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Journal of Chromatography B, 770 (2002) 177–181

JOURNAL OF CHROMATOGRAPHY B

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Enzymes immobilized on magnetic carriers: efficient and selective system for protein modification

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Abstract

In order to obtain an economical, efficient and selective system for glycoprotein modification we prepared reactors with immobilized neuraminidase or (and) galactose oxidase. High storage and operational stability of the enzyme reactors was obtained by their immobilization through the carbohydrate parts of the enzyme molecules to hydrazide-modified supports. Magnetic and non-magnetic forms of bead cellulose and poly(HEMA-co-EDMA) microspheres were used for immobilization. These reactors can be used almost universally for the activation of ligands and for labelling of substances having a carbohydrate moiety. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Magnetic sorbents; Neuraminidase; Galactose oxidase; Enzymes; Glycoproteins

1. Introduction

The application of immobilized enzymes is, in comparison with their soluble form, indisputably advantageous. The complete elimination of the enzyme from the reaction mixture, the absence of mixing and dilution problems, and the possibility of re-using the reactors is of distinct advantage. The storage properties and the pH stability of enzymes are often improved by immobilization. Glycoprotein enzymes immobilized orientedly through their carbohydrate moieties give conjugates with very good steric accessibility of their active sites. Increased thermal stability of their biological activity and lower sensitivity to inappropriate microenvironmental conditions have been reported [1].

The use of hydrazide-derivatized supports in the preparation of affinity carriers has often been reported [2–4]. Site-specific modification of biologically active glycoproteins (antibodies, enzymes) is achieved by oxidation of their carbohydrate moieties with sodium periodate [5]. The considerable sensitivity of glycoproteins to periodate often results in a significant decrease of their activity [6,7]. For this reason, the use of a sensitive method for the oxidation of biologically active molecule is advisable. After splitting the terminal neuraminic acid on the carbohydrate moieties by neuraminidase, the terminal galactose can be oxidised by galactose oxidase to generate aldehyde groups. These groups can react with the hydrazide groups of the support to form stable hydrazone bonds [8].

The necessity for centrifugation, undesirable dilu-

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00037-5

tion of the sample and loss of the carrier during washing often complicate the use of non-magnetic enzyme reactors. Supports with a magnetic core were chosen to avoid these problems [9,10]. This type of support can be used favorably for high-speed separation, for the elimination of mechanical damage by centrifugation, and for minimal detection of undesirable materials.

The main aim of this work was to prepare enzyme reactors for the specific and sensitive modification of glycoproteins for immobilization or specific labelling. Neuraminidase (sialidase) from *Clostridium perfringens* is an exoglycosidase, which removes α-linked *N*-acetylneuramine acid from the glycoside chain. The optimum pH depends on the type of buffer [11]. Galactose oxidase from *Dactylium dendroides* attacks D-galactose and some of its derivatives as well as polymers. Specific oxidation occurs at the C_6 position of the galactose molecule. The immobilized enzyme is stable in the presence of copper (II) ions [12]. The enzyme has to be re-activated before use by three pulses with D-galactose or potassium ferricyanide [13].

The oriented immobilization of the enzymes to hydrazide-derivatized supports was chosen to obtain highly active and stable reactors for various laboratory applications. This paper is mainly aimed at the preparation of reactors with immobilized neuraminidase. The oriented immobilization of galactose oxidase has been described previously [14].

2. Experimental

2.1. Chemicals

Galactose oxidase (EC 1.1.3.9.) from *Dactylium dendroides* (450 IU), neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (10 IU, 0.1–3 I U/mg solid), catalase (E.C.1.11.1.6) from bovine liver (2800 IU/mg), peroxidase (E.C.1.11.1.7) from *horseradish* (5000 IU), D-galactose, *o*-phenylenediamine (OPD), pig IgG (10 mg/ml) and seamless cellulose dialysis tubing were purchased from Sigma–Aldrich (St. Louis, MO, USA). D-Fucose (99%) was from Acros Organics (Geel, Belgium). The hydrazide derivatives of bead cellulose Perloza MT 200 (15 μmol adipic acid dihydrazide/ml sorbent) and of magnetic bead cellulose (20 μmol adipic acid dihydrazide/ml sorbent) were prepared at the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague (IMC Prague) [9]. Hydrazide derivatives of poly(HEMA-co-EDMA) microspheres (2 μmol hydrazide/g sorbent) and of magnetic poly(HEMA-co-EDMA) microspheres (4.8 μmol hydrazide/g sorbent) were also prepared at IMC Prague [10]. Macroporous bead cellulose Perloza MT 200 with particle diameter 80–100 μm was supplied by Lovochemie (Lovosice, Czech Republic). Magnetic macroporous bead cellulose (particle diameter 125–250 μm) was a laboratory product of IMC Prague. Natrium periodate was purchased from Reanal (Budapest, Hungary). The remaining chemicals were supplied by Lachema (Brno, Czech Republic) and were of analytical reagent grade.

2.2. Oriented immobilization of galactose oxidase to hydrazide-modified support

The oriented immobilization of galactose oxidase to a hydrazide-modified support, the determination of the galactose oxidase activity, and re-activation of the support with the immobilized enzyme have been described previously [14].

2.3. Oriented immobilization of neuraminidase to hydrazide-modified support

Neuraminidase (3 IU) was dissolved in 1 ml of 0.1 *M* potassium acetate buffer (pH 5.5). After 10 min incubation at 37 °C, sodium periodate (100 μl of 0.1 *M* $NaIO_4$) was added and the reaction mixture stirred in the dark at 4 °C for 30 min. The oxidation was terminated by pouring the mixture in 20 m*M* ethylene glycol (stirring for 10 min). Low-molecular-mass components were removed by dialysis into 0.1 *M* potassium acetate buffer with 0.25 *M* KCl for 24 h. Oxidized neuraminidase was stirred with 250 μl of activated support for 24 h at 4 °C. The support was then washed with 0.1 *M* potassium acetate buffer containing 0.05 *M* KCl (pH 4). The washing procedure was continued to zero enzyme activity in the supplement. After coupling the enzyme to the carrier, the preparation was treated with 0.2 *M* acetaldehyde in 0.1 *M* acetate buffer (pH 5.5) for 24

h to block the residual reactive hydrazide groups. Carrier with immobilized enzyme was equilibrated with 0.1 *M* potassium acetate buffer containing 0.05 *M* KCl (pH 4) and sodium merthiolate. The approximate amount of enzyme immobilized on the carrier was determined by measuring the respective enzymatic activities in supernatants before and after the reaction.

2.4. Determination of neuraminidase activity

The activity of the enzyme [15] was measured fluorometrically by determination of the released 4-methylumbelliferyl (4-MU) from the substrate 2-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid (4-MU-NANA). All reagents were tempered to 37 °C. The reaction was performed under the following experimental conditions: 0.1 *M* potassium acetate buffer pH 4, 2 m*M* 4-MU-NANA, stop buffer (0.2 *M* glycin–NaOH) pH 10.6. The activity of the enzyme was measured using the following procedure: 20 μl of 0.1 *M* potassium acetate buffer pH 4 and 40 μl of 2 m*M* 4-MU-NANA were added to 50 μl of the sedimented carrier. The reaction mixture was gently stirred for 30 min at 37 °C and 1.2 ml of stop buffer was added to the separated supernatant. The fluorescence was measured at 365/448 nm. One unit will hydrolyze 1.0 μmol of substrate 2-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid per minute at pH 5.5 at 37 °C [15].

3. Results and discussion

The active sites of enzyme molecules are situated in the protein part. In order to protect the activity of the immobilized molecules we used a carbohydrate chain for the immobilization strategy. Glycoprotein galactose oxidase or (and) neuraminidase were oxidized with sodium periodate under mild conditions [5]. The conditions during immobilization and storage of the galactose oxidase reactor were reported in Ref. [14]. We looked for the reaction conditions minimising the decrease in enzyme activity during oxidation. The activity of neuraminidase after oxidation and immobilization was measured fluorometrically by determination of the released 4-methylumbelliferyl (4-MU) [15]. We observed that pH in the range 4.0–4.8 and concentrations of natrium periodate between 0.01 and 0.001 *M* had no influence on the immobilization efficiency. The enzyme activity and binding efficiency remained unchanged. We observed a positive effect of potassium ions on neuraminidase activity during the immobilization procedure (Table 1).

In order to obtain enzyme reactors with a highly active and stable enzyme for daily use in the laboratory we tested four types of support. There were the magnetic form of macroporous bead cellulose (15 μmol hydrazide/ml support), the magnetic form of nonporous poly(HEMA-co-EDMA) microspheres with 2 μmol hydrazide/g support, and their non-magnetic forms. All types of carriers were used in the batch-wise arrangement. The resulting activities of the oxidation reactors are given in Table 2.

The stability of the enzyme reactors in regular use as well as during storage is a very important quality parameter. Freshly immobilized neuraminidase was stored in 0.1 *M* potassium acetate buffer with 0.25 *M* KCl, pH 4.0, at 4 °C. Galactose oxidase was stored in 0.1 *M* phosphate buffer with 2 m*M* $CuSO_4$, pH 6.0, at 4 °C. The storage stability of neuraminidase was observed for 30 days. Every seventh day the activity of an aliquot of the immobilized enzyme was determined. The results are summarized in Figs. 1 and 2. Detailed information on the storage and operational stability of galactose oxidase and reactivation of the reactor has been reported previously [14].

Neuraminidase immobilized on bead cellulose without a magnetic core showed good stability, with even a temporary increase in activity after immobilization (Fig. 2). The rapid decrease of neuraminidase activity immobilized on magnetic bead cellulose during regular use and during long-term storage was significant (Fig. 1). Here we discuss the negative

Table 1
Positive effect of potassium ions in binding buffer on neuraminidase activity

Type of binding buffer	Activity of immobilized enzyme (IU/g dry sorbent)
Na-acetate buffer, pH 4	0.29
K-acetate buffer, pH 4	1.65

Table 2
Activities of enzymes immobilized on hydrazide derivative supports

Type of support	Activity (IU/g dry support)	
	Galactose oxidase	Neuraminidase
Macroporous bead cellulose	611	35
Magnetic form of macroporous bead cellulose	585	30
Nonporous poly(HEMA-co-EDMA) microspheres	138	–
Magnetic form of nonporous poly(HEMA-co-EDMA) microspheres	112	–
Macroporous bead cellulose: co-immobilization	234	40

influence of the iron core on the biological activity of the enzyme.

The final activities of both immobilized enzymes over a 21-day period are presented in Fig. 3. The storage stabilities of enzymes immobilized on the nonmagnetic form of the supports were superior (98 and 102% of their original activity). The gentle decline in the enzyme activity of galactose oxidase and the loss of neuraminidase activity if immobilized on bead cellulose with a magnetic core may be caused by the toxic influence of iron ions. It appears that the sensitivity of these enzymes to the magnetic core varies.

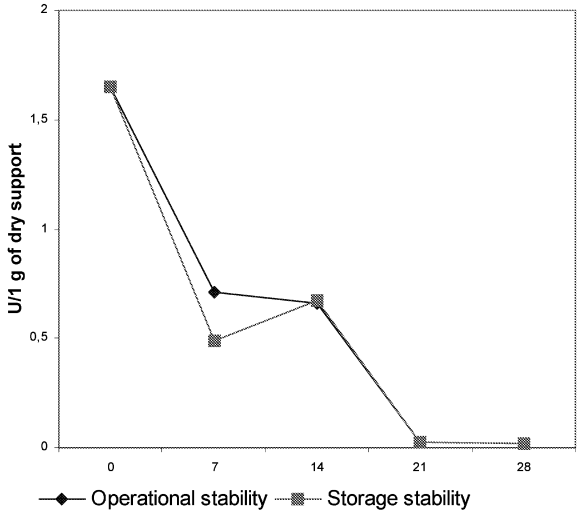

Fig. 1. Activity of neuraminidase immobilized on the magnetic form of bead cellulose.

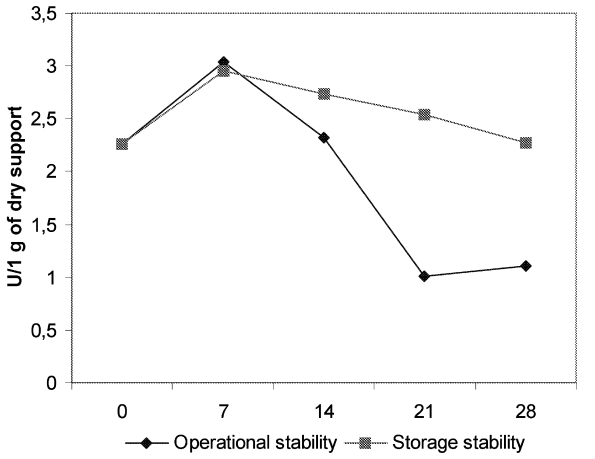

Fig. 2. Activity of neuraminidase immobilized on the nonmagnetic form of bead cellulose.

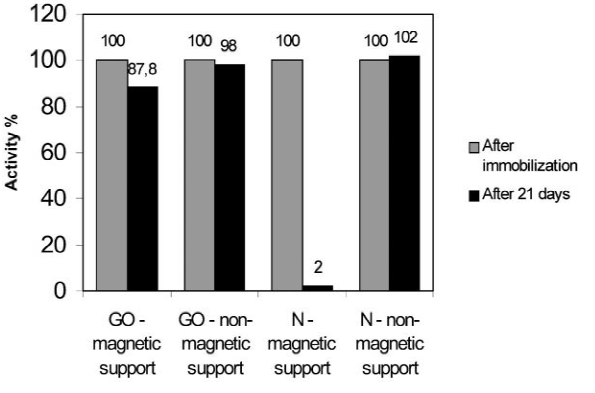

Fig. 3. Activity of the immobilized enzymes after 3 weeks of storage.

4. Conclusion

We have prepared highly active and stable reactors by the oriented immobilization of galactose oxidase and neuraminidase through the carbohydrate moeity. Immobilized galactose oxidase can be used for the enzymatic oxidation of glycoproteins for immobilization on hydrazide-derivatized supports or for coupling with agents that contain free amine or hydrazide residues. Immobilized neuraminidase can be used for the specific removal of *N*-acetylneuramine acid from the glycoside chain. Using co-immobilized galactose oxidase with neuraminidase, we can achieve a one-step modification of glycoproteins (for instance, antibodies) for oriented immobilization or for labelling. Enzymatic reactors with galactose oxidase and neuraminidase immobilized on macroporous bead cellulose exhibited good stability. The magnetic form of macroporous bead cellulose provides us with the best support for high-speed and gentle separation.

Acknowledgements

The authors gratefully acknowledge support from MŠM 253100002 and from the Grant Agency of the Czech Republic (203/00/P029, 230/00/A399 and 203/99/00). The authors also wish to thanks Dr. M.J. Beneš, Institute of Macromolecular Chemistry, Academy of Sciences, Prague, for his assistance.

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