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Note

Evaluation of man-tailored cellulose-based carriers in glucoamylase immobilization

Jolanta Bryjak,[a,*] Jolita Aniulyte[b] and Jolanta Liesiene[b]

[a]*Faculty of Chemistry, Wrocław University of Technology, Wybrzeże Wyspiańskiego 27, 50-373 Wrocław, Poland*
[b]*Department of Chemical Technology, Kaunas University of Technology, Radvilenu pl. 19, LT-50254 Kaunas, Lithuania*

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Abstract—Covalent immobilization of glucoamylase on the cellulose-based carrier Granocel was optimized by changing the anchor groups and the methods of activation/immobilization. Binding of the enzyme was via its primary amino groups. It was shown that using carbodiimide and divinyl sulfone for the activation of –COOH and –OH groups on the carrier resulted in the preparations with very low activity. A third method, using pentaethylenehexamine with glutaraldehyde, led to the attachment through a long spacer arm and to the preparations with the highest activity. Further optimization of the carrier's structure consisted of changing pore diameters and amount of functional groups on the carrier surface. The highest activity of bound glucoamylase was obtained by linking the protein via glutaraldehyde on NH_2-Granocel having high pore size and high number of functional groups. The immobilized enzyme was stable throughout extended storage and possessed higher thermal stability.

Keywords: Cellulose modification; Carrier; Granocel; Glucoamylase; Immobilization; Starch; Hydrolysis

The immobilization of enzymes is a widely used approach for obtaining reusable forms of biocatalysts that provide easy separation from products and convenient handling.[1–3] The most popular methods of obtaining water-insoluble enzyme derivatives are: adsorption and covalent coupling to solid matrices. The most important factors influencing immobilization processes are as follows: (i) carrier properties (material, particle diameter, pore size, available anchor groups and their amount); (ii) enzyme stability, available anchor groups on a protein surface; (iii) immobilization conditions (pH, ionic strength, protein concentration, carrier activators). The number of factors affecting the enzyme immobilization makes the 'trial and error method' the dominant way to select a carrier and an immobilization procedure for a given enzyme.[1–3] In such situations any information about the given enzyme, for example, penicillin acylase does not tolerate binding with the help of carbodiimide,[4] is helpful. Thus, the main point of this paper was to search for the kind of anchor groups and their activation method suitable for the enzyme immobilization. For this purpose, a technical glucoamylase preparation (Amigase; Gist-Brocades) was selected for its thermostability and popularity in immobilization as well as uses in starch hydrolysis[5–8] or as a catalytic element in biosensors for detection of starch and maltose.[9–11] Granocel, in the form of cellulose-based granules with diameter of 150–300 μm was used as the matrix. This material was previously used successfully for the preparation of stationary phases for chromatography[12–16] and for immobilization of enzymes.[17] The Granocel matrix was specially prepared to obtain carriers with three pore size diameters (Table 1). In order to make possible application of different covalent linkage methods, various functional groups were introduced into the matrix. In such ways three additional kinds of carriers: CM-Granocel, DEAE-Granocel, and NH_2-Granocel were obtained. The synthesis conditions and brief characteristics of the carriers used for immobilization of glucoamylase are presented in Tables 2 and 3.

* Corresponding author. Tel.: +48 71 320 26 77; e-mail: jolanta.bryjak@pwr.wroc.pl

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Table 1. Characteristics of the Granocel matrix

Matrix	Exclusion limit (for dextrans)	Specific volume [mL/g]	Water retention [g/g]
Granocel-500	10^5	1.7	2.6
Granocel-2000	10^6	2.1	6.5
Granocel-4000	2×10^6	2.8	7.2

According to the procedures used, glucoamylase was immobilized on activated matrixes. The procedures of immobilization were selected to target mainly protein amino groups during enzyme-carrier coupling. For a comparative study immobilization was also done on the well-known commercial carrier—Eupergit®C that has been identified as the carrier suitable for covalent attachment of enzymes for industrial applications.[2] As seen in Table 4, Eupergit C possessed the highest affinity of the enzyme to the carriers' surface that is manifested by the highest expected activity but the measured activity is not the highest. The best glucoamylase activity was obtained for NH_2-Granocel activated by glutaraldehyde. It is probably due to Granocel modification with PEHA that gave a 19-atom spacer arm and ensured minimal steric limitations. Immobilization via carbodiimide and divinyl sulfone on CM-Granocel and Granocel gave the preparation with very low activity. It was probably caused by the involvement of some amino acids, that are crucial for expression of the enzyme activity, in the formation of the covalent linkage. Alternatively, an exclusion (e.g., electrostatic repulsion) of the substrate molecules from the carriers surface possessing only hydroxyl or –OH and –COOH anchor groups may be the reason for low activity.

The storage stability of the enzyme-carrier preparations is an additional factor that determines their usefulness. As it is seen from the results in Table 4, all enzyme preparations are stable, when stored in buffer at 4 °C.

Table 2. Preparation and characterization of DEAE-Granocel

Carrier	Reaction mixture			Characteristics	
	Cellulose [g]	ClDEAE [g]	NaOH [g]	Nitrogen [%]	Ion-exchange capacity [mequiv/g]
DEAE-Granocel-2000/1.1	50	7.5	2.25	1.51	1.1
DEAE-Granocel-2000/0.9	50	5.0	1.75	1.27	0.9
DEAE-Granocel-2000/0.6	50	2.6	0.92	0.80	0.6
DEAE-Granocel-500/1.4	450	45	24.75	2.00	1.4

Table 3. Preparation and characterization of NH_2-Granocel

Carrier	Reaction mixture				Nitrogen [%]	
	Cellulose [g]	PEHA [g]	EClH [g]	4% NaOH [g]	Total	Primary amino groups
NH_2-Granocel-2000/1.1	50	3.4	5.1	62	1.1	0.8
NH_2-Granocel-2000/0.5	50	1.7	2.6	31	0.5	0.4
NH_2-Granocel-4000/1.0	50	3.4	5.1	62	1.0	0.8
NH_2-Granocel-4000/0.6	50	1.7	2.6	31	0.6	0.4
NH_2-Granocel-500/3.0	60	6.0	9.0	110	3.0	1.8

Table 4. Immobilization of glucoamylase on functionalized Granocel carriers (150 or 156 mg of protein per 1 mL of the carrier)

Carrier [–]	Bound protein [mg/mL]	Immobilization yield [%]	Expected activity[a] [U/mL]	Measured activity [U/mL]	Activity after 1 month of storage	
					[U/mL]	[%]
Glutaraldehyde activation						
NH_2-Granocel-500/3.0	11.814	7.8	1236	4.3	3.7	86.0
Carbodiimide activation						
CM-Granocel-2000	15.788	9.8	1936	0.1	0.1	100.0
Divinylsulfone activation						
NH_2-Granocel-500/3.0	15.901	10.3	1846	1.1	1.2	109.1
CM-Granocel-2000	20.494	13.2	1762	0.2	0.1	50.0
DEAE-Granocel-500/1.4	8.667	5.6	2004	1.6	2.2	137.5
Granocel-2000	15.742	10.2	1944	0.3	0.2	66.7
Immobilization via oxirane rings						
Eupergit®C	6.200	4.0	3186	2.7	2.3	85.2

[a] Expected activity calculated as subtraction of activity in the coupling mixture and eluate then recalculated per 1 mL of the carrier (activity balance).

Table 5. Immobilization of glucoamylase on NH_2- and DEAE-Granocel (42.6 or 43.0 mg of protein per 1 mL of the carrier)

Activator	Carrier [–]	Bound protein [mg/mL]	Immobilization yield (protein) [%]	Expected activity [U/mL]	Measured activity [U/mL]	Activity after 1 month of storage	
						[U/mL]	[%]
GLA	DEAE-Granocel-2000/1.1	8.80	20.7	729	3.1	3.1	100.0
	DEAE-Granocel-2000/0.9	8.40	19.7	720	3.0	2.3	76.7
	DEAE-Granocel-2000/0.6	0.20	0.5	167	2.2	1.6	72.7
	NH_2-Granocel-2000/1.04	2.20	5.2	321	12.2	10.1	82.8
	NH_2-Granocel-2000/0.57	0.20	0.5	19	3.6	3.3	91.7
	NH_2-Granocel-4000/1.10[a]	4.80	11.3	300	14.2	12.1	85.2
	NH_2-Granocel-4000/0.50[a]	0.60	1.4	89	5.3	5.6	105.7
DVS	DEAE-Granocel-2000/1.1	1.80	4.2	386	5.3	5.8	109.4
	DEAE-Granocel-2000/0.9	2.02	4.7	364	5.6	4.7	83.9
	DEAE-Granocel-2000/0.6	3.00	6.9	123	2.3	2.0	87.0
	NH_2-Granocel-2000/1.04	5.46	12.6	353	4.8	4.3	89.6
	NH_2-Granocel-2000/0.57	0.1	0.2	178	3.8	3.4	89.5
	NH_2-Granocel-4000/1.10	~0.01	<0.1	138	5.4	4.5	83.3
	NH_2-Granocel-4000/0.50	1.48	3.4	198	3.9	3.3	84.6

[a] The results were partly presented previously.[17]

Increased values of the immobilized activity after storage was also observed earlier.[18,19]

Irrespective of the mechanism of high or low activities of immobilized preparations, DEAE- and NH_2-Granocel seemed to be good carriers for glucoamylase covalent attachment. In the next set of experiments both matrices were used but with various amount of anchor groups and various pore size. Protein concentration in the coupling mixture was lowered more than three times as the amount of bound protein in the first set of experiments was relatively high and might be responsible for carrier's surface overloading and suppression of the enzyme activity. As can be seen in Table 5, the higher the pore size and the greater the number of anchor groups, the higher measured activity could be observed and these activities were significantly higher than in the case of Granocel-500 with lower size exclusion limit (Table 4). It is difficult to identify the main factor responsible for the increased activity: lower number of bound protein molecules or wider pore diameter.

NH_2-Granocel-4000/1.10 activated with glutaraldehyde can be regarded as the carrier of choice for glucoamylase immobilization. In Figure 1 the influence of the amount of protein in coupling mixture on the amount of bound protein and measured activities after glucoamylase immobilization is presented. The experimental data show that there is a strong limitation on the increase of protein concentration in the coupling solution.

For the best immobilized preparation obtained, the comparison of a reaction run in a batch mode with the native enzyme was done. The results, presented in Figure 2, show that low diffusion limitation was observed in the case of the immobilized enzyme, especially between 30 and 50 min of the process, expressed as lower product increments. It means that 19-atom spacer arm with glutaraldehyde as the activator is not so efficient to omit diffusional restrictions, when high molecular weight starch is used as the substrate.

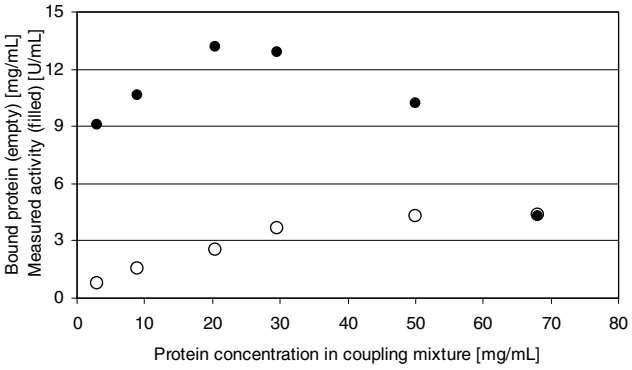

Figure 1. Dependence of bound protein (○) and measured activity (●) of immobilized glucoamylase versus protein concentration in the coupling mixture.

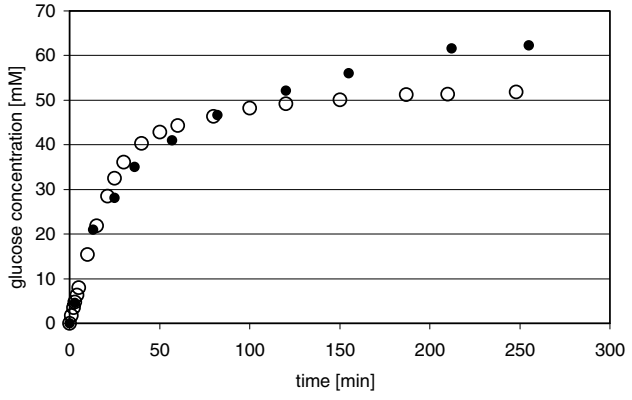

Figure 2. Progress curves of starch hydrolysis by native (○) and immobilized glucoamylase (●). Reaction conditions: stirred (200 rpm) batch reactor; 60 °C; 0.05 M acetate buffer, pH 4.5; substrate concentration 65.5 mM (native) and 69.4 mM (immobilized) of glucose equivalents.

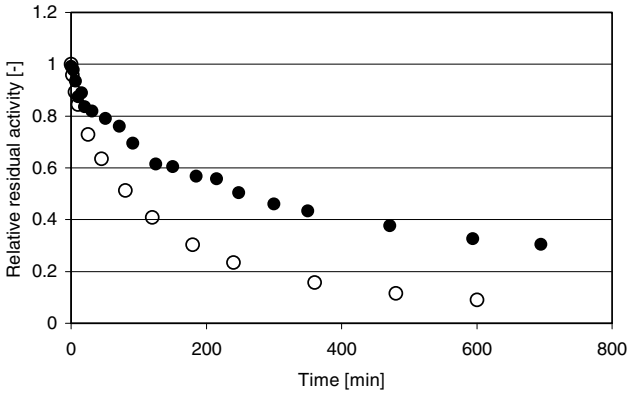

Figure 3. Dynamics of native (○) and immobilized (●) glucoamylase thermal inactivation in stirred (200 rpm) batch reactor at 60 °C (0.05 M acetate buffer, pH 4.5).

On the other hand, the higher substrate conversion was observed after longer time of the process when immobilized enzyme was used. It can be ascribed to the low stability of native glucoamylase at 60 °C.[20,21] As seen in Figure 3, the enzyme stabilization caused by covalent attachment results in retaining of 50% of its initial activity after 250 min incubation at 60 °C, whereas the native enzyme lost about 80% of activity under the same conditions.

The cellulose-based matrix Granocel with controlled morphology proved to be a promising carrier for the immobilization of enzymes. In the case of glucoamylase the carrier with the exclusion limit of approximately 10^6 is sufficient for obtaining highly active and stable preparations when NH_2-Granocel was activated with glutaraldehyde. The worst results were noted for the carriers contained –OH or –OH and –COOH groups, irrespective of the method of activation. These conclusions can be regarded as the rules with respect to glucoamylase immobilization.

1. Experimental

1.1. Materials

1.1.1. Chemicals. Liquid glucoamylase (Amigase, Batch 8187/SPE 0551) was kindly donated by Gist-Brocades (Netherlands). Glutaraldehyde (GLA), divinyl sulfone (DVS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (CDI), protein assay kit (P 5656), and Eupergit®C were purchased from Sigma (USA). Soluble starch, 1-chloro-2,3-epoxypropane (EClH) and chloroacetic acid were from Aldrich (USA), pentaethylenehexamine (PEHA) was purchased from Fluka (Germany), and *N*-chloroethyl-*N*,*N*-diethylamine (ClDEAE) was from Serva (Germany). Standard dextrans were produced by Pharmacia Ltd (Sweden). The other chemicals and glucose assay kit (Glukoza EO, glucose oxidase-peroxidase method) were of analytical grade and purchased from POCh (Poland).

1.1.2. Preparation of cellulose-based matrix Granocel. Cellulose matrix was prepared by saponification of diacetylcellulose using the procedure described previously.[14] Crosslinking of the matrix (9.5 ± 0.5%) was performed with EClH (0.7 mL per 1 g of cellulose) in 1 M sodium hydroxide. The suspension was stirred at 50 °C for 2 h. The resulting product was washed with 2% HCl and water.

Three basic Granocel matrices were prepared: Granocel-500, Granocel-2000, and Granocel-4000, which differed in pore size (Table 1). Exclusion limit of the matrix was evaluated by inverse gel-permeation chromatography[22] and water retention was determined by the centrifugation method.[23]

DEAE ligands were attached to the Granocel by the reaction with ClDEAE in the presence of sodium hydroxide. The amounts of reagents and characteristics of the products are presented in Table 2. The suspension was stirred at 50 °C for 1 h and then washed with water. The total anion exchange capacity was determined by titration with 0.1 M HCl.

For preparation of NH_2-Granocel, the matrix was suspended in the solution containing NaOH and PEHA. The reaction mixture was heated to 50 °C and then the required amount of EClH and a small quantity of sodium borohydride were added. The amounts of reagents and characteristics of products are presented in Table 3. After 3.5 h of the reaction at 50 °C the product was filtrated and washed with 0.5 M NaOH and water.

For the preparation of CM-Granocel, the matrix was suspended in 55 mL 40.0% NaOH (55 mL) and ethanol (125 mL). The mixture was heated and 36 g of chloroacetic acid was added. After 2.5 h of the reaction at 60 °C, the product was washed with 1.0% HCl and water. Ion-exchange capacity was 1.2 mequiv/g.

Primary amino groups were determined by desamination with sodium nitrite. Aminated cellulose was heated at 70 °C for 7 h in 0.1 M $NaNO_2$ and 0.2 M acetic acid. After desamination the cellulose was thoroughly washed with water and the nitrogen content was determined by Kjeldahl method.[24] The nitrogen content of primary amino groups was calculated as the difference of nitrogen content in cellulose before and after desamination.

1.2. Methods

1.2.1. Enzyme activity measurement. Enzyme activity was assayed in the presence of 1.25 wt % gelatinized soluble starch in 0.1 M acetate buffer, pH 4.5, and at 50 °C by initial reaction rate method. In predetermined time intervals the samples were withdrawn and the absorbance (670 nm, spectrophotometer Heliosα, Unicam) of released glucose was measured (enzymatic assay

kit). The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 mM of glucose per minute under the assay conditions.

Immobilized enzyme (0.2–2.0 mL of settled preparation) activity was measured in a thermostated batch reactor (200 rpm).

Protein concentration was determined spectrophotometrically at $\lambda = 280$ nm and/or by Lowry's method (Sigma procedure P 5656).

1.2.2. Immobilization of glucoamylase. Swollen carrier (5 mL) was rinsed five times with distilled water and with buffer appropriate to the kind of anchor groups to be activated as was described previously.[6] NH_2-groups were activated with glutaraldehyde (GLA) (pH 7.0), –COOH groups with carbodiimide (CDI) (pH 5.0), and –OH groups with divinyl sulfone (DVS) (pH 11). In the case of Eupergit C and carriers activated with DVS, pH value of coupling mixture was 8.5. The amount of bound protein (expected activity) was calculated as a result of the subtraction of the amount used for immobilization and washed off (mass/activity balance).

1.2.3. Starch hydrolysis in a batch reactor and thermal inactivation. The immobilized enzyme (2 mL of settled preparation) in 10 mL of acetate buffer was placed into a thermostated reactor (200 rpm) and the temperature was maintained at 60 °C. Then 10 mL of preheated substrate was added (2.5 wt %). After certain time intervals, the sample was taken, immediately diluted 15 times and glucose concentration was determined. A similar procedure was applied to the native enzyme. In order to compare processes with native and immobilized enzyme, the progress curves with the same initial activities (for first 5 min) of both forms of the enzyme were taken.

For the thermal inactivation a thermostated (60 °C) stirred batch reactor was used. For the experiment 50 mL 0.1 M acetate buffer pH 4.5 was allowed to reach a constant temperature then 50 μL of concentrated enzyme solution was added. After 10 s of vigorous mixing the first sample of 1 mL was withdrawn and cooled rapidly to 0 °C in an iced-water bath. At certain time intervals consecutive aliquots were taken, cooled, and stored in ice water until the activity measurement that was conducted after 1 h of storage. In the case of immobilized glucoamylase, 0.2 mL samples of settled preparation was added to preheated (60 °C) probes containing 9.8 mL of the buffer. After 15 s the first probe was withdrawn and hot buffer was removed rapidly by sucking and replaced by the buffer stored in an ice-water bath. At certain time intervals consecutive probes were taken.

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