Biochimica et Biophysica Acta, 571 (1979) 374—377

BBA Report

BBA 61364

AN ENZYME-POLYMER FILM PREPARED WITH THE USE OF POLY-(VINYL ALCOHOL) BEARING PHOTOSENSITIVE AROMATIC AZIDO GROUPS

SACHIO MIYAIRI*

National Chemical Laboratory for Industry, Hiratsuka 254, (Japan)

(Received September 14th, 1979)

Key words: Enzyme-polymer film; Immobilized enzyme; Poly(vinyl alcohol); Aromatic azido group; Photosensitive technique

Summary

Photochemical reaction of poly(vinyl alcohol) bearing aromatic azido groups was applied for immobilization of β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21.) in poly(vinyl alcohol) film. Photo-crosslinking and immobilization reactions proceeded by light irradiation for 25 min in air. The immobilized enzyme showed approx. 40% of its native enzyme activity with an apparent Michaelis constant of 3.9 mM. The Michaelis constant of the native enzyme was 2.3 mM. Some properties of the immobilized and native enzyme are compared.

Numerous methods for preparation of membranous immobilized enzymes for various applications have been reported [1—5]. Some photo-crosslinkable reagents, such as poly(ethylene glycol) dimethacrylate [6], copolymer having γ-stilbazolium groups [7] and 4,4′-diazidostilbene-2,2′-disodium sulfonate [8], were recently used for immobilization of enzymes or organelles in supporters. This report presents a novel and convenient method for the enzyme immobilization by using photo-crosslinking reaction of poly(vinyl alcohol) having photosensitive aromatic azido groups. By light irradiation aromatic azido groups yield highly reactive intermediate, nitrens, which directly insert into the C—H bond of —C—H structures in both enzyme molecule and supporting polymer to form secondary amines [9].

The photosensitive derivative of poly(vinyl alcohol) was prepared by esterification of poly(vinyl alcohol) (Kurare Co., Kurashiki, Japan; mean

*Present address: 1-1 Higashi, Yatabe-machi, Tsukuba-gun 300-21, Japan.

polymerization degree: 1700) with *p*-azidobenzoyl chloride according to the previous report [10]. *p*-Azidobenzoyl chloride was prepared from thionyl chloride and *p*-azidobenzoic acid derived from *p*-aminobenzoic acid [10, 11]. As poly(vinyl alcohol) becomes insoluble in water when highly esterified, partially esterified poly(vinyl alcohol) was prepared as a support of an enzyme. Degree of esterification of poly(vinyl alcohol) was calculated to be 6.4 mol% from the nitrogen content. β-Glucosidase (0.25 unit/mg) from sweet almonds was purchased from Tokyo Kasei Kogyo Co., Tokyo.

Immobilization of the enzyme was performed as follows. 100-ml of the esterified poly(vinyl alcohol) solution (20 mg/ml) was mixed with 10 ml of β-glucosidase solution (8 mg/ml). The mixture was then cast on a horizontal polyacrylate plate (20 × 20 cm) which was dried in air (relative humidity: 60%) for 20 h at 20° C. Thus, a translucent film of 135 ± 3 μm thick was obtained. The film was peeled off and irradiated in air (relative humidity: 60%) at 20° C for 25 min with near-ultraviolet light (max. 345 nm) given by a high-pressure mercury arc lamp of 500 W (USIO High Intensity Projection Lantern, Type 501C) through a filter (Toshiba glass filter, No. 35) which can eliminate almost all ultraviolet light (< 300 nm). 25-min irradiation appears sufficient for photo-crosslinking of the gel, as the infrared absorption spectra of the film showed complete disappearance of the peak at 2150 cm^{-1} (characteristic for azido group) after 25 min irradiation. During irradiation, the temperature of the film did not rise above 30° C. The film was cut into small pieces (1 cm square) and used as the immobilized enzyme. Enzyme content in the film was calculated to be 3.8% from the weights of dry supporter and β-glucosidase which was used for preparation of the film.

β-Glucosidase reaction was performed at 25° C and pH 5.7 (0.05 M sodium acetate buffer) with stirring, using 0.05 M *p*-nitrophenyl-β-D-glucopyranoside as a substrate [5]. 1 Unit of the enzyme is defined as the amount which forms 1 μmol *p*-nitrophenol per min.

The retention of the enzyme within the film was tested by suspending the film in 0.05 M sodium acetate buffer (pH 5.7) with stirring. Activity released into the buffer solution during the 1 h incubation was negligible.

Enzymatic properties of the immobilized enzyme are summarized in Table I, comparing with the native enzyme. Immobilization decreased ap-

TABLE I

PROPERTIES OF THE IMMOBILIZED AND NATIVE ENZYMES

	Enzyme preparation	
	Native	Immobilized
Optimum pH	5.6	5.8
Apparent Michaelis constant (mM)	2.3 ± 0.2	3.9 ± 0.4
V (units/g of enzyme)	250 ± 13	102 ± 6
Activation energy[1] (kcal/mol)	11.1[a] 7.3[b]	12.4[a] 7.7[b]
Thermostability[2] (%)	14	38

[1] The values were obtained at (a) 10—27° C and (b) 27—40° C.
[2] Expressed as relative activity remaining after treatment at 65° C for 30 min with 0.05 M sodium acetate (pH 5.7).

parent enzyme affinity for the substrate and maximum velocity. V of the poly(vinyl alcohol)-immobilized β-glucosidase is comparable with those of sericin- and fibroin-immobilized β-glucosidases [4, 5] and with that of the enzyme photo-crosslinked with bis-azido compound [8], and greater than that of a preparation immobilized onto collagen [3]. Activation energy was increased a little and apparent thermostability was increased considerably by immobilization.

Stability of the system was assayed by measurement of the activity of the immobilized enzyme once a day for 20 days. After each measurement, the film was stored in 0.05 M sodium acetate buffer (pH 5.7) containing 0.02 M *p*-nitrophenyl-β-D-glucopyranoside at 4°C. Released enzyme form the film was not detected during storage, and after 20 days, the remaining activity was 84% of the initial activity.

Poly(vinyl alcohol) film was also prepared by the above method without addition of the enzyme. Diffusion coefficient of the substrate in poly(vinyl alcohol) film was obtained by measurement of permeation rate of *p*-nitrophenyl-β-D-glucopyranoside through the film according to the previous report [5]. Partition coefficient of the substrate in the supporter was obtained by suspending the film in the substrate solution and measuring change of substrate concentration in the solution [5].

Diffusion coefficient of the substrate in poly(vinyl alcohol) film was obtained to be $(6.6 \pm 0.3) \times 10^{-7}$ cm^2/s at 25°C and pH 5.7 which is comparable with those in collagen and sericin films [3, 4], and larger than that in fibroin film [5]. Partition coefficient in poly(vinyl alcohol) film, estimated to be 0.71 ± 0.05 at 25°C and pH 5.7, is smaller than that in sericin film [4], and larger than those in collagen and fibroin films [3, 5].

Supposing that diffusion of substrates in films obeys Fick's second law of diffusion and enzyme reaction in films obeys Michaelis-Menten kinetics, Sundaram et al. [12] proposed the following Eqns. 1—3 to express reaction in films to a good approximation:

$$K_m\,(\text{app}) = \frac{K_m'}{PF} \tag{1}$$

where K_m (app) and K_m' are apparent and true Michaelis constants of immobilized enzymes, respectively, and P is partition coefficient. The function F relates to the extent to which the reaction in the film is diffusion-controlled, and is expressed as follows

$$F = \frac{\tanh \gamma l}{\gamma l} \tag{2}$$

where l (cm) is the thickness of the film and γ (cm^{-1}) is given by

$$\gamma = \left[\frac{k_c(\text{app})\,(E)_m}{4DK_m'}\right]^{1/2} \tag{3}$$

k_c (app) $(E)_m$ (M/s) is equivalent to the maximum reaction rate by 1 l of the immobilized-enzyme film, and D is diffusion coefficient. 1 l of the β-glucosidase-poly(vinyl alcohol) film was equivalent to 15.2 g of the enzyme.

By applying Eqns. 1—3 on the basis of the data of l, D, P, K_m (app)

and k_c (app) $(E)_m$ with respective experimental errors, K_m' was calculated to be 2.2 ± 0.6 mM. Though the deviation of K_m' is considerably large, the standard value of K_m' is close to K_m (2.3 mM) of the native enzyme. Thus, it might be considered that the enzyme was not much affected by the immobilization in the affinity for the substrate, and the apparent Michaelis constant was affected chiefly by diffusion and partition effects of the film.

As described, the immobilization process with the aromatic azido derivative of poly(vinyl alcohol) is simple and losses of enzyme and its support during preparation are negligible. Since aromatic azido group is sensitive to a wide wavelength range, a variety of light sources including sunlight are available.

These results suggest that this method could be generally applied for immobilization of other enzymes.

The author would like to thank Dr. Hideaki Tanaka of National Chemical Laboratory for Industry for preparation of the aromatic azido derivative of poly(vinyl alcohol).

References

1 Mattiasson, B. and Mosbach, K. (1971) Biochim. Biophys. Acta 235, 253—257
2 Naparstek, A., Thomas, D. and Caplan, S.R. (1973) Biochim. Biophys. Acta 323, 643—646
3 Sugiura, M. and Toyoda, H. (1977) J. Agric. Chem. Soc. Japan 51, 703—708
4 Miyairi, S. and Sugiura, M. (1978) J. Ferment. Technol. 56, 303—308
5 Miyairi, S., Sugiura, M. and Fukui, S. (1978) Agric. Biol. Chem. 42, 1661—1667
6 Tanaka, A., Yasuhara, S., Osumi, M. and Fukui, S. (1977) Eur. J. Biochem. 80, 193—197
7 Ichimura, K. and Watanabe, S. (1978) Chem. Lett. 1289—1292
8 Miyairi, S., Tanaka, H., Sugiura, M. And Fukui, S. (1979) Anal. Biochem. in the press
9 Reiser, A. and Wagner, H.M. (1971) in The Chemistry of the Azido Group, (Patel, S., ed.), pp. 441—501, Interscience Publishers, London
10 Tsuda, M., Tanaka, H., Tagami, H. and Hori, F. (1973) Makromol. Chem. 167, 183—190
11 Tsunoda, T., Tanaka, J. and Kobayashi, M. (1969) Bull. Technic. Assoc. Graphic Arts Japan 21—26
12 Sundaram, P.V., Tweedale, A. and Laidler, K.J. (1970) Can. J. Chem. 48, 1498—1504