SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
7 (2001)

STABILITY OF ENZYMES IMMOBILIZED IN ORIENTED WAY ON MAGNETIC AND NON-MAGNETIC BEAD CELLULOSE

Marcela SLOVÁKOVÁ[a1], Zuzana BÍLKOVÁ[a] and Jaroslav CHURÁČEK[b]
[a]Department of Biological and Biochemical Science,
[b]Department of Analytical Chemistry,
The University of Pardubice, CZ–532 10 Pardubice

Received September 28, 2001

The enzymes galactose oxidase and neuraminidase have been immobilized in an oriented way to hydrazide derivative of magnetic and non-magnetic macroporous bead cellulose. Binding was accomplished after the sugar components have been oxidatively activated by natrium periodate as the oxidating agent. The operational and storage stability of neuraminidase reactor was studied during four weeks and operational stability of galactose oxidase reactor during three weeks. The operational stability of galactose oxidase immobilized to magnetic macroporous bead cellulose was excellent in the environment of Cu^{2+} ions. Stability of immobilized neuraminidase to non-magnetic support had a similar course. The stability of neuraminidase immobilized to magnetic macroporous bead cellulose was decreasing rapidly.

---

[1] To whom correspondence should be addressed.

Introduction

Immobilization of enzymes often is a necessary prerequisite for their use in protein microanalysis and modification. The main advantage of immobilized enzymes is their reusability, which minimises costs and problems arising from preparation and handling of enzyme solution. Glycosylated enzymes take advantage of carbohydrate moieties as anchor sites for the immobilization providing conjugates with good steric accessibility of active sites [1]. The first step of oriented immobilization is chemical oxidation under mild conditions with sodium periodate, to generate aldehydes from vicinal diols present on the carbohydrate part. The product is then made to react with hydrazide functionalities present on the bead cellulose, forming stable hydrazone linkages [2].

The use of hydrazide derivative supports for the preparation of affinity carriers where the ligand is glycoprotein is often reported [3,4,5]. Disadvantages of non-magnetic supports, such as necessity of centrifugation, undesirable dilution of sample and loss of the carrier during washing often complicate the application of enzymatic reactor, especially in microanalysis and specific modifications. Magnetic hydrazide derivatives were chosen to avoid these problems [6,7].

The use of enzymes as analytical reagents for the specific determination of their respective substrates, inhibitors or activators is both well accepted and well documented [8,9]. Some enzymatic reactors under routine operational conditions have limited stability. In this respect, the enhanced stability of immobilized enzymes is an important factor. The most realistic way to test their storage and operational stability is to subject them to the storage and operational conditions that they will encounter in the analytical laboratory and to measure periodically their residual activity [10].

Optimization of immobilization conditions of galactose oxidase was published in an earlier report [11]. In this work, we describe operational stability of galactose oxidase and neuraminidase reactors. These reactors may be used for the enzymatic oxidation of glycoproteins designed for the oriented immobilization or to the labelling.

Experimental

Chemicals

Galactose oxidase (EC 1.1.3.9.) from *Dactylium dendroides* (450 I.U., 500-1500 I.U./mg, partially purified), neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (10 I.U., type V, 0.1 – 3 I.U./mg solid), catalase (E.C.1.11.1.6) from bovine liver (2800 I.U./mg), peroxidase (E.C.1.11.1.7) from horseradish (5 000 U),D-galactose, *o*-phenylendiamine (OPD), seamless cellulose dialysis tubing were

purchased from Sigma-Aldrich (St. Louis, MO, USA). D-Fucose (99%) was from Acros Organics (Geel, Belgium). Lucifer Yellow CH was purchased from Molecular Probes (Eugene, OR, USA). Hydrazide derivative of bead cellulose Perloza MT 200 (15 μmol adipic acid dihydrazide/ml of sorbent) and of magnetic bead cellulose (20 μmol adipic acid dihydrazide/ml of sorbent) were prepared at the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague (IMC Prague) [6]. Natrium periodate was purchased from Reanal (Budapest, Hungary). The other chemicals used were supplied by Lachema (Brno, Czech Republic) and were of analytical reagent grade.

Oriented Immobilization of Galactose Oxidase to Hydrazide Modified Support

(According to [12], slightly modified)
Galactose oxidase was covalently immobilized after natrium periodate oxidation by reaction with hydrazide groups of the support. The procedure of preparation of hydrazide derivative of bead cellulose has been described by Beneš *et al.* [13]. Galactose oxidase (350 I.U.) was dissolved in 2.5 ml 0.1 M acetate buffer (pH 5.5) containing addition of 2 mM $CuSO_4$ and 1 mM D-fucose. Then 100 I.U. of catalase were added. After 10 min of incubation at 37 °C and 15 min at 4 °C, 250 μl 0.01 M $NaIO_4$ was added and the reaction mixture stirred at 4 °C for 30 min. The reaction was stopped by the addition of 30 μl ethylene glycol and the mixture was further stirred for 10 min. Low molecular-weight components were removed by dialysis for 24 h or by gel filtration on a Sephadex G-25 column. The oxidized galactose oxidase was stirred with 1.5 ml of hydrazide derivative of bead cellulose for 24 h at 4 °C. To remove non-bound enzyme the support conjugated with galactose oxidase was washed with 0.1 M acetate buffer containing 0.5 M NaCl (pH 4). The washing procedures continued with 0.1 M phosphate buffer containing 2 mM $CuSO_4$ (pH 6) until reaching zero enzyme activity in the supernatant. After coupling the enzyme to the hydrazide support, 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. The support with immobilized enzyme was equilibrated with 0.1 M phosphate buffer containing 2 mM $CuSO_4$ (pH 6) and sodium merthiolate. Approximate amount of enzyme immobilized on the support was determined by measuring the respective enzymatic activities in supernatants before and after the reaction.

Determination of Galactose Oxidase Activity

The activity of immobilized galactose oxidase was measured spectrophotometrically by determination of the peroxidase-chromogen (OPD) assay based on

oxidation of D-galactose as a substrate [14]. The reaction was performed under the following experimental conditions: 0.1 M phosphate buffer, pH 6, with 2 mM $CuSO_4$, 1.5 M D-galactose, 0.8 mg peroxidase, 3.75 mg OPD. The following procedure was used: 50 μl 3M D-galactose and 100 μl the indicator mixture (1.6 mg of peroxidase, 7.5 mg of OPD in 0.1 M phosphate buffer, pH 6) were added to 100 μl the sedimented support. The reaction mixture was gently stirred for 30 min at 37 °C, one drop of HCl (36%) was added to the separated supernatant to stop the reaction, and the absorbance at 450 nm was measured. One unit will produce a ΔA_{425} of 1.0 per min at pH 6.0 at 25 °C, in a peroxidase and *o*-toluidine system. Reaction volume = 3.4 ml. Light path = 1 cm [14].

Stability of Immobilized Galactose Oxidase

Galactose oxidase was oxidized and immobilized according to the procedures described above. The enzymatic reactor was stored at 4 °C for 6 months. The enzymatic activity was determined 4, 11 and 18 days after immobilization.

Oriented Immobilization of Neuraminidase to Hydrazide Modified Support

The immobilization procedure of neuraminidase (sialidase) from *Clostridium perfringens* is identical with that of galactose oxidase except for the additives. Neuraminidase (3 I.U.) was dissolved in 1 ml 0.1 M potassium acetate buffer (pH 5.5). After 10 min of incubation at 37 °C sodium periodate (100 μl 0.1 M $NaIO_4$) was added and the reaction mixture was stirred in the dark at 4 °C for 30 min. The oxidation reaction was stopped by pouring the mixture in 20 mM ethylene glycol and stirring at 4 °C for 10 min. To remove low molecular-weight components, the oxidized enzyme was dialyzed in 0.1 M potassium acetate buffer with 0.25 M KCl addition for 24 h at 4 °C. The hydrazide modified bead cellulose (250 μl) was then rinsed and resuspended in a mixture containing oxidized neuraminidase. The binding procedure was carried out at 4 °C for 24 h. The support was then washed with 0.1 M potassium acetate buffer containing 0.05 M KCl (pH 4) to remove the free enzyme. The washing procedure continued until reaching zero enzyme activity in the supernatant. After coupling the enzyme to the support, 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. The support 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 support was determined by measuring the respective enzymatic activities in the supernatants before and after the reaction.

Determination of Neuraminidase Activity

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

Stability of Immobilized Neuraminidase

The operational and storage stability was determined every seventh day during 30-day storage at 4 °C.

Results and Discussion

Advantages of Magnetic Support

Positive properties of magnetic support were fully confirmed. The advantages, i.e., quick separation of the support from reaction mixture without centrifugation (Fig. 1), gentle manipulation with samples, elimination of lengthy centrifugation, minimal presence of undesirable impurities, economical benefits and no loss of the support during washing, make the material useful in daily laboratory practice. We have used hydrazide derivative 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). The supports were prepared at the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague (IMC Prague).

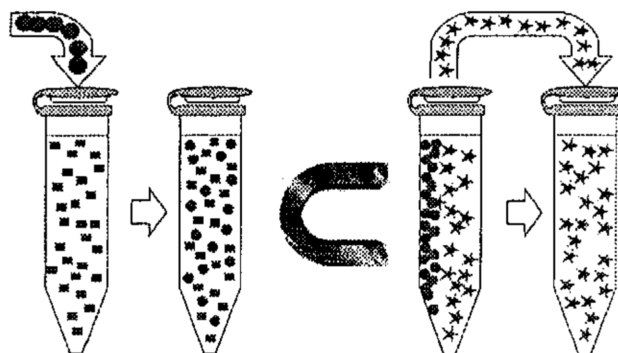


Fig. 1 The principle of magnetic separations of proteins

Stability of Galactose Oxidase in Enzymatic Reactor

Galactose oxidase from *Dactylium dendroides* attacks D-galactose and some of its derivatives and polymers. The oxidation leading to the aldehyde groups occurs at the C_6 position of galactose. The pH optimum in phosphate buffer is about 7.0. The immobilized enzyme is stable in the presence of copper(II) ions [16]. A detailed information about storage and reactivation of galactose oxidase was published in [11].

Galactose oxidase was immobilized to both magnetic and non-magnetic hydrazide derivative of bead cellulose. The activity of the non-magnetic and magnetic reactor immediately after enzyme immobilization is comparable and high (Table I).

Table I Enzymatic activity of reactors determined immediately after immobilization of the enzymes

Type of support	Activity of galactose oxidase, I.U g^{-1} of dry support	Activity of neuraminidase, I.U g^{-1} of dry support
Macroporous bead cellulose	611	35
Magnetic form of macro-porous bead cellulose	585	30

The operational stability of enzymatic reactors in analytical use represents a very important quality parameter. Freshly immobilized galactose oxidase was stored in 0.1 M phosphate buffer, pH 6.0 with 2 mM $CuSO_4$ at 4 °C. The enzy-

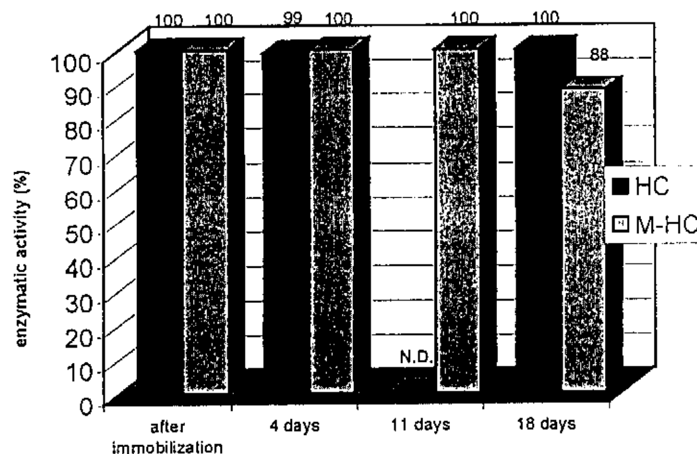


Fig. 2 Operational stability of galactose oxidase immobilized in oriented way to hydrazide derivative of bead cellulose (HC) and to magnetic form of this support (M-HC). Enzymatic reactors were kept in phosphate buffer containing 2 mM $CuSO_4$ (pH 6) at 4 °C.

matic activity of magnetic and non-magnetic reactors was determined 4, 11 and 18 days after immobilization, and the results are presented in Fig. 2. No significant loss of the activity was observed.

The enzymatic reactors were stored for 6 months, and then the activity was determined. The activity decreased to 26% and 22% compared to the activity after immobilization. The reactivation, which was performed by pulses of 3 M D-galactose [11], substantially increased the performance of the immobilized enzyme. The results are presented in Fig. 3.

Stability of Neuraminidase in Enzymatic Reactor

Neuraminidase (sialidase) from *Clostridium perfringens* is an exoglycosidase; it removes α-linked N-acetylneuramine acid from glycoside chain of glycoconjugates such as glycoproteins and glycolipids. Neuraminidase was isolated from many bacterias and tissues [17].

We have prepared an enzymatic reactor with neuraminidase from *Clostridium perfringens*. Neuraminidase is a glycoprotein, therefore the oriented immobilization through carbohydrate part of it molecule represents an advantageous modification. Neuraminidase was immobilized to both magnetic and non-magnetic macroporous beaded cellulose. The decrease of immobilized enzyme activity by 7% compared with soluble enzyme is ascribed to non-optimum conditions during immobilization

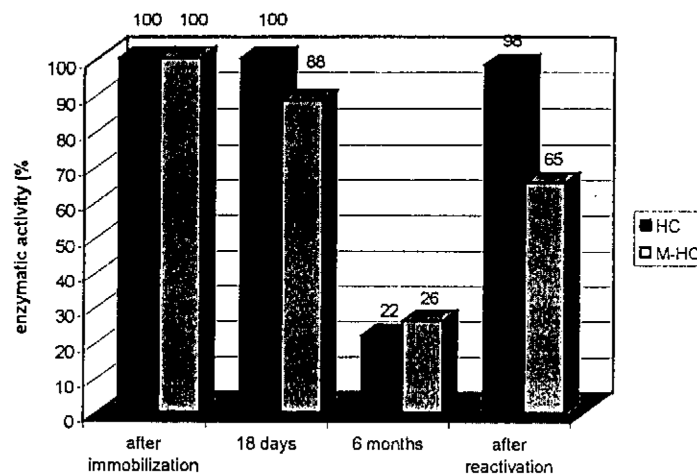


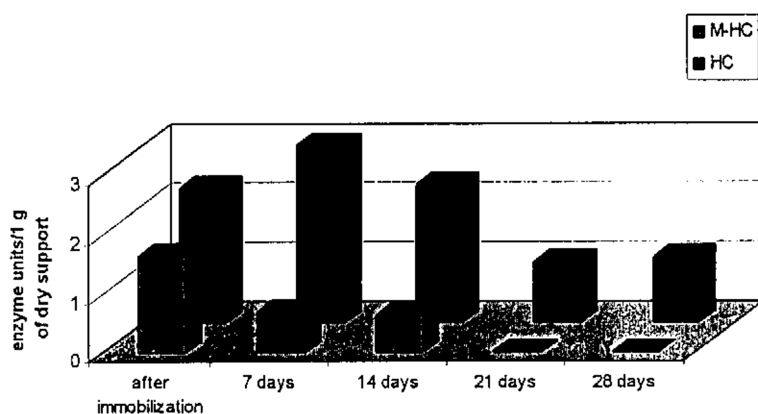
Fig. 3 Operational stability of galactose oxidase reactors. The reactors were kept 6 months in phosphate buffer containing 2 mM $CuSO_4$ (pH 6) at 4 °C. Three pulses of 3 M D-galactose were used for reactivation

[4,18,19,20]. Therefore, we looked for conditions that are more suitable. We observed that pH in the range 4.0 – 4.8 and concentrations of natrium periodate between 0.01 – 0.001 M did not influence neuraminidase immobilization and enzymatic activity of the reactor. We observed positive effect of replacing natrium acetate by potassium acetate buffer according to [9]. The subsequently prepared neuraminidase reactors were found to be very active (Table I).

The specific activities of enzymatic reactors were determined once a week during four weeks. The enzymatic reactors were stored in 0.1 M potassium acetate buffer pH 4.0 with addition of 0.25 M KCl at 4 °C. The courses of operational and storage stability are summarized in Figs 4A and 4B.

According to the results presented in Figs 4A and 4B, the storage and analytical use did not influence markedly the activity of non-magnetic reactor. The operational and storage stability of non-magnetic reactor was excellent, however, the stability of magnetic reactor showed a rapid decrease. This could be explained by possible inhibition of magnetic iron oxides present in the support to the enzyme. Possible loosening of iron oxides from cellulose during storage is not out of the question either.

A. Operational stability



B. Storage stability

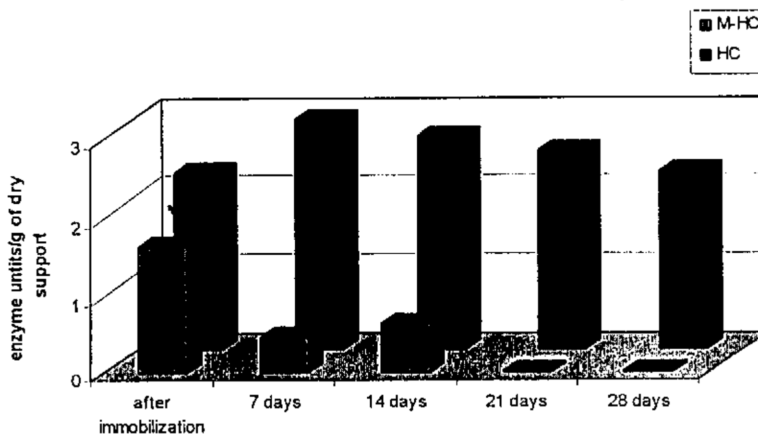


Fig. 4 Operational (A) and storage stability (B) of neuraminidase magnetic (M-HC) and non-magnetic (HC) reactors. The reactors were kept four weeks in 0,1 M potassium acetate buffer containing 0.25 M KCl at 4 °C

Conclusion

Both magnetic and non-magnetic galactose oxidase reactors showed extremely high operational stabilities. Differences in long-term stabilities of neuraminidase reactors were significant. The stability of non-magnetic neuraminidase reactor was

excellent, but that of magnetic neuraminidase reactor decreased rapidly in short time. By oriented immobilization of galactose oxidase and neuraminidase to magnetic and non-magnetic bead cellulose, we prepared highly active enzymatic reactors with good sterical accessibility of their active sites. Such immobilized enzymes are suitable for batch operations after which the immobilized enzymes can simply be separated and repeatedly used.

Acknowledgements

The project is sponsored by grants from Czech Ministry of Education, Youth and Sports 253100002 and Grant Agency of the Czech Republic 203/00/P029.

References

[1] Turková J., Kučerová Z., Vaňková H., Beneš M.J.: Int. J. Biochromatogr. **3**, 45 (1997).
[2] Morehead H.W., Talmadge K.W., O´Shannessy D.J., Siebert C.J.: J. Chromatogr. **587**, 171 (1991).
[3] O´Shannessy D. J.: J. Chromatogr. **510**, 13 (1990).
[4] Fleminger G., Solomon B., Wolf T., Hadas E.: Appl. Biochem. Biotech. **23**, 231 (1990).
[5] Ruhn P.F., Garver S., Hage D.S.: J. Chromatogr. A **669**, 9 (1994).
[6] Horák D., Karpíšek M., Turková J., Beneš M.: Biotechnol. Prog. **15**, 208 (1999).
[7] Horák D., Rittich B., Šafář J., Španová A. Lenfeld J., Beneš M.: Biotechnol. Prog. **17**, 447 (2001).
[8] Johnson J.M., Halsall H.B., Heineman W.R.: Anal. Chem. **54**, 1394 (1982).
[9] Dahodwala S.K., Weibel M.K., Humphrey A.E.: Biotechnol. Bioeng., **18**, 1679 (1976).
[10] Ngo T.T.: Int. J. Biochem. **11**, 459 (1980).
[11] Bílková Z., Slováková M., Horák D., Lenfeld J., Churáček J.: J. Chromatogr. (In press).
[12] Murayama Akira, Kohkichi Shimada and Tadashi Yamamoto: Immunochemistry b, 523 (1978).
[13] Beneš M. J., Adámková K., Turková J.: J. Bioact. Compat. Polym. **6**, 406 (1991).
[14] Avigad G., Amaral D., Asensio C., Horecker B.L: J. Biol. Chem. **237**, 2736 (1962).
[15] Paton B.C., Schmidt B., Kusterman-Kuhn B., Polus A., Harzer K.: Biochem. J. **285**, 481 (1992).

[16] Whittaker M.M., Ballou D.P., Whittaker J.W.: Bioch. **37**, 8426 (1998).
[17] Cassidy J.T., Jourdian G.W., Roseman S.: J. Biol. Chem. **240**, 3501 (1965).
[18] Wolfe C.A.C., Hage D.S.: Anal. Biochem. **219**, 26 (1994).
[19] Wolfe C.A.C., Hage D.S.: Anal. Biochem. **231**, 123 (1995).
[20] Hage D.S., Wolfe C.A.C., Oates M.R.: Bioconjug. Chem. **8**, 914 (1997).