APPLICATION OF PHOTO-CROSSLINKABLE RESIN TO IMMOBILIZATION OF AN ENZYME

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1. Introduction

The aim of this communication is to report a novel convenient method for entrapping enzymes or microbial cells using photo-crosslinkable resins. The technique consists of mixing a liquid photo-crosslinkable resin containing photo-sensitive functional groups located at a desired distance, an appropriate initiator and an enzyme solution or microbial cell suspension, followed by illumination with near-ultraviolet light for only a few minutes. This simple procedure produced tailor-made matrices in which enzyme molecules or microbial cells were successfully entrapped. Typical experiments performed with a photo-sensitive resin, polyethyleneglycol dimethacrylate of different chain-lengths and yeast invertase are described in this paper. Various criteria indicate that this new method is very useful for immobilization of enzymes and other biologically active macromolecules.

2. Materials and methods

Of a variety of photo-sensitive resins available now, polyethyleneglycol dimethacrylate was employed for the following reasons: the matrices formed by photo-crosslinking are mechanically strong chemically inert and yet permeable enough to substrate and enzyme reaction products. Esterification was carried out by refluxing a mixed solution of commercially available polyethyleneglycol of an appropriate chain length and excess methacrylate in toluene in the presence of small amounts of *p*-toluenesulfonic acid in the usual way. The details of the procedure will be reported elsewhere. Yeast invertase (β-D-fructo-furanoside fructo-hydro-lyase, EC 3.2.1.26) obtained from Tokyo Kasei Co., Tokyo, Japan, was used as a test enzyme. The standard procedure for entrapping invertase was as follows: One part of solid polyethyleneglycol dimethacrylate (referred to as 'oligomer') (fig.1) was mixed with 0.01 parts of an initiator, benzoin ethyl ether. The mixture was melted by warming at 50°C. To the molten oligomer, was added 1.5 parts of a solution of invertase in 0.1 M acetate buffer (pH 5.0) (enzyme, 0.0015 parts). The homogeneous liquid mixture was layered on a sheet of transparent polyester film. The layer (thickness, 0.4 mm) was covered with the same film and then illuminated with a Toshiba Chemical Lamp FL 20BL (wavelength range, 300–400 nm; maximum intensity at 360 nm) for 3 min. The illumination resulted in the photo-crosslinking of the oligomers at

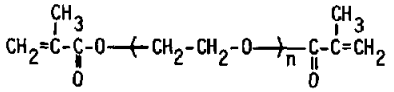

Fig.1. Structure of polyethyleneglycol dimethacrylate.

their methacrylate sites by a radical reaction mechanism. Invertase molecules were readily and completely entrapped inside the lattice of the resulting insoluble gel. During the illumination, the temperature of the enzyme-oligomer mixture did not rise above 40°C. The film thus formed was cut into small pieces (each, approx. 5 X 5 cm) and used as test sample of immobilized invertase.

Invertase activity was measured as follows: the pieces of gel containing the entrapped invertase were mixed with 10 ml of 100 mM sucrose solution in 0.1 M acetate buffer (pH 5.0). The enzyme reaction was carried out at 40°C for 10 min with agitation. The amount of glucose liberated was assayed by the coupled reactions of glucose oxidase and peroxidase [1].

3. Results

To establish adequate immobilization conditions, several factors which may affect the activity of the entrapped enzyme and the strength of matrices were examined. The relative activity of the entrapped enzyme assayed by this method was generally more than 40% of the original activity of the untreated enzyme. Under the optimal preparation conditions, the activity of the entrapped enzyme attained 60%. When the film entrapping invertase was ground to a fine powder, the activity of invertase in the powder reached the activity of the native counterpart. This would indicate that invertase was not damaged during the entrapping process.

Fig.2 shows the relationship between the chain length of the polyethyleneglycol part of the oligomer and the relative activity of the entrapped invertase. The relative activity increased with increasing chain length of the polyethyleneglycol moiety. The longer the length, the stronger the matrix of photo-cross-linked gel became. Leakage of enzyme was not observed in any case. Thus, we employed oligomer of 200 Å chain length for subsequent experiments. As shown in fig.3, entrapped invertase of almost identical activity was obtained by illumination times from 1 to 5 min. The ratio of the amount of invertase solution to the amount of oligomer as well as the enzyme concentration to be entrapped affected markedly the activity of the immobilized enzyme. A higher ratio of the enzyme to oligomer rendered the activity of the entrapped enzyme larger but lowered the strength of the matrices. The entrapped invertase catalyzed the hydrolysis of sucrose of various concentrations linearly with time for at least 60 min (fig.4, left). The

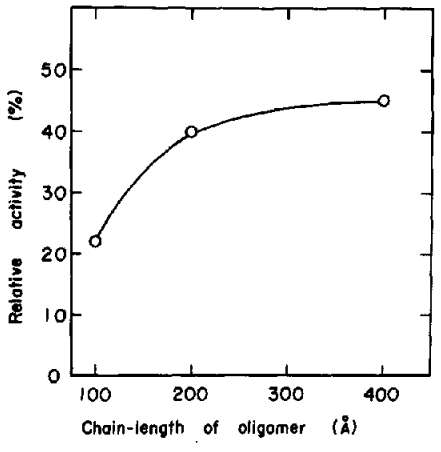

Fig.2. Effect of oligomer chain-length on relative invertase activity after immobilization. Oligomers having different lengths of the polyethyleneglycol moiety were used as starting materials. Polymerization procedure is given in the text. The activity of native invertase was taken as 100%.

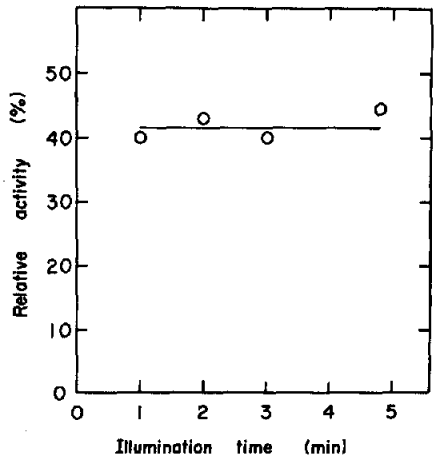

Fig.3. Effect of illumination time on relative invertase activity after immobilization. Oligomer of 200 Å chain-length was used as the starting material. The polymerization procedure is given in the text. The activity of native invertase was taken as 100%.

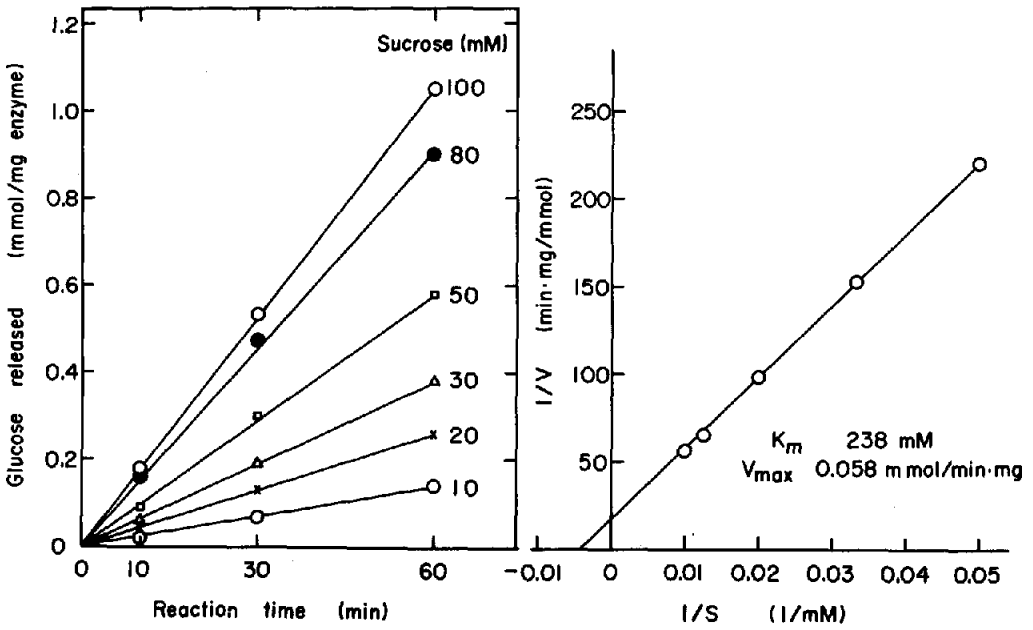

Fig.4. Time-course of glucose release using entrapped invertase. The enzyme was entrapped as described in the text using oligomer of 200 Å chain-length as the starting material.

apparent K_M value for sucrose of the immobilized enzyme was calculated as 238 mM from a Lineweaver-Burk plot (fig.4, right). This value, about 5-fold larger than that of the native enzyme (50 mM), would be ascribable to a limited diffusion rate of substrate and/or reaction products, as observed in many immobilized enzyme systems. The V_{max} of the entrapped enzyme was estimated as 0.058 mmol/min/mg-enzyme, which is comparable to that of the free enzyme (0.036 mmol/min/mg-protein) (fig.4, right). In comparison with a rather sharp optimal pH of the native enzyme (approx. pH 5.0), the immobilized form showed maximal activity over a somewhat wider pH range (about pH 5.0 to 5.8).

Comparison of the effect of temperature on the activity of native and entrapped invertase showed that the former was much more quickly inactivated by temperatures above 50°C. Long-term storage tests have shown that the immobilized invertase is much more stable than the free enzyme when stored in 0.1 M acetate buffer (pH 5.0) in a refrigerator. During the course of repeated (more than 30) batch reactions, the entrapped enzyme could be successfully used without any loss of initial activity.

4. Discussion

Of various principles currently available for immobilization of enzymes or microbial cells [2,3], inclusion of enzyme molecules inside gel matrices of synthetic polymers was first proposed by Bernfeld and Wan [4]. This method has very bright prospects both for practical uses and for theoretical analyses of enzyme behaviour and kinetics in structured systems.

The conventional techniques for inclusion of enzymes inside synthetic resins such as acryl polymers usually involve polymerization process(es) in the presence of the enzyme. Inclusion of enzyme or other biologically active macromolecules in synthetic or natural polymers offers the advantage of relatively milder conditions than necessary for other methods, such as covalently binding to insoluble matrices or intermolecular crosslinking of biologically active substances. However, much more convenient and versatile techniques would be desirable to entrap not only enzymes but also microbial cells and even organelles inside gel matrices of suitable physico-chemical properties.

The method presented here has the following

merits: (1) oligomers containing multiple photosensitive functional groups located at a desired distance can be prepared previously in the absence of enzyme or cells. If necessary, suitable ionic, hydrophobic or hydrophilic group(s) can be introduced into the oligomer at this stage; (2) the formation of polymer matrices by a photo-crosslinking reaction can be performed under extremely mild conditions. The reaction could be achieved without heating, shifting pH to extreme values or the use of chemicals that might modify the structures of enzymes, cells or organelles. The application of this convenient technique to immobilization of various enzymes, microbial cells and organelles such as microbodies and mitochondria will be reported elsewhere.

References

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