A SIMPLE ENTRAPMENT METHOD FOR IMMOBILIZING ENZYMES WITHIN CELLULOSE FIBERS

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1. Introduction

Immobilized enzymes have been intensively studied during recent years. The immobilization to a matrix includes chemical methods of covalent bond formation and cross-linking, physical methods of adsorption and entrapment, and various combinations [1]. Cellulose and cellulose derivatives are the most frequently employed carriers [2], although promising synthetic polymers have recently been developed [3]. Chemical methods of immobilization are rather tedious, as compared with the adsorption on ion exchange cellulose [4], with entrapment in cellulose triacetate fiber [5] or in membrane [6]. Among others, aminoacylase adsorbed on DEAE-sephadex has been successfully applied for optical resolution of DL-amino acids [7] and glucose isomerase adsorbed on DEAE-cellulose for producing fructose syrup [4].

The present work deals with a new simple method of entrapping glucose isomerase or β-galactosidase in cellulose fibre of highly hydrophilic nature. α-Cellulose solubilized in a mixture of *N*-ethyl-pyridiniumchloride and dimethylformamide, and containing solid enzyme as suspension, could be regenerated as enzyme containing fiber from water. The activity recovery of immobilized whole-cell glucose isomerase was 40–60% and half-life of 42 days in continuous column operation was obtained for glutaraldehyde treated fiber entrapped enzyme. We were also able to show that the activity recovery of immobilized soluble β-galactosidase could be markedly improved by recovering the fiber from 2.5% glutaraldehyde solution. The fiber entrapped β-galactosidase was stable over four times of reuse in batch hydrolysis of lactose.

2. Materials and methods

2.1. *Chemicals and enzymes*

α-Cellulose (D.P.880) was supplied by Rauma-Repola Oy, Finland. Dimethylformamide (Merck A.G.) was vacuum distilled before use. *N*-ethylpyridinium-chloride was prepared from pyridine and ethylchloride. Whole-cell glucose isomerase (EC 5.3.1.5) Maxazyme GI 14000 (from *Actinoplanes*, 12.7 μkat/g) was a gift from Gist-Brocades N.V. and β-galactosidase (EC 3.2.1.23) Lactase LP (from *Aspergillus niger*, 103 μkat/g) from Wallerstein Co.

2.2. *Enzyme assays*

For glucose isomerase activity the enzyme was incubated at 65°C in 1 ml of 0.25 M maleate buffer, pH 6.8, containing 0.1 M $MgSO_4 \cdot 7H_2O$ and 0.13 M KCl. After 10 min 1 ml of 10% glucose in above buffer containing in addition 0.002 M $CoCl_2 \cdot 6H_2O$ was added and the reaction was carried out for 20 min with magnetic stirring. The reaction was stopped by adding 10 ml of 0.1 M perchloric acid. Fructose pro-

duced was then determined by the cystein-carbazole method [8]. For blank glucose was added after perchloric acid.

β-Galactosidase activity was determined by a modified glucose oxidase-peroxidase method according to Hyrkäs et al. [9].

Activities are expressed as micro katals per gram of dry weight (105°C, 2 h) of enzyme cellulose fiber.

2.3. *Enzyme immobilization*

A mixture of 150 g of *N*-ethylpyridiniumchloride and 75 g of dimethylformamide was added to one liter Sovirel reactor equipped with mechanical stirrer, nitrogen inlet, reflux condenser, drying tube and glycerol jacket to maintain the required temperature. After heating to 90°C, 3 g of α-cellulose were added to the melt. Mixing was continued until total dissolution. The temperature was lowered to 70°C, a suitable quantity (e.g. 6 g) of solid enzyme was added, mixed well with a glass rod and the suspension was poured into 4 liters of water (23°C) with stirring. The regenerated cellulose fiber with the entrapped enzyme was washed with 3 × 4 liters of water and the excess water was removed by suction. The fiber could be further treated with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at 23°C for 1 h to 'fix' the cell wall and to cross-link enzyme molecules. The treated fiber was washed thoroughly with water.

2.4. *Enzyme reactor experiments*

Continuous isomerization of glucose was carried out in a jacketed glass column (1.5 × 15 cm) with a G3 glass filter at the bottom. The enzyme fiber was soaked in 1 M glucose of pH 7.2 for 30 min and packed into the column. The pH of substrate, 1 M glucose containing 0.003 M Mg^{++}, 0.003 M Co^{++}, 0.1% methyl-*p*-hydroxybenzoate and 0.01% propyl-*p*-hydroxybenzoate, was adjusted to 7.2 at 60°C with 0.2 N NaOH just before passing to the column. Column temperature was maintained at 60°C by a water jacket.

Batch hydrolysis of lactose was carried out at 45°C for 4 h in a small reactor equipped with a glass stirrer. Immobilized β-galactosidase (4.1 μkat, equivalent to 39.4 mg of free enzyme, corresponding to 0.79% of weight of lactose) was added to 100 ml of 5% lactose in 0.1 M acetate buffer, pH 4.5, for hydrolysis at 45°C.

3. Results and discussion

Cellulose may be chemically converted to soluble derivatives such as cellulose triacetate used by Dinelli and Morisi [5] for enzyme entrapment or solubilized cellulose may be regenerated as enzyme containing pure cellulose fiber as described here. Several types of solvents have been suggested for cellulose [10]. However, the reaction conditions are generally too severe for enzyme immobilization. The use of *N*-ethylpyridiniumchloride was first suggested as a solvent for cellulose by CIBA [11]. Husemann and Siefert [12] used a melt of *N*-ethylpyridiniumchloride for homogenous reactions of cellulose in solution. The salt melts at 118–120°C, but with the addition of 50% of dimethylformamide, pyridine or dimethylsulfoxide the melting point is lowered to about 77°C. Cellulose dissolves well in such salt solvent mixture. After addition of dry enzyme, the dissolved cellulose may be regenerated from water as fiber or beads to entrap the enzyme. The pH of this solvent system is about 6.5–7.5, as compared with the highly alkaline conditions of most other methods.

3.1. *Glucose isomerase*

Immobilized glucose isomerase activity was proportional to the quantity of enzyme added until the added quantity was equal to that of cellulose (table 1). When the quantity of enzyme was further tripled, the entrapped activity did not increase enough to warrant higher enzyme concentrations. The activity recovery of immobilized glucose isomerase was 40–60%. This compares favorably with the typical recovery rates of less than 40% obtained by other immobilization methods [13]. We believe that this is largely due to the highly hydrophilic nature of fiber.

Glutaraldehyde treatment for cell wall 'fixation' and enzyme cross-linking did not decrease activity, but increased substantially the column stability. Lyophilized cross-linked enzyme cellulose fiber retained about 50% of activity. Giovenco et al. [14] reported the loss of all activity on lyophilization of cellulose triacetate entrapped glucose isomerase.

The cross-linked immobilized glucose isomerase was partially dried in vacuum at 45°C for approx. one hour to approx. half of original weight. This preparation was used for continuous isomerization

Table 1
Effect of enzyme quantity on the activity of the immobilized enzyme

Glucose isomerase Enzyme added (g/3 g cellulose)	Activity (μkat/g)	β-Galactosidase Enzyme added (g/3 g cellulose)	Activity (μkat/g)
1	0.7	1	1.9
2	2.2	2	2.9
3	3.5	3	4.3
5	4.7	4	7.3
6.3	5.3	7.7	11.7
9	5.8		

of 1 M glucose in the column. The system was operated continuously for 46 days at 60°C. The operation conditions and results are shown in fig.1. During the 46 days, no compacting of the column was observed. Conversion of 43% was maintained unchanged for 25 days, followed by a slow decrease until after 42 days half of the inital activity remained. Saini and Vieth [15] reported a half-life of 50 days for collagen entrapped glucose isomerase and Dinelli and Morisi [5] obtained operational stability for 150 days at 45°C on cellulose triacetate entrapped enzymes. Others [16,17] have reported shorter half-lives.

3.2. *β-Galactosidase*

The activity of immobilized β-galactosidase was proportional to the quantity of β-galactosidase added within the total range of concentrations (table 1). With the addition of 7.7 g of enzyme to 3 g of cellulose an activity of 11.7 μkat/g dry weight was achieved. In most earlier experiments by other immobilization techniques activities of the same order or less have been obtained [18–20]. The activity recovery of 5–7% was, however, low as compared to that obtained with glucose isomerase. This is believed

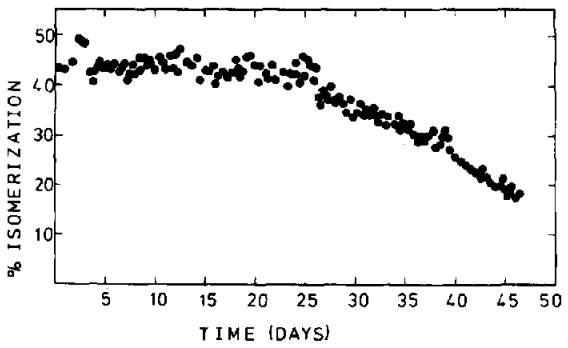

Fig.1. Continuous isomerization of glucose with immobilized glucose isomerase (substrate 1 M glucose, flow rate 6 ml/h, pH 7.2, 60°C, initial total activity 4.8 μkat).

Table 2
Batch hydrolysis of lactose with free and immobilized β-galactosidase

Time (h)	Free enzyme	Degree of hydrolysis (%)			
		Immobilized enzyme No. of the consecutive batches			
		1	2	3	4
1	26.9	22.8	22.0	21.9	21.6
2	39.5	31.8	31.7	30.7	31.2
3	47.2	39.0	36.9	38.5	39.0
4	52.7	44.3	45.1	44.2	44.6

to be due to solubilization of enzyme in water during regeneration of cellulose. Consequently, when regeneration was carried out from 2.5% glutaraldehyde, the activity recovery was doubled. On the other hand, glutaraldehyde treatment of β-galactosidase cellulose fiber resulted in 12% loss of activity.

The conditions and the results of 4 consecutive hydrolyses are shown in table 2. As can be seen, the degree of hydrolysis remained constant during the experimental period as compared with about 17% decrease with β-galactosidase absorbed on phenol-formaldehyde resin and cross-linked with glutaraldehyde [9]. The somewhat lower hydrolysis rate obtained with the immobilized enzyme is likely due to diffusion limitation. This, however, is of no consequence, because of the stability of the enzyme.

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