Biotechnology Letters Vol 6 No 12 765-770 (1984)

POLYETHYLENE GLYCOL-MODIFIED ENZYMES TRAP WATER ON THEIR SURFACE AND EXERT ENZYMIC ACTIVITY IN ORGANIC SOLVENTS.

K. Takahashi, H. Nishimura, T. Yoshimoto, M. Okada, A. Ajima, A. Matsushima, Y. Tamaura, Y. Saito and Y. Inada.

Laboratory of Biological Chemistry, Tokyo Institute of Technology Ookayama, Meguroku, Tokyo 152, Japan.

Summary: Polyethylene glycol-modified enzymes dissolved and had high enzymic activity in organic solvents. A trace amount of water was found to be necessary for the activity. It was reasoned that the amphipathic polymer covalently attached to enzymes kept water molecules around them. This was supported by findings that : (1) high enzymic activity was found in water- immiscible solvents, whereas activity was never observed in water-miscible solvents; (2) enzymic activity was inhibited by increasing the concentr- ation of dimethyl sulfoxide in benzene; (3) activity of lipase was inhibited by a water-miscible alcohol substrate, but was steadily elevated by increasing the concentration of a water-immiscible alcohol substrate; (4) water was not absorbed from benzene solution containing a modified enzyme by molecular sieves, while it was easily absorbed in the presence of a water-miscible organic solvent, dimethyl sulfoxide.

INTRODUCTION

It has generally been believed that enzymes are only active in aqueous media, presumably because of denaturation in the hydrophobic environment. We have succeeded for the first time to make enzymes active and soluble in organic solvents (1-6), by exploiting the amphipathic nature of polyethylene glycol, attached covalently to the surface of enzymes. Recently, we have noticed that a trace amount of water is essential for the activity. Zarks and Klibanov reported that water molecules enhance the flexibility of an enzyme(7). In the present paper, effects of water, solvents and substrates on the activity of polyethylene glycol-modified enzymes in organic solvents were investigated.

MATERIALS AND METHODS

Enzymes were obtained as follows. Lipoprotein lipase cystallized from Pseudomonas fluorescens was obtained from Amano Pharmaceutical Ltd. (Nagoya, Japan). Horseradish peroxidase and bovine liver catalase were purchased from Miles Laboratories Ltd. (Good Wood, South Africa) and Sigma Chemical Co. (St. Louis, Mo.), respectively. Chymotrypsinogen from bovine pancreas was prepared by the method of Kunits and Northrop (8). The modifier, 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine

(activated PEG_2), was kindly synthesized by Seikagaku Kogyo Co. Ltd. (Tokyo, Japan) by the method described previously (9). Other reagents were of analytical grade.

Enzymes were modified with activated PEG_2 and enzymic activities in organic solvents were measured by the methods described previously (1-6). The degree of modification of amino groups in the enzymes, lipase, catalase, peroxidase and chymotrypsinogen, was 55, 42, 70 and 83%, respectively. Activities measured with lipase, peroxidase, catalase and chymotrypsin were pentyl pentanate synthesis, oxidation of o-phenylenediamine with hydrogen peroxide, decomposition of hydrogen peroxide and N-benzoyltyrosine butylamide synthesis in organic solvents, respectively.

For the experiment on water absorption from benzene solution, 3 ml of benzene solution with or without 3 mg of modified lipase was mixed with about 0.1 g of molecular sieve 3A as water absorbent. After shaking for 1 min, the water content in the supernatant was determined by measuring the decrease of infrared absorption at 3330 and 3500 cm^{-1}.

RESULTS AND DISCUSSION

We have previously shown that enzymes become soluble and active in benzene following modification of amino groups with polyethylene glycol. In order to further understand the underlying mechanism, activities of polyethylene glycol-modified enzymes in various organic solvents were determined. As is shown in Table 1, the highest activity among organic solvents tested was found in water-immiscible benzene in every case, and little activity was detected in water-miscible organic solvents such as dioxane, acetone, ethanol and dimethylformamide, in which the polyethylene glycol-modified enzymes were quite inefficient. It is implied that water molecules have to be associated with enzyme molecules to manifest activity.

TABLE 1. Enzymic activity of PEG2-modified enzyme in organic solvents

Solvent	Relative activity (%)			
	Lipase	Catalase	Peroxidase	Chymotrypsin
Water-immiscible group				
Benzene	100	100	100	100
Toluene	87	38	-	-
Chloroform	67	90	-	-
Water-miscible group				
Dioxane	23	0	0	-
Acetone	0	0	0	0
Ethanol	-	0	0	0
Dimethyl formamide	0	-	-	-

Table 2 shows the effect of water content on the activity of modified lipase in benzene and dioxane. An increase of water content caused an elevation of activity in benzene, but in dioxane the activity was markedly decreased. Since water is not involved in the ester synthesis reaction itself, water

may be required for other reasons, such as keeping proper conformation of enzymes. Both benzene and dioxane have very low dielectric constants (2.3 and 2.2, respectively), but the latter is very miscible with water (in contrast to the former). Therefore, water molecules might be efficiently concentrated around the enzyme molecule in benzene via the polyethylene glycol attached covalently to the enzyme surface, but not in dioxane.

TABLE 2 Effect of water content on the activity of pentyl pentanate synthesis by modified lipase in benzene and dioxane

Water conc. (mM)	Activity(/umole/min/mg of protein) in Benzene	in Dioxane
9	0.84	0.19
16	1.56	0.06
23	1.60	0.05
30	1.92	0.04

The concentration of water was determined by Karl-Fischer technique.

From this reasoning, to get high activity of the modified enzyme, the organic solvent should be very immiscible with water. Otherwise, water can not be efficiently concentrated around the enzyme molecule by polyethylene glycol. This is clearly demonstrated in Fig 1. When various concentrations of two types of organic solvents, water-miscible and -immiscible, were added to benzene, the ester synthetic activity of modified lipase was affected in two distinct ways. With water-miscible organic solvents (acetone and dimethyl sulfoxide) the activity was steadily decreased, and diminished to nil with 18 and 11% (v/v), respectively, as it is depicted by -▲- and -△-. With water-immiscible organic solvents, (diethyl ether and n-hexane) there was even a small increase in activity with increasing concentrations of the solvents, as is shown by -●- and -○- for diethyl ether and n-hexane, respectively. The presence of trace amount of water-miscible organic solvents in benzene may forcibly remove water from the enzyme surface.

This notion was further supported by an experiment using various concentrations of alcohols, one of the enzyme substrates, shown in Fig. 2. When ethanol was used to synthesize the ethyl ester of lauric acid (-●-), the initially increasing activity decreased rapidly beyond 8% (v/v) of the alcohol in benzene. On the other hand, when water-immiscible lauryl alcohol was used to synthesize the lauryl ester of lauric acid (-○-), the enzymic activity increased steadily with the concentration of the alcohol added up to 80% (w/v). Thus a higher enzymic activity is obtained when both the substrate and the organic solvent are immiscible with water.

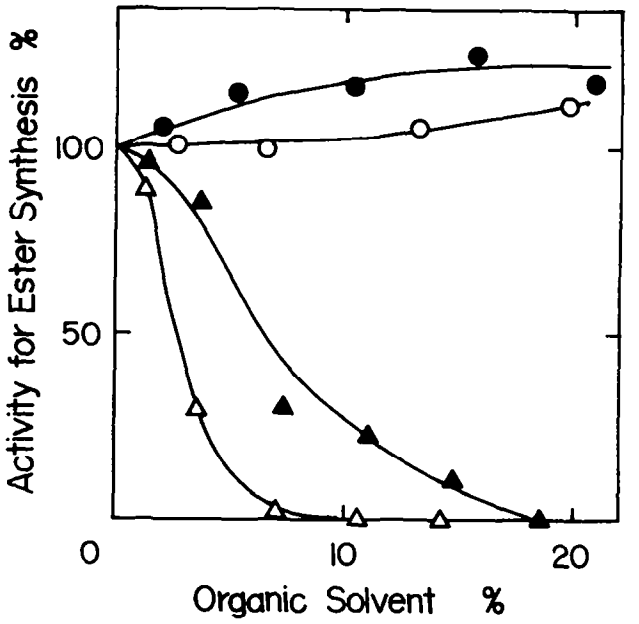

Fig. 1 Effect of organic solvents on ester synthetic activity of modified lipase in benzene containing 30 mM water. The control activity (100%) for pentyl pentanate synthesis from 0.75M pentanol and 0.50 M pentanoic acid was 1.92 /umoles/min/mg protein.
-●- diethyl ether; -o- n-hexane; -▲- acetone; -Δ- dimethyl sulphoxide

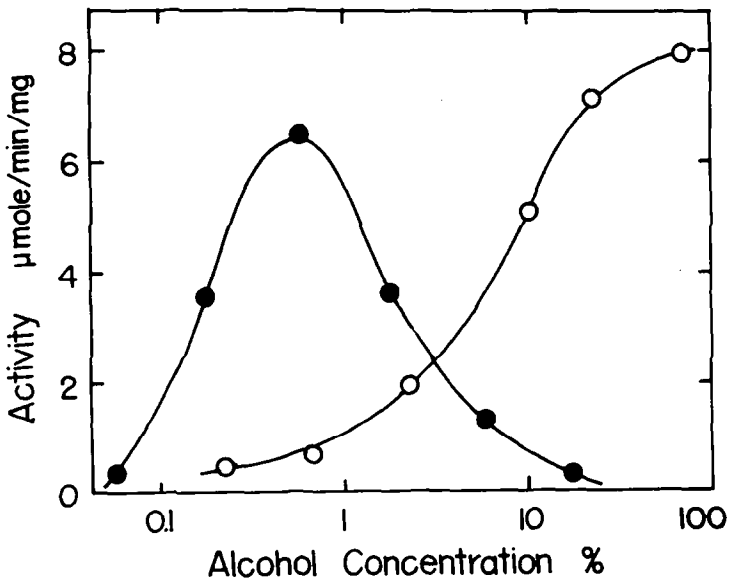

Fig. 2 Effect of alcohol substrates on ester synthetic activity of the modified lipase in benzene containing 30 mM water.
-●- ethanol; -o- lauryl alcohol. The concentration of the other substrate, lauric acid, was fixed at 9.7% (w/v).

It is strongly suggested by infrared spectroscopy that water molecules areindeed attached to the surface of polyethylene glycol-modified enzymes in water-immiscible benzene. The results are shown in Table 3.

TABLE 3 Absorption of water from benzene solution by molecular sieve 3A

Benzene sol.	Absorbed water (%)	
	- Dimethyl sulfoxide	+ Dimethyl sulfoxide
- Enzyme	41	23
+ Enzyme	1	22

The same amount of water was dissolved in benzene with or without the modified lipase. In the absence of dimethyl sulfoxide, molecular sieve 3A absorbed 41% of water from benzene after shaking for 1 min, while only 1% was absorbed from benzene containing the modified lipase. In contrast, in the presence of water-miscible dimethyl sulfoxide (5%), about the same amount of water was absorbed from the benzene solution, regardless of the presence of the modified enzyme.

From these data, we would like to emphasize the suitability of the amphipathic polymer for studying enzymic reactions in a hydrophobic environment. This would make a new horizon in fields of biochemistry, enzymology, biotechnology and chemical engineering.

REFERENCES

1.Takahashi, K., Nishimura, H., Yoshimoto, T., Saito, Y. and Inada, Y., (1984) Biochem. Biophys. Res. Commun., 121, 261-265.

2.Inada, Y., Nishimura, H., Takahashi, K., Yoshimoto, Y., Saha, A.R. and Saito, Y., (1984) Biochem. Biophys. Res. Commun., 12, 845-850.

3.Yoshimoto, T., Takahashi K., Ajima, A., Tamaura, Y. and Inada, Y., (1984) Biotechnol. Lett., 6, 337-340.

4.Takahashi, K., Yoshimoto, T., Ajima, A., Tamaura, Y. and Inada, Y., Enzyme, in press.

5.Matsushima, A., Okada, M. and Inada, Y., FEBS Lett., in press.

6.Pobiner, H., (1961) Anal. Chem., 3, 1423-1426.

7.Zaks, A. and Klibanov, A.M., (1984) Science, 224, 1249-1251.

8.Kaunitz, M. and Northrop, J.H. (1936) J.Gen.Physiol., 19, 1467-1473.

9.Nishimura,H., Takahashi, K., Sakurai,K., Fujinuma, K., Imamura, Y., Ooba, M. and Inada, Y., (1983) Life Sci., 33, 1467-1473.