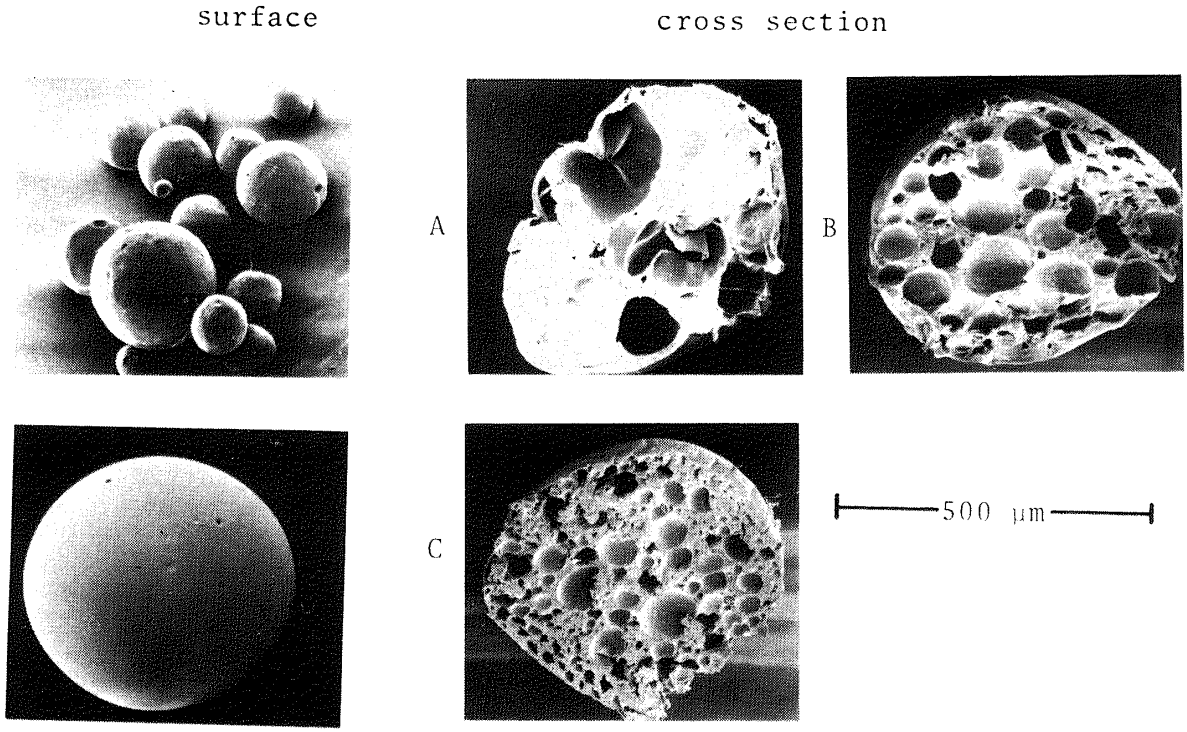

Fig. 1. Scanning Electron Microscopic Photographs of the Surface and Cross Section of Microcapsules

Concentration of styrene polymer (%): A, 10; B, 15; C, 20.

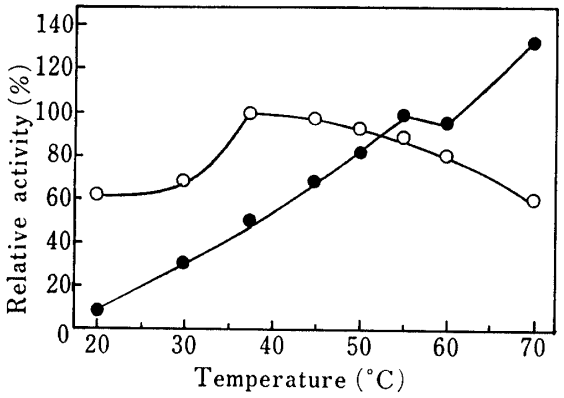

Fig. 2. Temperature Dependency of Enzyme Activity

Weight of microcapsules, 400 mg; concentration of substrate, 2 mmol/l.
○, native enzyme; ●, microencapsulated enzyme (350—420 μm).

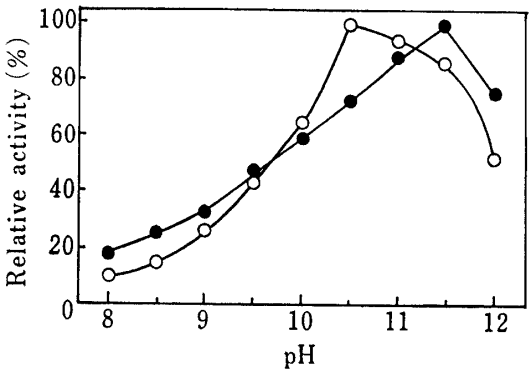

Fig. 3. pH Dependency of Enzyme Activity

Weight of microcapsules, 400 mg; concentration of substrate, 2 mmol/l.
○, native enzyme; ●, microencapsulated enzyme (350—420 μm).

so the reaction of microcapsules was no longer controlled by the diffusion of substrate through the wall.

The optimum pH values of the medium for the reaction were 10.5 and 11.5 for the native enzyme and the microencapsulated enzyme, respectively, as shown in Fig. 3. The pH shift toward to the alkaline side observed in the microcapsules could be interpreted in terms of the action of gelatin contained in the microcapsules. In the alkaline region, gelatin is negatively charged since the isoelectric point of gelatin is 5.5. To neutralize the negative charge electrically, the concentration of hydrogen ions in the microcapsules becomes higher,

which results in a more acidic internal environment. This effect might move the apparent optimum pH of the reaction slightly to the alkaline side.

Reaction Kinetics of Microencapsulated Enzyme

The reactivity of microcapsules was compared with that of native enzyme at the same concentration of enzyme contained in the reaction system. As expected, the reaction of microcapsules was much slower than that of the native enzyme, as shown in Fig. 4. The reaction rate of microcapsules increased with decreasing size of microcapsules due to the fact that specific surface areas of microcapsules increase with decreasing size. The microcapsule density data in Table I indicate that the porosity of microcapsules increased with decreasing size. This finding also accounts for the results in Fig. 4, since the internal diffusivity of substrate might increase with increasing porosity.

The Lineweaver–Burk plots, expressed by equation (1), of the native enzyme and the microencapsulated enzyme are shown in Fig. 5.

$$\frac{1}{V_o} = \frac{1}{V_m} + \left(\frac{K_m}{V_m}\right)\frac{1}{S_o} \tag{1}$$

K_m is the Michaelis constant and V_m is the maximum reaction rate. When $K_m > S_o$, equation (1) can be simplified to equation (2), representing a first-order reaction.

$$V_o = V_m S_o / K_m \tag{2}$$

When $K_m < S_o$, a zero-order rate equation (3) is obtained.

$$V_o = V_m \tag{3}$$

The kinetic parameters, *i.e.* V_m and K_m, were determined from the intercept of the ordinate and the slope of the straight line in Fig, 5. Both parameters were larger for the microencapsulated enzyme than for the native enzyme, as listed in Table II. This finding suggested that the reaction rate of microcapsules was not determined by the intrinsic enzyme reaction, but by another mechanism. When the concentration of the substrate was larger than K_m, it was found that the native enzyme obeyed a zero-order reaction but the microcapsules did not give a zero-order or a first order reaction, as shown in Figs. 6 and 7. The findings in Figs. 6 and 7 are consistent with those in Fig. 5.

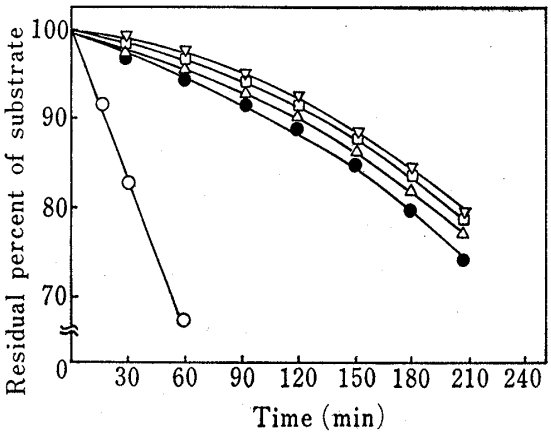

Fig. 4. Reaction Profiles of Native and Microencapsulated Enzyme

Temperature, 37.5°C; substrate concentration, 2 mmol/l.
○, native enzyme;
●, microencapsulated enzyme 120—210 μm;
△, microencapsulated enzyme 210—350 μm;
□, microencapsulated enzyme 350—420 μm;
▽, microencapsulated enzyme 420—500 μm.

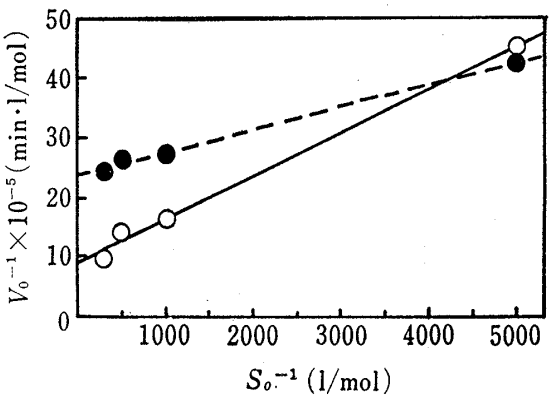

Fig. 5. Lineweaver–Burk Plots of Kinetic Data

Temperature, 37°C; agitation speed, 250 rpm.
●, native enzyme ○, microencapsulated enzyme (350—420 μm) 930 mg.

Table II. Kinetic Parameters of Native and Microencapsulated Enzyme

Enzyme	$K_m \times 10^4$ (mol/l)	$V_{max} \times 10^6$ (mol/min·l)
Native enzyme	1.64	4.24
Microencapsulated enzyme	9.54	12.5

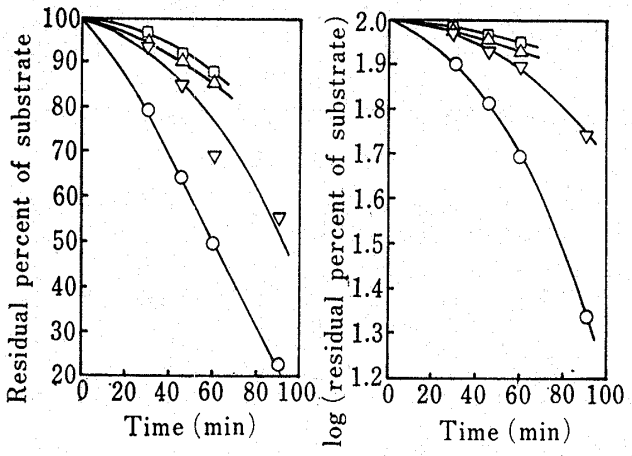

Fig. 6. Reaction Rates of Microencapsulated Enzyme as a Function of Substrate Concentration

Temperature, 37°C; agitation speed, 250 rpm; microcapsules used (350—420 μm), 930 mg.
Substrate concentration (mmol/l): ○, 0.2; ▽, 1.0; △, 2.0; □, 3.0.

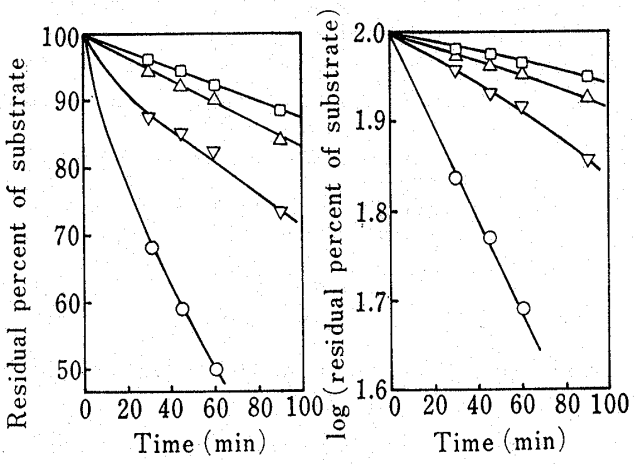

Fig. 7. Reaction Rates of Native Enzyme as a Function of Substrate Concentration

Temperature, 37°C; agitation speed, 250 rpm.
Substrate concentration (mmol/l): ○, 0.2; ▽, 1.0; △, 2.0; □, 3.0.

The effects of concentration of enzyme contained in the system on the initial reaction rate can be seen in Fig. 8. A linear relationship between the initial reaction rate and the concentration of enzyme was found when microcapsules with equal amounts of polystyrene were used. Such a correlation was not observed with microcapsules containing different amounts of the polymer. This finding can be interpreted by recalling the scanning electron microscopic photographs of the internal microcapsule structure in Fig. 1. When the amount of polystyrene used in the preparation of microcapsules is increased, the internal texture of the resultant microcapsules becomes denser, and the size of the primary minute capsules contained in the parent capsule becomes smaller. The characteristic structure might contribute to decreasing the internal diffusion rates of the substrate and the product, leading to a slower reaction. As expected, it was found that the reaction rate of microcapsules decreased from 2.45 to 0.45×10^{-6} (mol/min·l) with increasing amount of polystyrene from 12 to 24 g in the formulation for preparing the microcapsules. These findings suggested that the diffusion of the product or the substrate in the microcapsule might be a rate-determining step in the reaction with the microencapsulated enzyme.

It was found that the initial reaction rate also depended on the agitation speed of the system, as shown in Fig. 9. The reaction rate increased with increasing agitation speed of the system in the range of 150 to 250 rpm.

In Fig. 10, the effect of removal of the microcapsules from the system at 60 minutes after the start of the reaction process is shown by dotted lines. It was found that, even after the microcapsules were removed from the system, the reaction still proceeded gradually, although the reaction rate was greatly reduced. This finding suggested that a small amount of enzyme leaked out of the microcapsules during the reaction. A few pin holes on the surface of the microcapsules (Fig. 1) might allow leakage of the enzyme. This leakage was promoted by increasing the agitation of microcapsules, which resulted in a pressure difference between

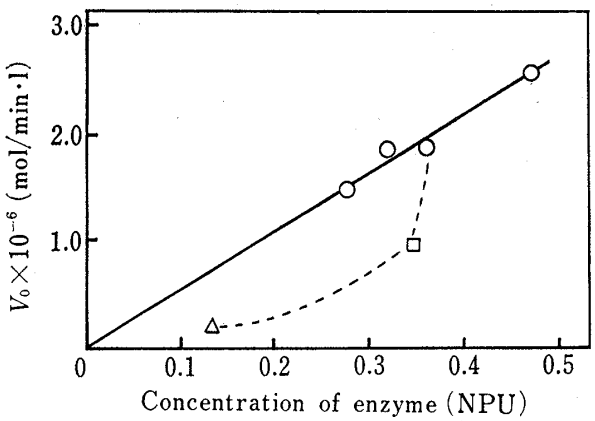

Fig. 8. Relationship between Initial Reaction Rate and Concentration of Enzyme

Concentration of styrene polymer (%): ○, 10; △, 15; □, 20.

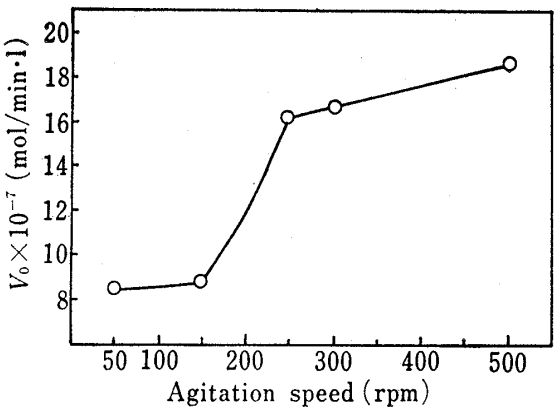

Fig. 9. Initial Reaction Rate as a Function of Agitation Speed

Particle size of microcapsules used, 350—420 μm; weight of microcapsules, 400 mg; concentration of substrate, 2 mmol/l.

outside and the inside of the microcapsules, inducing a capillary flow of enzyme from the pin holes of the microcapsules.[8]

In conclusion, it was clarified that the reaction of microcapsules was controlled by both the internal diffusion of substrate and product, and by the capillary flow of enzyme from the microcapsules. The increasing effect of agitation on the reaction rate became insignificant at speeds above 250 rpm, since the intrinsic agitation speed of the microcapsules was no longer proportional to the stirrer speed.

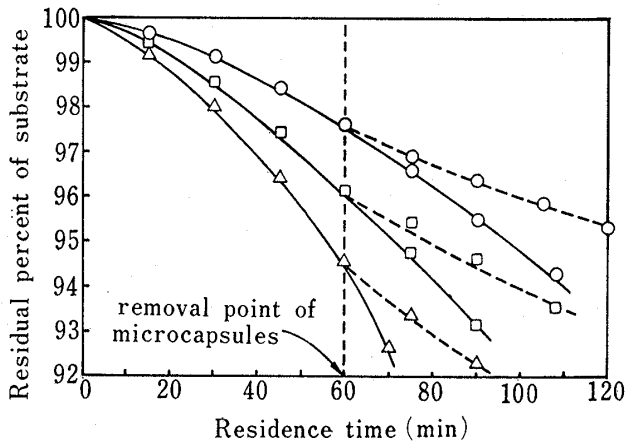

Fig. 10. Effect of Removal of Microcapsules from the System on Reaction Rate

Temperature, 37°C; agitation speed, 250 rpm; concentration of substrate, 2 mmol/l.
Weight of microcapsules used (350—420 μm): ○, 300 mg; □, 400 mg, △, 500 mg. --- Residual percent after removal of microcapsules.

Stability of the Microencapsulated Enzyme

The effect of repeated usage of microcapsules on the reactivity of the enzyme was investigated. In Fig. 11, the reaction profiles are shown as a function of the times of usage of microcapsules. The reaction rate at the second usage was higher than at the first one, but it decreased on subsequent usage. The relative initial reaction rate of each run with respect to that of the first usage is also plotted against the number of usages in Fig. 11. The reactivity at the second usage was the maximum. The microcapsules were used repeatedly without drying, so at the second usage, where the internal texture of microcapsule may have become loosened, the diffusibity might have increased. The reduction of reactivity caused by usage more than three times might be due to loss of the enzyme by leakage from the microcapsules. At the fifth usage, the microcapsules still retained 60% of the reactivity at the first usage.

Fig. 12 shows the changes in the initial reaction rate for native enzyme and microencapsulated enzyme stored at 60°C for various times. The reactivity of native enzyme decreased with aging time. The activity decreased to 50% and 25% of the initial activity after storage for 30 minutes and 2 hours, respectively, whereas the microcapsules still retained 98% and 48% of the initial reactivity, under the same storage conditions, respectively. Unexpectedly,

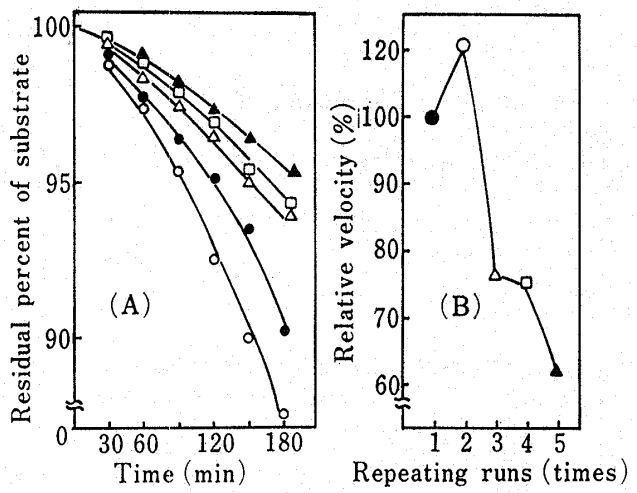

Fig. 11. A) Reaction Profiles of Microencapsulated Enzyme as a Function of Repeating Time
B) Relationship between Initial Reaction Rate and Times of Usage
●, first usage; ○, second; △, third; □, fourth; ▲, fifth.

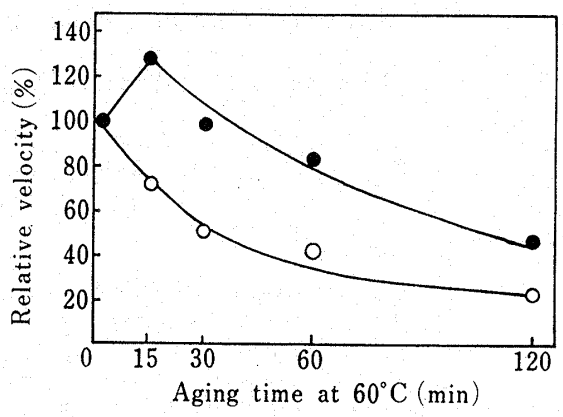

Fig. 12. Effect of Aging on Initial Reaction Rate
○, native enzyme; ●, microencapsulated enzyme.

the activity of microcapsules increased to 125% of the initial activity after storage for 15 minutes. The porosity of the internal texture of microcapsule might have been slightly increased by heating, increasing the permeabilities of substrate and product. This effect might outweigh the inactivation of enzyme by heat to increase the initial apparent reaction rate.

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