Journal of Solid-Phase Biochemistry, Vol. 5, No. 4, 1980

IMMOBILIZATION OF β-GALACTOSIDASE ON BENZOQUINONE-ACTIVATED BEAD-CELLULOSE[1]

MOONJIN CHUN,[2] GISELA DICKOPP, and MANFRED SERNETZ[3]

Institut für Biochemie und Endokrinologie, FB 18
Justus-Liebig-Universität Giessen
D-6300 Giessen, Federal Republic of Germany

Accepted April 30, 1980

β-galactosidase from *Escherichia coli* was immobilized on porous bead cellulose by a benzoquinone coupling method. Optimum conditions for activation and coupling were investigated, and the kinetic parameters of the immobilized enzyme described. The binding capacity was 15.6 mg/g of wet conjugate, corresponding to 109 mg/g dry matrix. A saturation activity of 4100 U/g dry cellulose beads was achieved. The apparent Michaelis constant of the immobilized β-galactosidase at pH 7.6 for orthonitrophenyl-galactopyranoside was 2.4×10^{-3} mol/liter, as compared to 2.4×10^{-4} mol/liter of the native enzyme. The stability of benzoquinone-activated bead cellulose and of immobilized β-galactosidase were also determined.

INTRODUCTION

Until now cellulose has been regarded as of little significance as a matrix for biotechnical applications because of its poor flow properties due to the fibrous structure or powder form. Recently, however, porous bead cellulose has been introduced as a new promising matrix (1–5). This study presents the immobilization of β-galactosidase as a high molecular weight enzyme on porous bead cellulose using a benzoquinone coupling method (6), which had not previously been applied to bead cellulose. The kinetic experiments were performed using miniaturized analytical stirred batch reactors (7).

MATERIALS AND METHODS

β-galactosidase (β-D-galactoside-galactohydrolase (E.C. 3.2.1.23)) from *Escherichia coli*, free of albumin, was purchased from Boehringer

[1]Supported by a grant Se 315/6 of the Deutsche Forschungsgemeinschaft. This work was presented in part at the 5th Enzyme Engineering Conference, Sept. 29, 1979, Henniker, New Hampshire, U.S.A.

[2]Present address: Department of Agricultural Chemistry, Korea University, Seoul 132, Korea.
[3]Author whom all correspondence should be addressed.

0146-0641/80/1200-0211$03.00/0

GmbH, Mannheim. The crystalline suspension in 2.2 M ammonium sulfate with a specific activity of 32.5 U/mg for lactose was dialyzed against 0.1 M potassium-phosphate buffer, pH 7.6. *p*-Benzoquinone z.S. and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from E. Merck, Darmstadt. Porous bead cellulose, lot No. C 503 and C 527/2, was a gift from the Institute of Macromolecular Chemistry, Czechoslovakian Academy of Sciences, Prague. The gel beads, suspended and swollen in water, had a diameter of 40 to 140 μm and a declared water content of 85%, which served for the conversion of wet to dry weight for comparison with other matrices. The preparation was stored at 4°C in a refrigerator.

Activity Determinations

Stirred batch reactor experiments were performed in 10 ml glass vessels as analytical reactors (7) with magnetic stirrers. Experiments were carried out using an 0.2 ml enzyme-matrix suspension (10 g/liter, wet) and a 1.0 ml ONPG solution, with final concentrations ranging from 0.33 to 1.36 mM in 0.1 M K-phosphate buffer, pH 7.6. The reactions were stopped by addition of 1.0 ml 0.25 M NaOH, and recorded in semimicro flowthrough cuvettes on a spectrophotometer PMQ II (Zeiss) at 405 nm. Calculations were performed using an extinction coefficient at pH 9.0 of $\varepsilon = 4.1 \times 10^3\ M^{-1}\ cm^{-1}$. The specific activity of β-galactosidase for ONPG was 123 U/mg at 26°C.

Activation

Swollen cellulose beads (10 g wet) were washed on a filter paper funnel with 0.1 M K-phosphate buffer. The beads were then titrated to pH 10.0 with NaOH, containing 20% ethanol, and subsequently suspended for activation in 100 ml of this buffer, containing 0.1 M benzoquinone. The reactants were stirred gently for 4–6 h at room temperature. The beads were washed again with ethanol buffer, then with water, and finally with the buffer used for coupling.

Immobilization

Immobilization of dialyzed β-galactosidase was performed by adding 0.5 ml of an adequate dilution of the enzyme dialyzate (0.2–2.6 g/liter) to 100 mg (wet weight) activated beads. The mixture was allowed to react with gentle shaking for 24 h at room temperature. The preparation was then washed on a glass funnel for 50 min with 20 ml 0.1 M K-phosphate buffer, pH 7.6, 10 ml 1.0 M KCl, and again with 70 ml K-phosphate buffer.

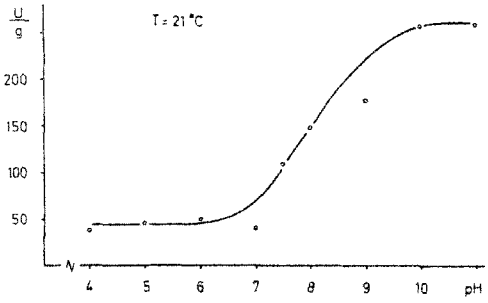

FIG. 1. pH-dependence of the benzoquinone activation, measured from bound enzyme activity; 100 mg wet beads were incubated with benzoquinone, washed, and reacted with 1 ml β-galactosidase, 616 mg/liter, for 22 h.

RESULTS AND DISCUSSION

Optimization of the Activation Procedure

Figure 1 shows the enzyme activity of the preparation as a function of the pH of the activation reaction in the range pH 4–11. Activation becomes effective above pH 7.5 and is optimum at pH 10.0. The benzoquinone activation at pH 10.0 is almost complete after 4 h (Fig. 2), and thus activation times of 4–6 h were regarded as convenient. The influence of benzoquinone concentration on the activation was determined at pH 10.0 and at 4.5 h incubation with benzoquinone concentrations ranging from 10 to

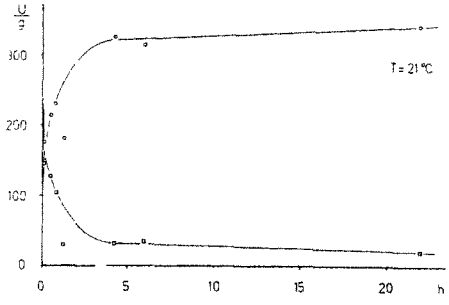

FIG. 2. Time dependence of benzoquinone activation at pH 10.0, measured from bound (○) and eluted (□) enzyme activity; 100 mg wet beads were coupled with 1 ml β-galactosidase, 696 mg/liter, in 0.1 M K-phosphate buffer, pH 7.6, for 48 h.

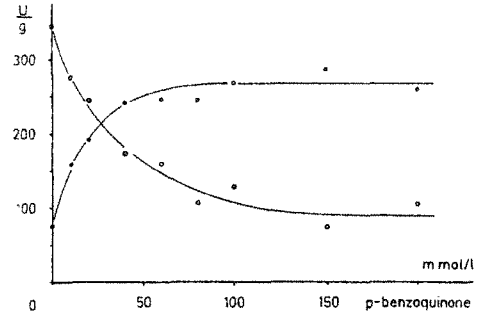

FIG. 3. Concentration dependence of benzoquinone activation of bead cellulose, measured from bound (●) and eluted (○) enzyme activity; 100 mg wet beads were coupled with 1 ml β-galactosidase, 847 mg/liter, for 24 h at pH 7.6.

200 mM. Figure 3 shows the bound and eluted β-galactosidase activity as a function of the benzoquinone concentration, with an optimum at 100–150 mM *p*-benzoquinone.

Optimization of Coupling

After activating cellulose beads with 100 mM benzoquinone at pH 10.0 for 4.5 h, it was observed that coupling with β-galactosidase (847 mg/liter) for 22 h was most effective in the range of pH 7–8. The time dependence of coupling with 960 mg/liter β-galactosidase at pH 7.6 was determined by removing and washing aliquots of 50 mg cellulose beads from the rotator at different times. Figure 4 shows the bound and eluted activity as function of

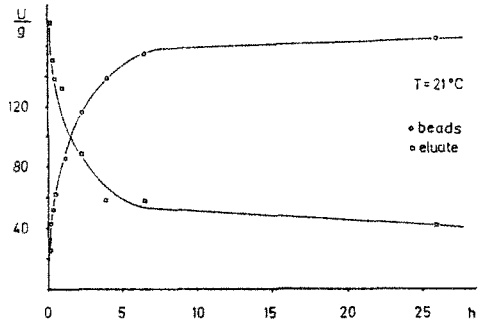

FIG. 4. Time dependence of coupling, measured from bound (○) and eluted (□) activity.

the coupling time. Coupling is almost complete after about 7 h, but the gel evidently contains reactive groups even after 24 h.

Kinetic Data of Immobilized β-Galactosidase

The activities of the conjugate as a function of the substrate concentration and enzyme loading are shown in Fig. 5. Similarly, the activities as a function of the enzyme load ranging from 13 to 109 mg/g dry cellulose under substrate saturation are shown in Table 1.

Figure 6 shows the apparent Michaelis constant of the β-galactosidase cellulose conjugate as a function of the enzyme loading at pH 7.6. The increase from $K_M^{native} = 2.3 \times 10^{-4}$ M ONPG of the native enzyme to $K_{M,app}^{imm} = 2.4 \times 10^{-3}$ M ONPG at high load reflects the influence of diffusion limitation (7,8,11,14). Similarly, a 1.9-fold increase was reported for β-galactosidase immobilized on cellulose membranes (9) and increase by a factor of 2.8 for invertase on porous bead cellulose (10).

Saturation activity is reached at 4130 U/g and maximum binding at 109 mg/g dry conjugate (Fig. 7). Comparatively for immobilization of β-galactosidase to tricarbanilated cellulose by glutaraldehyde coupling, activities of 93 U/g, corresponding to about 2 mg enzyme/g cellulose, were reported (15).

The enzyme load was calculated by balancing the activities applied in the binding assay and eluted after 21 h coupling (Tables 1 and 2).

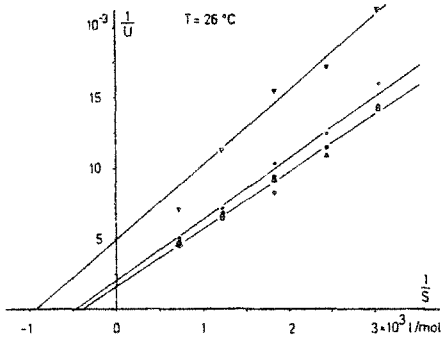

FIG. 5. Activity of immobilized β-galactosidase (U/g wet cellulose) as a function of the substrate concentration and with the enzyme-to-matrix ratio in the binding assay as parameter; □, 13.4; △, 11.4; ○, 8.4; ●, 4.7; ▼, 1.6 U/mg; Lineweaver-Burk-plot.

TABLE 1. **Activity of Immobilized β-Galactosidase as a Function of the Enzyme Loading of the Cellulose Matrix (Dry Weight) in the Binding Assay**

Binding assay, β-galactosidase/g dry cellulose C 503						Immobilized β-galactosidase				
Activity			Protein							
Total (U/g)	Eluted (U/g)	Bound (U/g)	Total (mg/g)	Eluted (mg/g)	Bound (mg/g)	Maximum activity[a] (U/g)	App. spec. activity (U/mg)	Binding yield[b]	Activity yield[c]	K_M^{imm} (mM)
1680	70	1610	13.7	0.6	13.1	1400	107.0	0.96	0.87	1.1
4970	280	4690	40.6	2.1	38.5	3500	90.9	0.94	0.75	2.1
9030	630	8400	73.5	4.9	68.6	4130	60.2	0.93	0.49	2.4
13,580	2170	11,410	110.6	17.5	93.1	4130	44.4	0.84	0.36	2.4
22,610	9240	13,370	184.1	74.9	109.2	4130	37.8	0.59	0.31	2.4
			maximum		109.2	4130	123.0	($T = 26°C$)		

[a] Maximum activity in U/g dry cellulose under substrate saturation.
[b] Binding yield as ratio of bound activity to total activity applied.
[c] Activity yield as relative apparent specific activity.

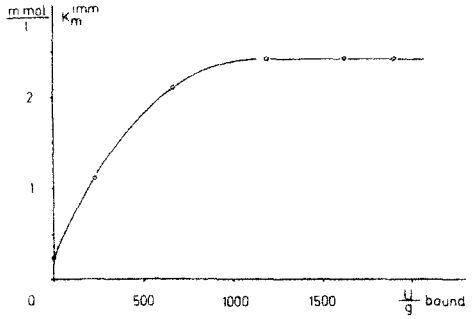

FIG. 6. Apparent Michaelis constant as a function of enzyme load.

The effectively bound amount of 109 mg protein/g dry cellulose corresponds to 15.6 mg/g wet cellulose beads. This is comparable with data obtained with β-galactosidase bound to oxirane-acrylic beads (11) on a wet weight basis.

Stability Determinations

These experiments concerned both the stability of the benzoquinone activation of the cellulose matrix and the stability of the activity of the conjugate (Table 2 and Fig. 8). The activated cellulose was stored in 0.1 M K-phosphate buffer, pH 7.6, with 0.1 g/liter NaN_3, both at room temperature (21°C) and in the refrigerator (0°C) over 54 days. Thereafter, β-galactosidase was coupled at two concentrations for 24 h (Table 2).

The decrease in protein binding depends differently on the temperature and on the enzyme concentration. At low loading of 1530 U/g in the

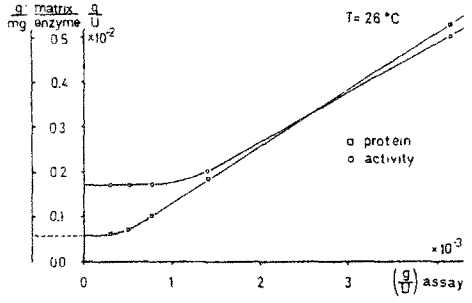

FIG. 7. Activity (○) and protein load (□) per g wet cellulose conjugate as a function of the enzyme-to-matrix ratio in the binding assay (double reciprocal plot).

TABLE 2. **Stability of Benzoquinone Activated Bead Cellulose C 503 as a Function of Time and Storage Temperature, Determined for Two Enzyme-to-Matrix Ratios in the Binding Assay**[a]

		Binding assay, β-galactosidase/g dry cellulose C-530						Immobilized β-galactosidase				
Storage		Activity			Protein							
Time (d)	Temp. (°C)	Total (U/g)	Eluted (U/g)	Bound (U/g)	Total (mg/g)	Eluted (mg/g)	Bound (mg/g)	Maximum activity (U/g)	App. spec. activity (U/g)	Binding yield	Activity yield	K_M^{imm} (mM)
0		1600	70	1530	13.0	0.5	12.5	1330	107.0	0.96	0.83	1.1
0		13,000	2100	10,900	105.3	16.7	88.6	3940	44.4	0.84	0.30	2.4
54	21	1530	800	730	12.7	6.7	6.0	530	87.9	0.48	0.35	0.6
54	21	12,700	9600	3100	102.1	77.4	24.7	1870	75.7	0.24	0.15	1.3
54	0	1530	270	1260	12.7	2.4	10.3	1130	109.7	0.81	0.74	1.2
54	0	12,700	7100	5600	102.1	57.6	44.5	2940	66.0	0.44	0.23	2.1

[a]Definitions as in Table 1.

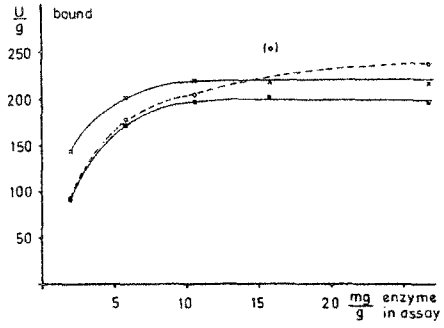

FIG. 8. Stability of bound β-galactosidase (U/g wet cellulose) as a function of the enzyme-to-matrix ratio in the binding assay; ×, initial activity, ●, activity after 60 days at 0°C, ○, activity of conjugate and supernatant.

coupling assay, the protein binding decreases within 54 days at 21°C to about 50%, whereas storage at 0°C retains at least 85% of the initial activity. With high loading of 12,700 U/g, only about 30% of the initial value is reached at 21°C, whereas storage at 0°C yields about 50%. When compared on the basis of activities, the stability of the benzoquinone activated cellulose seemingly yields better data. Thus under storage at 21°C, the maximum activity decreases to about 45% of its initial value and to about 75–85% at 0°C.

The results of the apparent stability of the β-galactosidase-cellulose-conjugate are given in Fig. 8 for five enzyme-to-matrix ratios in the binding assay and after storage at 0°C over 60 days. At high load (>10 mg/g), 91–95% is retained, whereas at a load of 2 mg/g, the activity decreases to 64%. In comparison, the activity of the native enzyme falls in the same time to about 30% under storage without NaN_3. These findings agree with the observation and calculation made originally by Ollis (14), that diffusional constraints lead to higher apparent stabilities. The sum of the activities of the conjugate and of the supernatant show that at high enzyme load, some enzyme is eluted during storage without appreciable loss of the activity of the conjugate at saturation and due to limitation by diffusion.

Table 3 summarizes and compares maximum protein load achieved for our β-galactosidase-cellulose conjugate with data reported for the binding of different proteins to cellulose, agarose, and polyacrylamide gel beads for benzoquinone and other coupling reactions. The binding of β-galactosidase to benzoquinone-activated cellulose on the basis of the wet weight of the matrix is about 16 mg/g. This is equivalent to epoxy-activated polyacrylamide beads (7). On a molar comparison, β-galactosidase exhibits the

TABLE 3. **Maximum Protein Loading and Activities Achieved for the Immobilization of Enzymes to Polysaccharide and Polyacrylamide Beads (Dry Weight)**

Matrix	Method of coupling	Enzymes	M.W.	Maximum protein load (mg/g)	(mmol/g)	Maximum activity (U/g)	References
Agarose beads (Sepharose 4B)	*p*-Benzoquinone activation	Chymotrypsin	25,000	72	2.88		(13)
		Ribonuclease	14,000	78	5.57		
Cellulose beads (C-503)		β-Galactosidase	540,000	109	0.202	4100 (26°C)	(8)
				16 (wet)			
Cellulose beads	Diisocyanate-cross-linking	Glucoamylase	96,000	220	2.32	2000 (55°C)	(1)
	DEAE-ionic bond	Glucoisomerase				400 (60°C)	(1)
	Guanidino-ionic bond	Yeast invertase	270,000				(10)
Oxirane-acrylic beads (2878 C)	Epoxy-preactivation	β-Galactosidase	540,000	40	0.074	1780 (26°C)	(7)
				16 (wet)			
		Albumin	69,000	139	2.01		(7)
		γ-Globulin	156,000	122	0.782		(7)

lowest load due to its high molecular weight of about 0.2 and 0.07 mmol/g dry matrix. This reflects the decrease of the inner surface available for binding as function of the molecular weight of the ligand.

Thus benzoquinone activation of porous bead cellulose represents a very attractive activated matrix for biotechnical and industrial application. As compared to fibrous or crystalline cellulose (15), porous gel beads possess a much higher inner surface for binding, and combine convenient chemical and mechanical stability with good flow properties. Due to its hydroxy groups, the bead represents a very hydrophilic, inert matrix with a wide potential for chemical modification and sufficient stability against bacterial degradation. The activation with benzoquinone likewise is a very simple, cheap and effective procedure. 1,4-Benzoquinone is not very toxic and is relatively safe. Moreover, it becomes a nondeleterious substance during the immobilization procedure, because the reaction products obtained might have a 2.5-substituted hydroquinone structure. Therefore, it should be hygienically unobjectionable and apt for industrial application (12).

REFERENCES

1. CHEN, L. F., and TSAO, G. T. (1976) Biotechnol. Bioeng. 18 : 1507.
2. ŠTAMBERG, J., and PEŠKA, J. (1978) Bead Cellulose, Institute of Macromolecular Chemistry, Prague, p. 15.
3. PEŠKA, J., ŠTAMBERG, J., and HRADIL, J. (1976) Angew. Makromol. Chem. 53 : 73.
4. PEŠKA, J., ŠTAMBERG, J., HRADIL, J., and ILAVSKY, M. (1976) J. Chromatogr. 125 : 455.
5. PEŠKA, K., ŠTAMBERG, J., and PELZBAUER, Z. (1978) Cell. Chem. Technol. 21 : 419.
6. PORATH, J., and AXÉN, R. (1976) Immobilization of enzymes to agar, agarose, and Sephadex supports. *In* Methods in Enzymology, Vol. 44, COLOWICK, S. P., KAPLAN, N. O. (eds.), Academic, New York, p. 19.
7. HANNIBAL-FRIEDRICH, O., CHUN, M., and SERNETZ, M. (1980) Biotechnol. Bioeng. 22 : 29.
8. CHUN, M. (1979) Thesis, Giessen.
9. SHARP, A. K., KAY, G., and LILLY, M. D. (1969) Biotechnol. Bioeng. 11 : 363.
10. DICKENSHEETS, P. A., CHEN, L. F., and TSAO, G. T. (1977) Biotechnol. Bioeng. 19 : 365.
11. CHUN, M., and SERNETZ, M. (1980) β-Galactosidase immobilized on benzoquinone-activated bead cellulose and on oxirane-acrylic beads. Proc. V Enzyme Engineering Conference, Henniker, N.H., U.S.A., July 29–August 3, 1979 (Enzyme Engineering, Vol. 5, WEETALL, H. H., and ROYER, G. P. (eds.), Plenum, New York, 1980).
12. WINDHOLZ, M. (1976) The Merck Index, 9th ed., Merck & Co., Inc.
13. BRANDT, J., ANDERSSON, L.-O., and PORATH, J. (1975) Biochim. Biophys. Acta 386 : 196.
14. OLLIS, D. F. (1972) Biotechnol. Bioeng. 14 : 871.
15. BEDDOWS, C. G., MIRAUER, R. A., and GUTHRIE, J. T. (1980) Biotechnol. Bioeng. 22 : 311.