Radiat. Phys. Chem. Vol. 27, No. 4, pp. 311-316, 1986
Int. Jnl. Radiat. Applic. Instrum., Part C
Printed in Great Britain.

0146-5734/86 $3.00 + .00

IMMOBILIZATION OF CELLULASE AND CELLOBIASE BY RADIATION-INDUCED POLYMERIZATION

O. Z. Higa, N. L. del Mastro and A. C. Castagnet

Instituto de Pesquisas Energéticas e Nucleares - CNEN/SP
Cidade Universitária, São Paulo, Brazil

ABSTRACT

An application of irradiation technology for the immobilization of cellulase and cellobiase using hydrophilic glass-forming monomers was performed. Some experiments to observe the effect of additives as silicates and polyethylene glycol in the enzyme entrappment are reported. In all the cases, the enzymatic activity was maintained by more than fifteen batch enzyme reactions.

KEYWORDS

Immobilization; enzyme; cellulase; cellobiase; radiation polymerization; 2-hydroxyethyl methacrylate.

INTRODUCTION

Among the number of reasons to immobilize enzymes the most common is simply to facilitate recovery or separation of the product from the catalyst. Compared with other techniques for enzyme immobilization, the radiation polymerization method for the preparation of the matrix is facile and the products show good activity, stability and easy regeneration (Dobó, 1970; Hoffman, 1977; Kawashima and Umeda, 1974). In the studies of enzyme immobilization by radiation-induced polymerization at low temperature, hydrophilic glass-forming monomers, such as 2-hydroxyethyl methacrylate, has been employed by most of the researchers. The support is biochemically innert and the immobilization is accomplished by entrappment. Through the polymerization, the enzyme is partially occluded inside the polymer matrix, which offers little resistance to substrate diffusion (Kaetsu, Kumakura and Yoshida, 1979).

Since the irradiation pre-treatment has been used as a means for solubilization of cellulosic raw materials, the interest in the research on the enzymatic hydrolysis of various cellulosic materials by immobilized cellulase has grown (Yoshida, Kumakura and Kaetsu, 1979; Kumakura and Kaetsu, 1983). In this work, some experiments on the entrapping of cellulase and cellobiase into hydrophilic gels, with and without additives, are reported.

MATERIALS AND METHODS

Hydrophilic glass-forming monomers were used: 2-hydroxyethyl methacrylate (HEMA) from Rohm and Haas Co., Philadelphia; hydroxyethyl acrylate (HEA) and hydroxypropyl acrylate (HPA) from The Dow Chemical Co., Michigan. Commercial solutions (180 mg protein/ml) of Trichoderma reesei cellulase and cellobiase from NOVO Industri A.S., Denmark, were used diluted in 0.05 M citrate buffer, pH 4.8. Double entrapping immobilization was tried by using 0.25 g/ml silica gel G (Merck, Darmstadt); 0.07 g/ml QUSO, microfine silica (Philadelphia Quartz Co, Philadelphia) and 0.25 g/ml polyethylene glycol, PEG 6000, (Atlas, São Paulo), as additives. Other reagents were commercial type of special grade.

The entrappment was carried out by mixing the solutions and monomers at different combinations in 0.7 x 15 cm glass tubes, being the final volume 2 ml (density about 1). In the case of double entrapping, the solutions were poured into the glass vessels containing the additives. The samples were kept frozen at -78°C and irradiated by gamma rays from a ^{60}Co source up to 10^4 Gy (about 0.6 kGy/h). The resultant immobilized enzyme composites were sliced in pellets of 1 - 2 mm in thickness. The slices were washed 5 times with water and dried at 37°C or at room temperature.

After the polymerization, a qualitative test (ninhydrin) to detect the attachment of enzyme

into the polymer matrix was performed on the dried pellet.

The cellulase activity was determined by incubating immobilized samples of 125 mg (dry weight) in citrate buffer with the substrate, 1% carboxymethyl cellulose (CMC), for 1 h at 50°C. The cellobiase or β-glucosidase activity was determined with samples of 70 mg in 1% salicin (30 min, 50°C). The reducing sugar produced was measured using the dinitrosalicylic acid reagent (DNS) as described by Mandels (1976). The protein released from the biocomposites was determined in the buffered medium at 50°C after incubation of 1 h (cellulase) or 30 min (cellobiase) by the method of Miller (1959) based on the classic method of Lowry. After each enzymatic or proteic determination, the samples were washed and kept wet at room temperature.

RESULTS AND DISCUSSION

Using the irradiation technique at low temperature, no damage to the enzymes samples was observed by checking the enzymatic activity before and after the irradiation.

Pellets of immobilized enzymes produced by radiation-induced polymerization were qualitatively assayed for protein content. The violet color caused by the ninhydrin reaction was a good indicator of the occlusion grade of the proteic enzyme throughout the matrix. This previous ninhydrin assay correlated well with the subsequent biochemical determination of enzymatic activity carried out with the polymers. The dried immobilized particle changed from a rigid matrix to an expanded porous one, when immersed in the aqueous suspension of substrate.

The changes of the enzymatic activity with repeated use for the cellulase reaction with CMC as a function of monomer percentage in the biocomposite are shown in Figs. 1a and 1b. The figures describe experiments with 10% and 20% of cellulase and in both cases, preparations showing better activity were obtained with 60% HEMA in their composition. A very stiff polymer was formed with higher quantity of monomer. With lower concentrations of HEMA the polymer remained with a more porous structure. As it was demonstrated before by Kaetsu, Kumakura and Yoshida (1979), there is a decreasing in activity due to leakage of the enzyme from the matrix with large porosity. That could have occured in preparations of 40% of monomer.

In order to achieve a better retention of enzyme in the support, the joint of some additives was tried. Silicates in finely divided form, as silica gel and QUSO, present a large surface area and are able to absorb proteins. Similarly, PEG is a polymer capable of precipitating proteins from their solutions without denaturating them. Figure 2 shows the influence of the

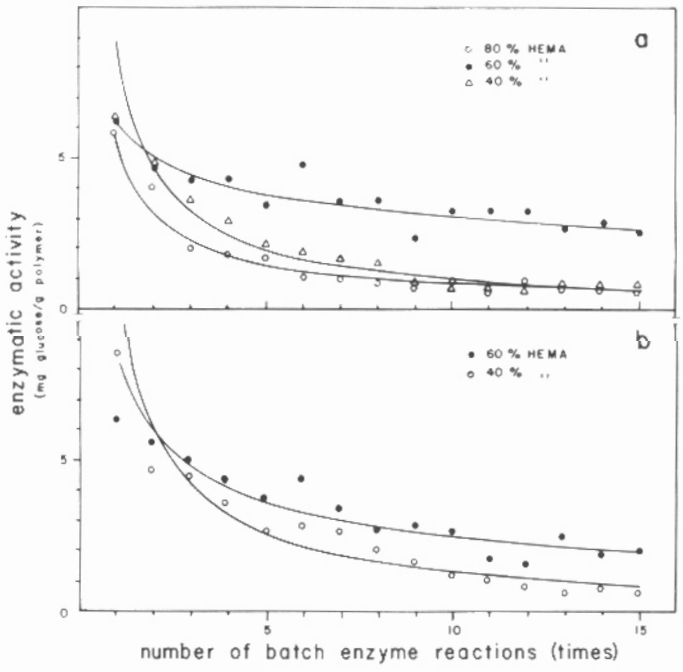

Fig. 1. Enzymatic activity as a function of repeated use of immobilized cellulase at different monomer concentration. Substrate concentration: 1% CMC. Enzyme concentration: a) 10%; b) 20%.

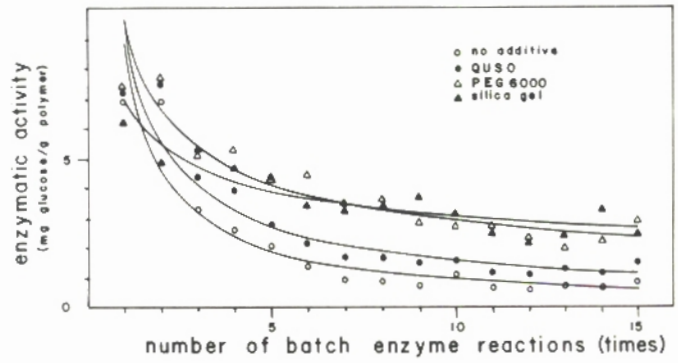

Fig. 2. Effect of additives on the enzymatic activity of immobilized cellulase. Enzyme concentration: 33%. Monomer concentration: 40%. Additives: silica gel, 0.25 g/ml; QUSO, 0.07 g/ml; PEG, 0.25 g/ml.

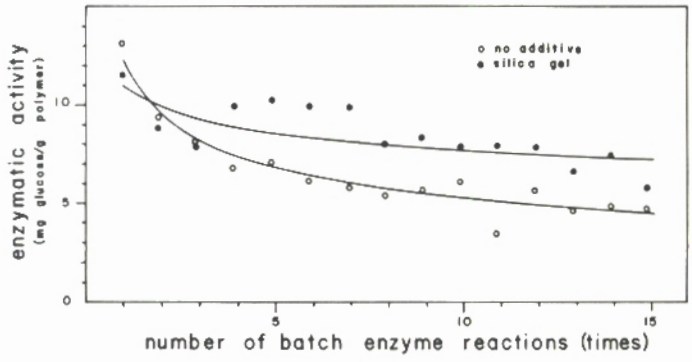

Fig. 3. Effect of silica gel on the enzymatic activity of immobilized cellobiase. Enzyme concentration: 10%. Monomer concentration: 60%. Substrate concentration: 1% salicin. Silica gel, 0.25 g/ml.

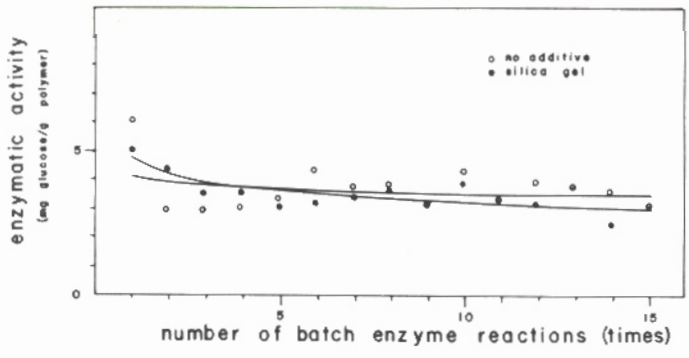

Fig. 4. Variation of enzymatic activity along sucessive batch reactions in a copolymer system. Cellulase concentration: 10%. Monomer composition: HEMA, 50%; HEA, 10%. Silica gel, 0.25 g/ml.

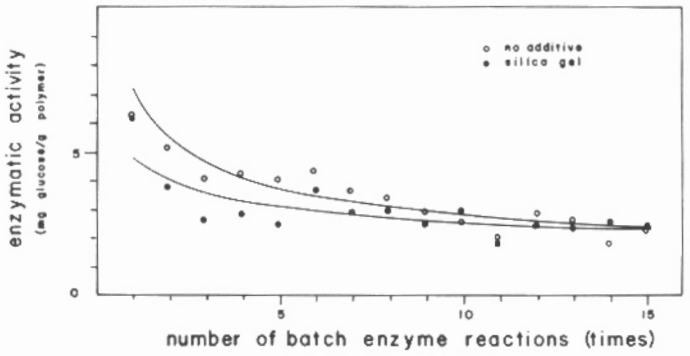

Fig. 5. Variation of enzymatic activity along sucessive batch reactions in a copolymer system. Cellulase concentration: 10%. Monomer composition: HEMA, 50%; HPA, 10%. Silica gel, 0.25 g/ml.

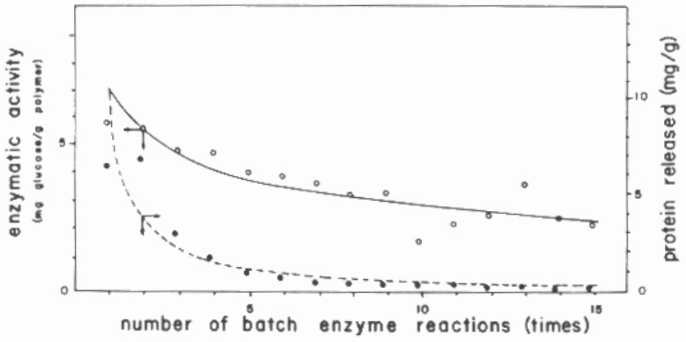

Fig. 6. Comparison between the variation of enzymatic activity and protein release of immobilized cellulase. HEMA concentration: 60%. Enzyme concentration: 10%. Substrate: 1% CMC

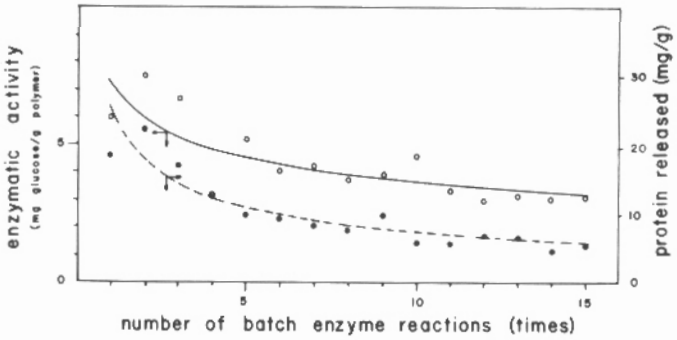

Fig. 7. Comparison between the variation of enzymatic activity and protein release of immobilized cellobiase. HEMA concentration: 60%. Enzyme concentration: 2%. Substrate: 1% salicin.

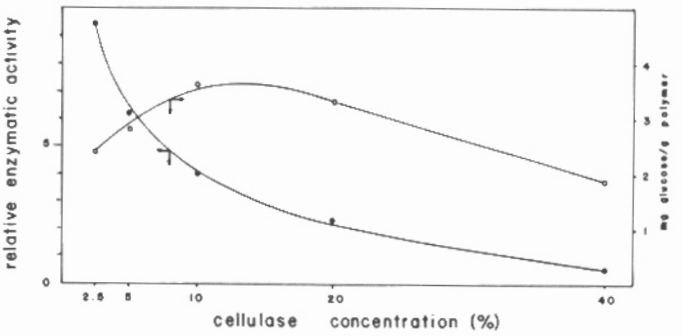

Fig. 8. Relationship between the relative enzymatic activity and the glucose yield of immobilized cellulase in the 6th batch reaction as a function of enzyme concentration.

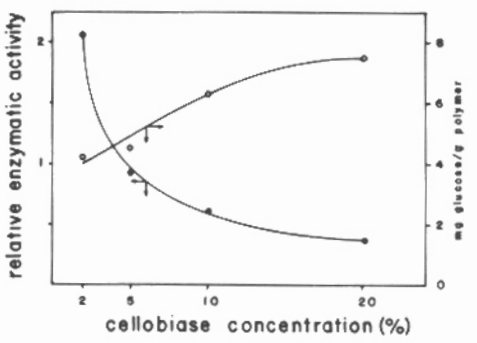

Fig. 9. Relationship between the relative enzymatic activity and the glucose yield of immobilized cellobiase in the 6th batch reaction as a function of enzyme concentration.

mentioned additives in the enzymatic activity maintenance, with matrices containing 40% HEMA. The presence of silica gel as well as PEG in the polymeric support made possible a better prevention of cellulase leakage than in the case of QUSO or none addition. Similar results were also obtained for immobilized cellobiase with the addition of silica gel, using 60% monomer concentration, as shown in Fig. 3.

In the case of a mixture of monomers, resulting in a higher total monomer concentration, the addition of silica gel showed no increasing in the capacity of retention of cellulase activity as shown in Figs. 4 and 5. The combination of HEMA-HEA appeared more promising in preventing the decrease of enzyme activity than HEMA-HPA, but behaves almost the same as HEMA alone. The capacity of additives in improving the enzyme trapping could depend on the monomer concentration.

The enzyme leakage from the 1st to 15th repeated use was evaluated by means of protein determination in the medium after incubation of the polymer slices (Figs. 6 and 7). From the 6th batch enzyme reaction, a stationary value of protein leakage was attained for any enzyme as measured once a day. At the same time, the immobilized enzymes present a constancy in their activity, in spite of the diminution of the protein content. In the case of two or more enzymatic reactions performed in the same day, a distinct decline of activity was observed. Thus, it seemed that between daily intervals, some kind of regeneration of the enzymatic activity in the matrix took place.

The relation between enzyme concentration and enzymatic activity of immobilized cellulase and cellobiase was established. Since the quantity of entrapped enzyme in the biocomposite was proportional to the enzyme concentration initially mixtured, the relative enzyme activity in each determination can be defined as the enzymatic activity (mg glucose produced/ g polymer) divided by the respective concentration. Figures 8 and 9 show the values of both relative and total enzymatic activity of the polymeric slices in the 6th enzyme batch reaction. For the two enzymes, the relative enzyme activity decreased regularly with the increasing of enzyme concentration. Nevertheless, in practice, total cellulase activity reached a maximum at about

10% enzyme concentration. For the cellobiase, a maximum value was not apparent until 20% enzyme concentration, the higher concentration tested so far.

REFERENCES

Dobó, J. (1970). Application of radiation polymerization for the production of water-insoluble enzyme preparations. Acta Chimica Academiae Scientiarum Hungaricae, 63 (4), 453-456.

Hoffman, A. S. (1977). Application of radiation processing in biomedical engineering. A review of the preparation and properties of novel materials. Radiat. Phys. Chem., 9, 207-219.

Kaetsu, I., M. Kumakura and M. Yoshida (1979). Enzyme immobilization by radiation-induced polymerization of 2-hydroxyethyl methacrylate at low temperatures. Biotechnol. Bioeng., 21, 847-861.

Kawahima, K. and K. Umeda (1974). Immobilization of enzymes by radiopolymerization of acrylamide. Biotechnol. Bioeng. , 16, 609-621.

Kumakura, M. and I. Kaetsu (1983). Immobilization of cellulase by radiation polymerization. Int. J. Appl. Radiat. Isot., 34 (10), 1445-1450.

Mandels, M., R. Andreotti and C. Roche (1976). Measurement of saccharifying cellulase. Biotechnol. & Bioeng. Symp., 6, 21-33.

Miller, G. L. (1959). Protein determination for large numbers of samples. Analyt. Chem., 31 964.

Yoshida, M., M. Kumakura and I. Kaetsu (1979). Immobilization of cellulase in the swollen microsphere by radiation polymerization. Polymer J., 11 (12), 915-919.