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Activation of cellulose-based carriers with pentaethylenehexamine

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Abstract. A method for the activation of a cellulose-based matrix resulting in obtaining active primary amino groups via a long spacer arm was developed. The method is based on coupling pentaethylenehexamine (PEHA) to the polymeric matrix through epoxy groups. It was shown that modification of cellulose with PEHA is a straightforward and convenient procedure that has the advantages of high coupling efficiency. The primary amino group density on the carrier surface can be easily adjusted by changing the content of epoxy groups in the matrix or by altering the amount of PEHA in the reaction mixture.

The lectins Concanavalin A and Wheat Germ Agglutinin were employed as ligands for the immobilization onto the PEHA-activated carrier using glutaraldehyde. It was shown that the spacer arm affected ligand coupling kinetics as well as the chromatographic behaviour of the adsorbents obtained. Covalent immobilization of enzyme glucoamylase on PEHA-activated cellulose was done. It was found that covalent binding via glutaraldehyde offers satisfactorily stable and active preparations.

Key words: cellulose activation, spacer arm, lectin-affinity adsorbents, enzyme immobilization.

INTRODUCTION

In order to immobilize proteins covalently under gentle conditions to a surface of any polysaccharide-based matrix, an activation of the matrix is required. Numerous activation chemistries are now available to couple proteins through the amino, thiol, aldehyde, or carboxyl groups [1–3]. The decision about the activa-

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tion method of carriers is based on the functional groups on the surface of the matrix and of the protein. The activated complex then has generated a covalent linkage between the protein and the carrier, resulting in protein immobilization [4].

The coverage with proteins on the solid-phase surface will affect working capacities up to a point where steric hindrance may diminish the performance. Therefore, a spacer arm is interposed between the matrix and the protein to facilitate the efficiency. Spacer arms must be designed to maximize binding without non-specific binding effects [5].

Matrices based on cellulose are frequently used as a carrier for protein immobilization because of their biocompatibility and hydrophylicity. Moreover, cellulose has good potential for varied derivatization due to the presence of three hydroxyl groups in every monomeric unit [6]. There are well-known methods for the activation of cellulose-based matrices with cyanogen bromide [7], bisepoxirane [8], organic sulphonyl chlorides [9], or carbodiimidazole [10].

In this work a method of the activation of a cellulose-based matrix with pentaethylenehexamine (PEHA) was developed. The method gives active primary amino groups via a long spacer arm. PEHA-activated cellulose was evaluated for the preparation of lectin-affinity adsorbents, as well as for the immobilization of enzymes.

EXPERIMENTAL

Materials

Cellulose-based matrix Granocel was prepared by saponification of diacetylcellulose (Roshal, Russia, 55.0% bond acetic acid) by the procedure described previously [11]. Pentaethylenehexamine and glutaraldehyde were purchased from Fluka (Steinheim, Germany), and 1-chloro-2,3-epoxypropane (epichorhydrin) was from Aldrich (Poznan, Poland). Liquid glucoamylase (Amidase, Batch 8187/SPE 0551) was kindly donated by Gist-Brocades (The Netherlands). The lectins Concanavalin A (type V) and Wheat Germ Agglutinin (WGA), the glycoproteins glucose oxidase (GOD) and fetuin, as well as sodium borohydride and Bradford reagent were purchased from Sigma (Munich, Germany). All other chemicals and solvents were of analytical-reagent grade.

Methods

Two-step preparation of PEHA-cellulose

Epoxidation of the cellulose-based matrix

A 25 g amount of sucked cellulose-based matrix Granocel was suspended in 30 mL of 5% NaOH solution, the required amount of epichlorhydrin (EClH) and 30 mg of sodium borohydride were added, and the mixture was stirred at 40 °C for 2 h. Then the activated matrix was thoroughly washed with water.

The concentration of epoxy groups was determined as follows: 1 g of epoxydated cellulose was immersed in 10 mL of 1.3 mol L^{-1} sodium thiosulphate and the obtained sodium hydroxide was titrated with 0.1 mol L^{-1} HCl keeping the pH near 7.0. The 1 mL of HCl used for titration corresponds to 100 μmol of epoxy groups.

Coupling of the PEHA spacer

One gram of sucked epoxidated cellulose matrix was mixed with 2 mL of PEHA, and the mixture was stirred at 40 °C for 2 h. The product was washed with 0.5 mol L^{-1} NaOH and water.

One-step preparation of PEHA-cellulose

Fifty grams of sucked cellulose Granocel was suspended in the solution containing the required amounts of 4% NaOH solution and PEHA. The reaction mixture was heated up to 50 °C and then the required amount of epichlorhydrin and also sodium borohydride $NaBH_4$ were added. The reaction mixture was stirred for 3.5 h at 50 °C. The product was washed with 0.5 mol L^{-1} NaOH and water. The amounts of reagents and characteristics of products are presented in Results and Discussion.

Evaluation of nitrogen content and primary amino groups

The primary amino groups were determined by desamination with sodium nitrite. Therefore PEHA-cellulose was heated at 70 °C for 7 h in a solution containing 0.1 mol L^{-1} of $NaNO_2$ and 0.2 mol L^{-1} of acetic acid (30 mL per g of cellulose). After desamination the cellulose was thoroughly washed with water and the nitrogen content was determined by the Kjeldahl method. The nitrogen content of the primary amino groups was calculated as a difference between the nitrogen content in the cellulose before and after the desamination.

Activation of PEHA-cellulose with glutaraldehyde

Five grams of sucked PEHA-cellulose was mixed with 9.4 mL of 0.05 mol L^{-1} phosphate buffer, pH 8.5, and 2.7 mL of 25% (v/v) glutaraldehyde. The pH was regulated with NaOH up to 9.3. The reaction mixture was stirred at room temperature for 2.5 h. After reaction, the activated cellulose was filtrated and washed with 0.05 mol L^{-1} phosphate buffer, pH 7.5.

Blocking of the unreacted aldehyde groups

$NaBH_4$ was used for the reduction of the residual aldehyde groups. One gram of the support was immersed into 2 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.4), adding 2 mg $NaBH_4$. The suspension was kept in a refrigerator for 1 h. Afterwards the adsorbent was washed with the same buffer.

Immobilization of the lectins

One gram of the sucked activated support was immersed into 3 mL solution of either lectin ConA or lectin WGA (10 mg mL^{-1}) in 0.05 mol L^{-1} phosphate buffer containing 5×10^{-3} mol L^{-1} of Mg^{2+} (pH 7.5). Afterwards the sorbent was washed with the coupling buffer containing 0.5 mol L^{-1} of NaCl to eliminate protein–protein interactions. The washing supernatants were collected and the protein concentration was measured by the Bradford method.

Immobilization of glucoamylase

The swollen carrier (5 mL) was rinsed 5 times with distilled water and the 0.1 mol L^{-1} phosphate buffer (pH 7.0) as was described previously [12]. Activation of the NH_2-groups by glutaraldehyde (pH 7.0) was stopped by sucking out all the liquid and rinsing with distilled water and buffer. After filtering, the activated carrier was suspended in 10 mL of protein in the same buffer. The excess protein was washed off with 0.1 mol L^{-1} phosphate buffer, the same buffer with 0.5 mol L^{-1} NaCl, and 0.1 mol L^{-1} acetate buffer (pH 5.0). All the eluates were collected and analysed for the presence of protein and activity. The enzyme activity was assayed in the presence of 1.25% gelatinized soluble starch (pH 4.5, 50 °C) and the released glucose was measured by a glucose oxidase–peroxidase enzymatic assay kit (Glucoza EO, POCh Gliwice, Poland). The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 mmol L^{-1} glucose per minute. Protein concentration was determined by Lowry's method (Sigma procedure P-5656).

RESULTS AND DISCUSSION

Activation of the cellulose-based matrix with PEHA

In this work macroporous cellulose Granocel [13] was used as a matrix (particle size of 200–315 μm). As it was determined by means of inverse gel-permeation chromatography, the pores of the matrix Granocel-4000 are accessible to molecules of molecular mass up to 2×10^6.

Carriers based on cellulose Granocel can be produced easily in a wide variety of particle diameter, surface area, and pore size and they have a good potential for varied derivatization. In order to indroduce primary amino groups via a spacer arm, the activation of the Granocel matrix with PEHA was studied. The activation procedure comprises two steps: (i) epoxy activation of cellulose and (ii) coupling of PEHA to epoxy groups. The synthesis pathway is shown in Fig. 1, a–c.

The epoxy groups were introduced by means of a well-known method based on the reaction of cellulose with epichlorohydrin in the presence of sodium hydroxide [14, 15]. The results in Table 1 show the dependence of the density of epoxy groups on the ratio EClH/Cel in the reaction mixture.

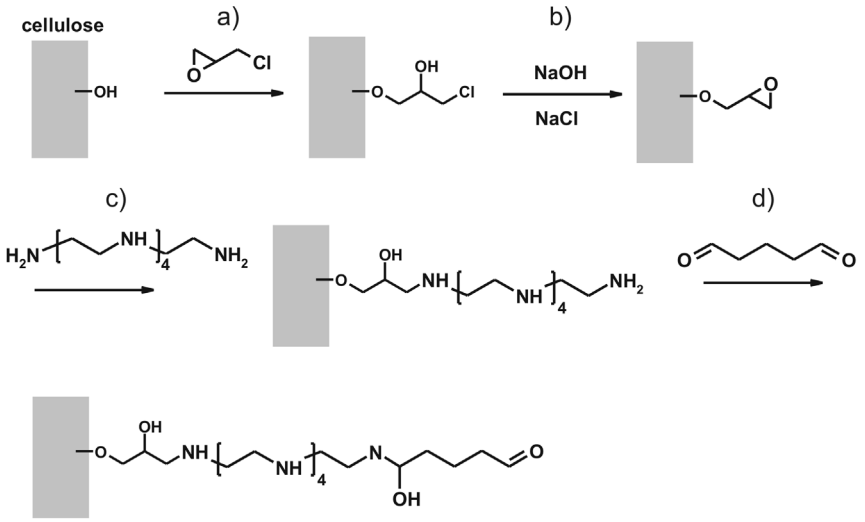


Fig. 1. Synthesis pathway of the modified cellulose support: (a) surface reaction of the cellulose matrix with epichlorhydrin (EClH), (b) formation of reactive epoxide with NaOH, (c) attachment of the spacer pentaethylenehexamine (PEHA), and (d) activation with glutaraldehyde.

Table 1. Effect of the ratio EClH/Cel in the reaction mixture on the epoxy groups density in cellulose

EClH/Cel, mol g^{-1}	Density of epoxy groups, μmol g^{-1}
0.01	144
0.02	375
0.03	405
0.04	490

Afterwards the reaction of epoxydated cellulose with PEHA was performed (Fig. 1, c) according to the procedures described in Methods. The dependence of the content of primary amino groups on the epoxy groups density was investigated keeping the ratio PEHA/Cel constant at 2 mL g^{-1}. Results presented in Table 2 show that the content of primary amino groups may be regulated by adjusting the density of epoxy groups.

Table 2. Effect of the epoxy groups density on the content of primary amino groups

Density of epoxy groups, μmol g^{-1}	Total nitrogen, %	Nitrogen of primary amino groups, %
144	0.43	0.09
375	1.19	0.46
405	2.59	1.81
490	4.63	2.11

PEHA/Cel = 2 mL g^{-1}.

The density of the primary amino group required for protein immobilization depends on the protein. Usually a low density of active groups is preferable in order to avoid multipoint attachment of the protein. This activation method allows getting a carrier with a desired content of the primary amino group.

While epoxy groups are not very stable, the PEHA coupling should preferably be performed directly after the epoxydation step. In order to simplify the procedures, it is possible to join the epoxydation and the PEHA coupling steps together (see Methods). In this case the reaction mixture contains both EClH and PEHA.

An immobilized PEHA spacer adds a 19-atom arm containing hydroxyl and amino groups onto which a further immobilization of the proteins may be performed. The presence of spacer molecules ensures that steric limitations during the protein immobilization are kept to a minimum, which is especially important considering the size of the protein molecules. A second benefit arising from the use of a spacer is that the protein is kept away from the particle surface, thus protein–surface interactions could be minimized.

The primary and secondary amino groups of the PEHA spacer arm are of weak basicity. This means that they are not dissociated at pH above 6.5–7. Therefore, the possibility of non-specific ionic interaction between amino groups and proteins is negligible. A long hydrocarbon arm chain could be involved into hydrophobic interactions. However, it was found [5] that sometimes some additional forces provided by a spacer arm are needed for the stronger ligand binding.

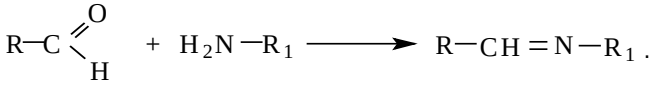
The method developed is not restricted just to cellulose-based matrices, but may be used for other matrices containing free hydroxyl groups, such as agarose and dextran.

PEHA-activated cellulose was evaluated for the preparation of lectin-affinity adsorbents, as well as for the immobilization of enzymes.

Immobilization of lectins on PEHA-cellulose

PEHA-activated cellulose was used as a carrier for the preparation of lectin-affinity adsorbents. With the aim to immobilize any lectin, the primary amino groups of the spacer arm may be activated with glutaraldehyde. The reaction of primary amino groups of the PEHA spacer arm with glutaraldehyde results in aldehyde end-groups onto which a further immobilization of specific ligands may be performed (Fig. 1, d).

The covalent attachment of the lectin onto the aldehyde-activated supports takes place at the primary amino groups of the lectins. This reaction results in obtaining the so-called Schiff's base:



The subsequent reduction with sodium borohydride led to the stabilization of the bonds between the protein and the polysaccharide, and to the reduction of the residual aldehyde groups [4].

Two supports, PEHA(0.37)-Cel and PEHA(1.1)-Cel with 0.37% and 1.1% nitrogen, respectively, were prepared for lectin immobilization. The content of primary amino groups was 0.10 and 0.16 mmol g^{-1}, respectively. Two lectins of different molecular weight, such as Concanavalin A (ConA, MM = 108 kDa) and Wheat Germ Agglutinin (WGA, MM = 36 kDa) were employed as ligands for the immobilization onto the PEHA-activated carrier.

The same matrix Granocel activated with sodium periodate (OXY-Cel) was used for comparison. OXY-Cel contains aldehyde groups introduced directly into the cellulose chain without any spacer.

As Fig. 2 demonstrates, the immobilization behaviour of ConA on the two supports is different. The faster kinetics and the higher amount of the coupled ligand were achieved on the PEHA(1.1)-Cel support with the spacer arm. The coupling of WGA is identical on both supports. The immobilization kinetics mainly depends on the accessibility of the active groups of the support to ligand molecules. Thus, the spacer arm evidently increases the accessibility of voluminous molecules of ConA to the active groups of the support.

ConA and WGA adsorbents were evaluated for the sorption of glucose oxidase and fetuin, respectively (Table 3). The effect of the spacer arm on the chromatographic behaviour of the adsorbents in the column was followed as well. Although the lectin density on PEHA-Cel and OXY-Cel is very similar, the glycoprotein sorption capacity is significantly higher for PEHA-activated supports. The best recovery (93%) was reached on PEHA(0.37)–Cel(ConA) with a low ligand density (7.5 mg mL^{-1}).

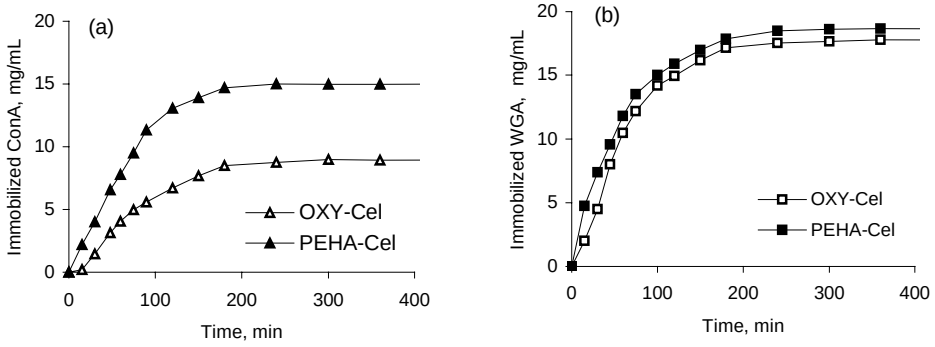


Fig. 2. Immobilization kinetics of ConA (a) and WGA (b) on OXY-Cel and PEHA(1.1)-Cel supports.

Table 3. Chromatographic performance of the lectin affinity adsorbents

Adsorbent	Ligand density, mg mL^{-1}	Glycoprotein sorption capacity		Recovery, %
		mg mL^{-1}	%, of max*	
PEHA(1.1)-Cel(WGA)	18.5	2.6	10.5	66
OXY-Cel(WGA)	17.5	1.3	8.7	70
PEHA(0.37)-Cel(ConA)	7.5	7.4	62.9	93
OXY-Cel(ConA)	9.0	3.1	22.0	70

* Theoretical maximum sorption capacity was calculated from the ligand density considering one binding site per ligand molecule.

Immobilization of glucoamylase on PEHA-Cel

The characteristics of PEHA-Cel used for the enzyme immobilization are presented in Table 4.

According to the procedures described in Methods, glucoamylase was immobilized on activated matrices using glutaraldehyde as an activator. The procedures of immobilization were selected to apply protein amino groups during the enzyme–carrier coupling.

The usefulness of PEHA carriers for enzyme immobilization is characterized by four main parameters: the amount of bound protein, enzyme activity, immobilization yield, and storage stability. The results in Table 5 show that the immobilized glucoamylase exhibits high specific activity. The higher the amount of the anchor groups was, the better the activity observed. PEHA-Cel activated with glutaraldehyde seemed to be a good carrier for glucoamylase covalent attachment. Immobilized glucoamylase has satisfactory stability in the buffer and at 4 °C.

Table 4. Preparation and characterization of PEHA-Cel

Carrier	Reaction mixture				Nitrogen, %	
	Cellulose, g	PEHA, mL	EClH, mL	4% NaOH, mL	Total	Primary amino groups
PEHA(1.1)-Cel	50	3.4	5.1	62	1.10	0.3
PEHA(0.5)-Cel	50	1.7	2.6	31	0.50	0.2

Table 5. Immobilization of glucoamylase on cellulose carriers

Carrier	Bound protein, mg mL^{-1}	Activity, U mL^{-1}	Immobilization yield (protein), %	Immobilization yield (activity), %	Activity after 1 month storage, U mL^{-1}
PEHA(1.1)-Cel	4.8	14.2	11.3	0.402	12.1
PEHA(0.5)-Cel	0.6	5.3	1.4	0.150	5.6

CONCLUSIONS

The activation of the polysaccharide-based matrix with pentaethylenehexamine is a straightforward and convenient method, which results in active primary amino groups coupled to the matrix via a long spacer arm. PEHA-activated carriers may be used for the preparation of biospecific sorbents as well as for enzyme immobilization.

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Tselluloosipõhiste kandjate aktiveerimine pentaetüleenheksamiiniga

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On uuritud tselluloosipõhiste kandjate aktiveerimist pika vahelüli kaudu primaarsete aminorühmadega, milleks pentaetüleenheksamiin (PEHA) on epoksürühmade abil seotud polümeerse maatriksi külge. Ligandid on immobiliseeritud PEHA-aktiveeritud kandjale glutaaraldehüüdiga ja saavutatud kromatograafiaks vajalik statsionaarse faasi püsivus.