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PREPARATION AND BEHAVIORS OF ENZYMES IMMOBILIZED BY POLYMER-POLYMER COMPLEXES†

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Invertase was immobilized by the polymer-polymer complexes between poly(methacrylic acid) and its complementary polymers. Obtained enzymes exhibited sufficiently high activities with enhanced stabilities against enzyme-inhibitors.

Among many preparation methods of immobilized enzymes the physical entrapping method in the polymeric gel matrices is of special interest, since it often duplicates situations existing in the subcellular compartments which permit entry of low molecular weight substances without allowing outflow of enzymes.[1-3)]

We have recently reported a novel immobilization method of enzymes by use of radio-frequency cold plasma.[4)] This method is characterized by the polymerization of aqueous monomers containing enzymes by the plasma exposure of the duration as short as 30-90 sec, followed by the postpolymerization at room temperature or below (plasma-initiated polymerization[5-7)]). Here, the plasma induces essentially the surface reaction, and a host of chemically active species such as electrons, ions and radicals in the gas plasma can hardly penetrate into the medium . Therefore, it is a useful method for the entrapment of bioactive substances including enzymes.

The present article concerns a new entrapping method of enzymes using the polymer-polymer complexes prepared from two complementary macromolecules.

It is well established[9-10)] that poly(methacrylic acid)(PMAA) undergoes intermacromolecular association with poly(ethylene glycol)(PEG), poly(N-vinylpyrrolidone)(PVPdn), poly(ethyleneimine)(PEI) and forms complexes insoluble in water. The complexes are considered to have scrambled network structures due to the interpolymer bridging between interacting sites. Therefore, if the complexation is performed in the presence of enzyme, the polymer constituents may incorporate the enzyme in the process of the complexation. Since the complex sensitively changes its conformation according to the changes in pH and metal ion concentrations, the immobilized enzyme obtained is expected to exhibit the activity with unique specificities.

Invertase was of commercial origin from yeast(Wako Chemical Inc., stabilized with 50% of glycerine). Saccharose was used as substrate in this experiment. PEG, PVPdn and PEI were used after purification of commercial

†(study of mechanochemical reaction XII)

samples. PMAA with viscosity-average molecular weight of 78×10^4 was obtained by the radical polymerization.

The polymer complexes containing invertase were prepared by the following procedure: 80 ml of 4 wt% NaCl solution were added to a mixture of 2 ml of 0.46 M aqueous solution of PMAA and 2 ml of the invertase sample. Two ml of 0.46 M PEG (or PVPdn, PEI) were then added slowly to the PMAA solution with stirring. The white precipitate of the complex formed simultaneously with the addition of PEG, but was allowed to stand for 30 min with stirring after the addition of PEG was finished. At this stage, NaCl was added so that the complex could be salted out to form a sufficiently large bunch of the solid polymer complex. The product was subsequently filtered, washed well with aqueous NaCl, and dried in vacuo at room temperature overnight. The amount of the invertase entrapped into the complex was assayed by the UV spectral peak at 270 nm as well as by the nitrogen analysis. The assay was carried out in 0.2 M acetate buffer(pH 1.0 - 5.7) and in 0.05 M phosphate buffer(pH 6.9 - 8.1) at 25°C. The reaction was followed polarimetrically by taking 3 ml portions of aliquot from the supernatant as described previously.[4)]

A variety of PEG, PVPdn, and PEI with various molecular weights were employed to immobilize the invertase. The yield of the polymer complex, percentage of the enzyme entrapped, and appearance of the complexes were summarized in Table 1.

It is apparent that the immobilization using the polymer complexes can be satisfactorily performed. The complexes prepared from PEG-PMAA and PVPdn-PMAA were found to be stable in buffers below pH 5.0 and 6.0, respectively, but they dissolved at the higher pH values. In contrast, the complex prepared from PEI-PMAA was stable in buffers of pH 1.8 or higher.

Fig. 1 shows typical percentage hydrolysis versus time relationships of the enzyme immobilized by PEG-PMAA and PVPdn-PMAA complexes. It is seen that the complexes have sufficiently high activities, exhibiting good reproducibility in the repeated uses. However, it was found that the complexes

Table 1
IMMOBILIZED ENZYMES BY POLYMER-POLYMER COMPLEXATION

EXP.NO.	MOL.WT.	YIELD** (%)	ENZYME INCORPORATED (%)	APPEARANCE
I - 1	PEG 20000	86	45	WHITE LARGE PARTICLE
I - 2	PEG 20000*	76	30	
I - 3	PEG 83000	75	≃ 25	
I - 4	PEG 830000	67	< 25	
II - 1	PVPdn 40000	110	55	WHITE FINE PARTICLE
II - 2	PVPdn 360000	104	60	
II - 3	PVPdn 360000*	103	60	
III - 1	PEI 60000	46	42	WHITE LARGE PARTICLE
III - 2	PEI 80000	39	40	

* NaCl was added after complexation
** accounted no enzyme weight

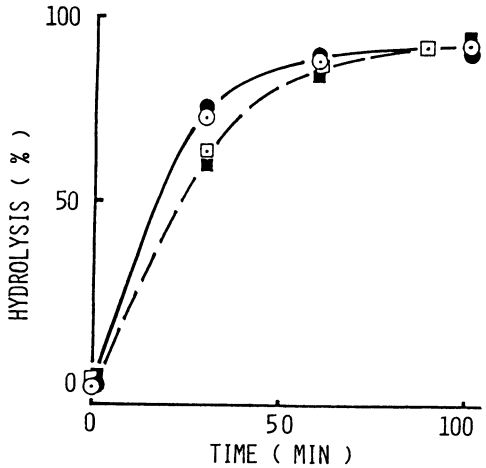

Fig.1 Percentage hydrolysis versus time relationship for the invertase immobilized by the polymer-polymer complexes.
PEG(20000)-PMAA: —⊙— 1st, — ● — 2nd
PVPdn(360000)-PMAA: ---⊡--- 1st, ---■--- 2nd
saccharose; 10wt%, 10ml, pH: 4.6, 25°C
complex 0.05g

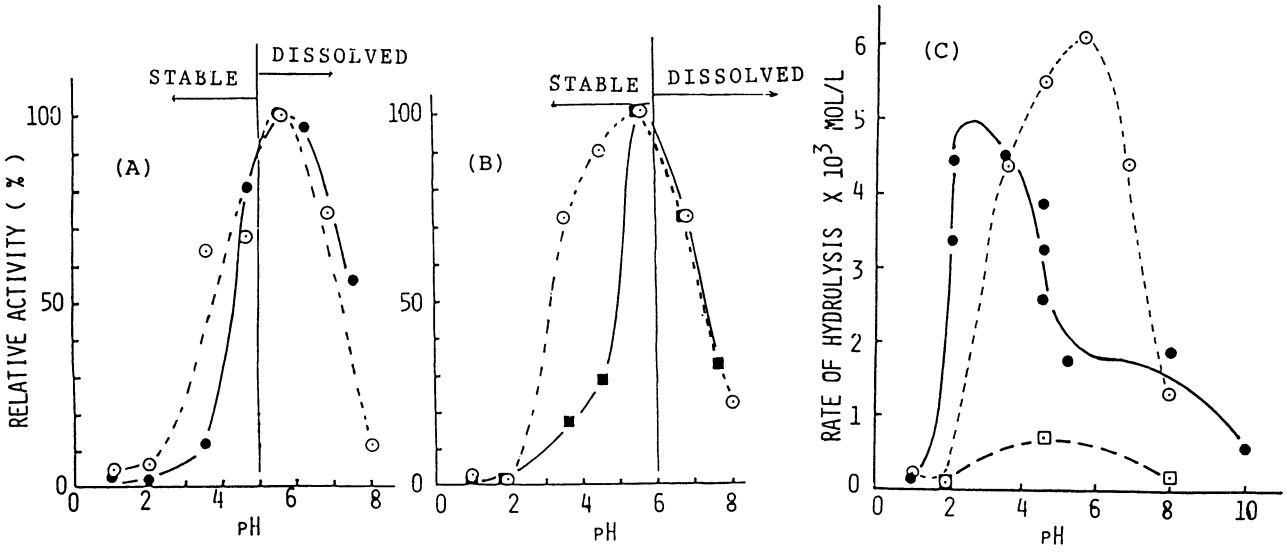

Fig.2 PH-activity curves of free and immobilized invertase.
(A) —●— : PEG(20000)-PMAA, 0.05g, (B) —■— : PVPdn(360000)-PMAA, 0.05g
(C) —●— : PEI-PMAA, 0.02g, ---⊡--- : PVBMA-PAMPS, 0.1g
---⊙---: free invertase, 0.02 ml, saccharose 10 wt%, 10 ml pH 4.6, 25°C

from PEG particularly with molecular weight of 83000 and 830000 lost the activity rapidly by repeated uses due to leakage of the enzyme from the network of the complexes. This result was apparently related to their high swellability in the buffer. Thus, it is necessary to employ the polymer constituents with appropriate molecular weights.

Fig. 2 shows pH-activity curves of the enzyme entrapped into the complexes. Fig. 2A and B indicate that the pH-activity curves are not changed drastically by the entrapment, especially in the pH regions where the complexes became soluble. However, considerable changes in the activity profiles of the complexed enzymes were noted between pH 2.0 and 5.0. The shrinkage of the complexes seems to be related to this result, since the complexes of this type is known to increase the bridgings of the inter-polymer hydrogen bonding with increasing hydrogen ion concentration and assume more compact conformations in water.[8-11] This conformational shrinkage may probably decrease the effective contact of the enzyme with the substrate and/or render the enzyme to abandon the optimum conformation for the biocatalytic reactions.

On the other hand, pH profile of the activity obtained with the PEI-PMAA complex was quite different. In this case the immobilized enzyme exhibited high activity in the low pH region rather than in the neutral one with a maximum activity at pH 1.0, while free invertase lost almost 100% of its activity under identical conditions(Fig. 2C). The relative activities of the immobilized enzymes were calculated as 6-10% for PEG-PMAA, PVPdn-PMAA and 20% for PEI-PMAA complexes compared with free enzyme. The enzyme was immobilized using anotner type of the complex from the polycation and polyanion —— poly(vinylbenzyltrimethylammonium chloride)(PVBMA) and poly(2-acrylamido-2-methyl propane sulfonic

acid)(PAMPS); however, it exhibited very low activity in the wide range of pH as shown in Fig. 2C.

An interesting feature of the immobilization by the polymer complex is that they can provide marked protective effects against enzyme inhibitors. From Fig. 3 it is apparent that the PVPdn-PMAA complexes provide a large protective effect against Hg^{2+}, Cu^{2+}, and aniline under the conditions that the free invertase lost the activity rapidly.

Hg^{2+} was the most noxious for the complexed and free enzyme. Complete denaturation occured at 10^{-4} M Hg^{2+} for the free enzyme, while the complexed enzyme kept 60% of the activity under the same condition. Cu^{2+} had a slight beneficial effect. The most pronounced protective effect was observed by the PEI-PMAA complex. As shown in Fig. 4 the presence of 10^{-4} M Hg^{2+} completely lost the activity of free invertase, however practically no noxious effect was sustained by the same amount of Hg^{2+} when the entrapment was made by the complex. Here, a slight beneficial effect was again obtained in the immobilized enzyme. Thus, the immobilization by PEI-PMAA complex was found to enhance the stability c.a.,100 times against inhibitory effect of Hg^{2+}.

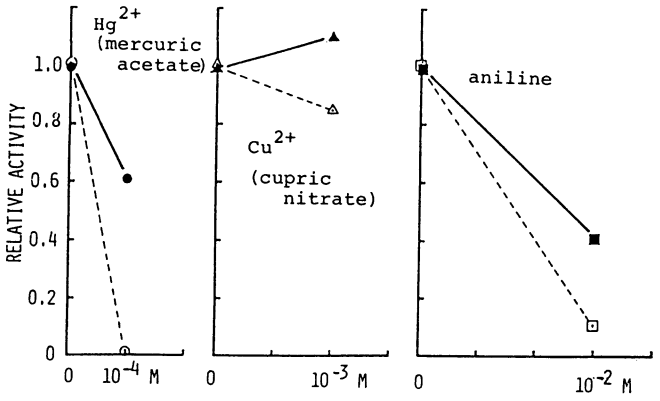

Fig.3 Influences of inhibitors for immobilized and free invertase. ·····: free invertase 0.02ml ——— : immobilized by PVPdn(360000)-PMAA 0.02g saccharose; 10wt%, 10ml, pH: 4.6, 25°C

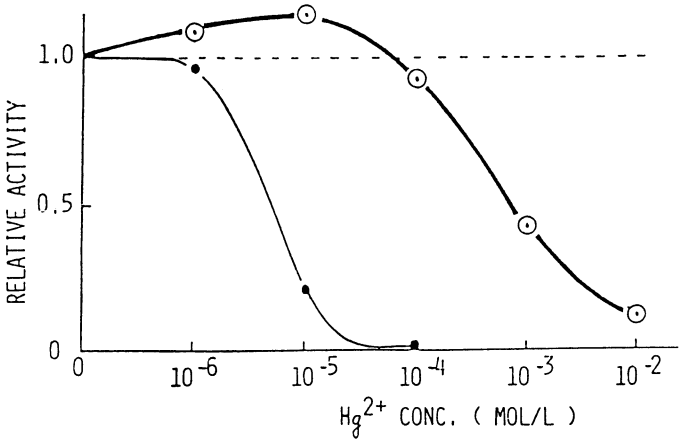

Fig.4 Relative activity-Hg^{2+} concentration relationship for immobilized and free invertase. - ● - free, - ⊙ - immobilized by PEI-PMAA complex, 0.02g, saccharose 10wt% 10 ml, pH 4.6, 25°C.

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