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FIBRIN MEMBRANE ENDOWED WITH BIOLOGICAL FUNCTION

V. MULTIENZYME COMPLEX OF URICASE, CATALASE, ALLANTOINASE AND ALLANTOICASE

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Summary

The enzymes (uricase (EC 1.7.3.3), allantoinase (EC 3.5.3.4), and allantoicase (EC 3.5.2.5) which participate in degradation of purine bases, were embedded separately in fibrin membranes formed by fibrinogen-fibrin conversion with thrombin. All of these enzymes together with catalase were also embedded in a single fibrin membrane to make an immobilized multienzyme complex. The multienzyme complex in fibrin membrane thus prepared had an ability of degradation of uric acid to urea and glyoxylic acid via allantoin and allantoic acid. The stability of immobilized uricase or catalase embedded in fibrin membrane upon lyophilization was also tested in a comparison with non-immobilized enzymes.

Introduction

Immobilization of an enzyme, L-asparaginase, has been carried out using a fibrin membrane formed by conversion of fibrinogen to fibrin by thrombin [1]. L-Asparaginase has been used as an antitumor agent for the treatment of human leukemia. Fibrin has also been used to immobilize chloroplast ATPase [2] which consists of seven subunits (α_2, β_2, γ, δ and ϵ) and has a molecular weight of 360 000. Living cells such as *Chlorella*, sea urchin eggs and *Paramecia* have also been embedded in fibrin membrane without impairing their functions [3]. The principal advantages of a fibrin polymer as a matrix are as follows: the formation of fibrin from fibrinogen with thrombin proceeds under physiological conditions (at neutral pH, room temperature and in aqueous solution)

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and the crosslinking reaction between fibrin molecule is readily catalyzed by blood coagulation factor XIII.

Bouin et al. [4] prepared immobilized enzymes of glucose oxidase and catalase covalently coupled to aminopropyl-triethoxysilane derivative. Hervagault et al. [5] studied the kinetics of immobilized bienzyme system of xanthine oxidase and uricase.

The present paper deals with multienzyme complex of uricase, catalase, allantoinase and allantoicase embedded in fibrin membrane. Uric acid, which is a purine derivative, was degraded to urea and glyoxylic acid via allantoin and allantoic acid by fibrin membrane containing these enzymes.

Experimental

Uricase (10.7 units/mg) from yeast and catalase (3900 units/mg) from bovine liver were obtained from Oriental Yeast Co. and Sigma Chemical Co., respectively. Allantoinase and allantocaise were partially purified from *Candida utilis* IFO-0626 [6]. *C. utilis* cells were cultivated in a nutrient medium containing 0.3% uric acid for 90 min to induce the synthesis of enzymes. The cell suspension was treated with ethylacetate and the cell extract was treated with ammonium sulfate (0.2—0.65 saturation) to precipitate enzymes. The precipitate was dissolved in 20 mM acetate buffer (pH 6.0) and dialyzed against the same buffer solution. The protein solution thus prepared had the enzymic activities of allantoinase (0.61 units/ml) and of allantoicase (0.15 units/ml). Human fibrinogen (89% clottable) and human thrombin (70 units/mg) were supplied by Green Cross Co.

Immobilization of enzymes with fibrin membrane was carried out as described previously [1,2]. One volume of fibrinogen (30 mg/ml) in 0.05 M phosphate buffer (pH 7.0) containing 0.3 M NaCl was mixed well with one volume of the sample enzyme(s) in 20 mM acetate buffer (pH 6.0) and then one-tenth volume of thrombin (0.9 mg/ml) dissolved in 0.1 M $CaCl_2$ containing 20 mg/ml cysteine was added. The mixture in the test tube was incubated for 1 h at 37°C. The coagulant formed was taken out from the mixture and pressed between filter papers to make a thin membrane. The exudation produced during this step was completely removed and the membrane washed with water for 12 h to remove free enzyme(s).

Measurements of activity of enzymes were carried out as follows: (1) uricase. The degradation of uric acid (1.0 mM in 20 mM borate buffer, pH 8.5) was monitored by the decrease of absorbance at 293 nm (Ref. 7). (2) Catalase. The degradation of H_2O_2 (37 mM in 50 mM phosphate buffer, pH 7.0) was monitored by the decrease of absorbance at 240 nm (Ref. 8). (3) Allantoinase. The conversion of allantoin (6 mM in 10 mM phosphate buffer, pH 7.6) to allantoic acid was determined by the method of Wang et al. [9]. Allantoic acid formed was degraded with 0.5 N HCl and its degradation product, glyoxylic acid, was reacted with phenylhydrazine. After oxidation of phenylhydrazone with potassium ferricyanide, the absorbance at 520 nm was measured [10]. (4) Allantoicase. The formation of glyoxylic acid from allantoic acid (0.67 mM in 67 mM phosphate buffer, pH 7.3) was estimated by the method described

above, except ethanol was used to stop the reaction instead of trichloroacetic acid [11].

The concentration of hydrogen peroxide or uric acid was determined by measuring the absorbance at 240 or 293 nm, respectively, and molar extinction coefficients of 43.6 $M^{-1} \cdot cm^{-1}$ for H_2O_2 and $1.26 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for uric acid were used for the calculation.

Results and Discussion

Uricase, catalase, allantoinase and allantoicase were separately embedded in fibrin membrane formed by fibrinogen-fibrin conversion by thrombin. The enzymic activity of each enzyme was measured separately after the addition of immobilized enzyme to the respective substrate solution. The results are shown in Fig. 1 in which the panels a, b, c, and d, represent the reaction curves of uricase, catalase, allantoinase and allanoicase, respectively. In the case of immobilized uricase or catalase, the amount of substrate, uric acid or hydrogen peroxide, decreased with increasing incubation time (panels a and b). At a given time, 40 min for uricase of 12 min for catalase, each immobilized enzyme was withdrawn. The reactions did not proceed at all after the removal of the enzyme. The reintroduction of each immobilized enzyme to its substrate solution caused a decrease of the amount of substrate once again. In the case of allantoinase and allantoicase, the amount of the reaction product, allantoic acid or glyoxylic acid, increased linearly with incubation time (panels c and d). At a given time, each immobilized enzyme was withdrawn and the enzymic reaction

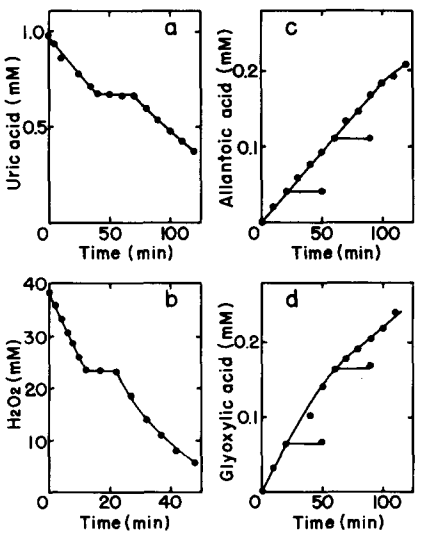

Fig. 1. Enzyme activities of immobilized uricase, catalse, allantoinase and allantoicase. Change in amount of substrates or products after each immobilized enzyme was added to respective substrate solution. Panel a: immobilized uricase. During the period between 40 and 70 min, the membrane was withdrawn from uric acid solution. Panel b; immobilized catalase. During the period between 12 and 22 min, the membrane was withdrawn from H_2O_2 solution. Panel c: immobilized allantoinase. After 20 or 60 min, the membrane was withdrawn. Panel d: immobilized allantoicase. After 20 or 60 min, the membrane was withdrawn.

did not proceed afterwards. These results indicate that each enzyme is completely embedded in the fibrin membrane. The rate of decomposition of uric acid with the immobilized uricase was enhanced by the coexistence of catalase in the fibrin membrane, because immobilized catalase catalizes the decomposition of an inhibitor, H_2O_2, formed from uric acid by the enzymic action of uricase.

Tanford et al. [12] reported that lyophilization of catalase caused complete loss of the enzymic activity due to dissociation of the catalase molecule into subunits. In the present paper, the effect of lyophilization on the enzymic activity of the immobilized catalase or the immobilized uricase was tested in comparison with that of non-immobilized enzymes. The results are shown in Table I. Non-immobilized catalase lost most of its activity upon one single lyophilization step. Immobilized catalase, however, retained 50% of the enzymic activity after four repeated lyophilization steps. A similar phenomenon was observed for immobilized uricase; 72% of enzymic activity was retained after four repeated lyophilization steps for immobilized uricase. This resistance of the immobilized enzyme embedded in fibrin membrane against lyophilization was explained as follows. The fibrin network supports the subunit structure of the molecule of catalase or uricase which consists of 4 subunits, and prevents the molecule from dissociation into subunits by lyophilization. The kinetic study of the immobilized uricase gave a K_m value for uric acid of $3.0 \cdot 10^{-4}$ M, which is higher than $K_m = 1.9 \cdot 10^{-5}$ M obtained for non-immobilized uricase.

The next series of experiments were concerned with multienzyme complex embedded in fibrin membrane. A mixute of 1.0 mg uricase, 1.0 mg catalase, 0.61 units allantoinase and 0.15 units allantoicase was added to the fibrinogen solution and thrombin solution was added to produce the fibrin membrane. The immobilized enzyme in the fibrin membrane thus prepared had an ability to degrade uric acid to glyoxylic acid and urea via allantoin and allantoic acid. The result is shown in Fig. 2. After the multienzyme complex in fibrin membrane was added to the uric acid solution, the amounts of substrate and its products were measured with time. Uric acid, the initial substrate, was converted to allantoin and H_2O_2 by uricase present in the multienzyme complex and as a consequence, uric acid concentration decreased with incuba-

TABLE I

EFFECT OF LYOPHILIZATION ON THE ENZYME ACTIVITY OF IMMOBILIZED CATALASE AND URICASE COMPARED TO NON-IMMOBILIZED CATALASE AND URICASE

Lyophilization stepes	Enzyme activity (%)			
	Catalase	Immobilized catalase	Uricase	Immobilized uricase
0	100	100	100	100
1	4	100	78	99
2	2	96	65	88
3	0	60	55	84
4	0	50	42	72

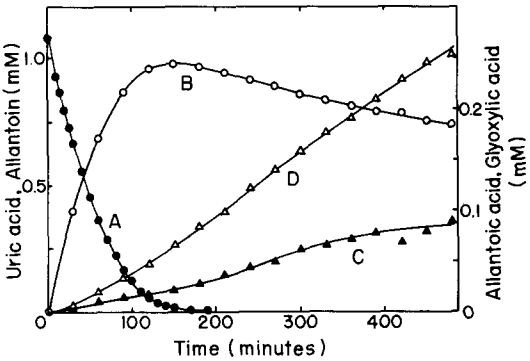

Fig. 2. Change in concentration of substrates or products after adding multienzyme complex in fibrin membrane containing uricase, catalase, allantoinase and allantoicase to 1 mM uric acid in 10 mM phosphate buffer (pH 7.6). Curve A: concentration of uric acid. Curve B: concentration of allantoin. Curve C: concentration of allantoic acid. Curve D; concentration of glyoxylic acid.

tion time (curve A). Concentration of allantoin increased and reached a maximum at 150 min (curve B). Allantoinase converted allantoin to allantoic acid and as a consequence, the allantoic acid concentration increased with time (curve C). Since allantoicase split allantoic acid to glyoxylic acid and urea, the concentration of glyoxylic acid increased with time (curve D). This multienzyme complex had activities of 1.48, 0.10 and 0.073 units/g fibrin of uricase, allantoinase and allantoicase, respectively.

These results may provide an excellent technique for the conversion of uric acid in blood or in cartilaginous tissues into glyoxylic acid and urea.

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