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Oriented immobilization of galactose oxidase to bead and magnetic bead cellulose and poly(HEMA-co-EDMA) and magnetic poly(HEMA-co-EDMA) microspheres

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Abstract

In order to obtain an active and stable oxidation reactor for daily use in biochemical laboratory we decided to immobilize galactose oxidase orientedly through a carbohydrate chain to the magnetic carriers. We used hydrazide derivatives of non-magnetic and magnetic bead cellulose and of magnetic and non-magnetic poly(HEMA-co-EDMA) microspheres. Activation of the enzyme molecules was done by sodium periodate in the presence of supplements (fucose, $CuSO_4$, catalase). Orientedly immobilized galactose oxidase presents high storage stability and lower susceptibility to inappropriate microenvironmental conditions. Reactor reactivated by three pulses of D-galactose retained practically 100% of its native activity after 6 months. The positive properties of both magnetic carriers were entirely confirmed.

Keywords: Oriented immobilization; Magnetic sorbents; Enzymes; Galactose oxidase

1. Introduction

The high efficiency of many processes in nature and their low energy demand depend on activities of highly effective and specific catalysts — enzymes. The application of enzymes in their native form in biochemical and biomedical fields is not always suitable and optimal. Binding of enzymes on a solid support is an advantageous modification of their application. Choosing a suitable method of enzyme immobilization also enables an increase in the stability without negative influence on their catalytic activity [1]. Binding of molecules in areas of active sites or in their vicinity can lead to a decrease or complete loss of activity [2]. As a result of the immobilization of the glycoproteins through a carbohydrate chain, the access to the active sites and the stabilization of protein structures is ensured [3].

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Hydrazide derivatives of porous and nonporous carriers are suitable supports for immobilizing glycoproteins [4–7]. Binding can be accomplished after sugar components have been activated using specific oxidizing agents, e.g. sodium periodate [8], or oxidizing enzyme, e.g. galactose oxidase [9], or mixtures of neuraminidase and galactose oxidase [10]. Both approaches lead to the formation of aldehyde groups which can react with hydrazides [4]. The resulting condensation product is a stable hydrazon [11]. Chemical oxidation with sodium periodate frequently used in practice depends on many factors, such as concentration of $NaIO_4$, pH, reaction time and temperature. Oxidation of carbohydrate chain with $NaIO_4$ was followed under various conditions [5,8,12–17]. Reaction conditions under which the biological activity of various glycoproteins remained unaffected were studied. Oxidation results in aldehyde groups at terminal C6, but also at C3 and C4 of the saccharide molecule [14]. Oxidation of the diol groups is possible even to the carboxyl groups. A further disadvantage consists in the oxidation of some sensitive amino acid residues, such as cysteine, cystine, methionine, tryptophan, tyrosine and histidine [18]. The biological activity of glycoproteins may be negatively affected if they contain one of the above-mentioned amino acids localized in the area of biological activity [19]. Also the average amount of the reactive aldehyde groups occurring during the oxidation of the glycoprotein (antibody) ranges between 3 and 4, but can reach at least 9 under conditions of slight oxidation [14]. Deformation of molecule conformation is possible during the immobilization due to the multiple connections, which can affect the biological activity of these substances.

Enzymatic oxidation is a second possibility to generate aldehyde groups on a glycoside chain. Specific oxidation occurs under moderate conditions and does not affect the active substances. It is suitable to split *N*-acetylneuramine acid with neuraminidase in glycoproteins of mammalian origin. This makes the saccharide molecules accessible to oxidation [20]. Solomon et al. [10] immobilized both enzymes on Eupergit C-ADH sorbent and they used such a reactor for specific oxidation of antibodies.

Based on the above-mentioned facts and on our experience with periodate oxidation of biological active proteins we decided to prepare orientedly immobilized galactose oxidase with a high activity and stability. In order to obtain an oxidation reactor with properties suitable for daily laboratory practice, hydrazide derivatives of various types of sorbents (porous, nonporous and magnetic) were used. This newly designed oxidizing reactor will be used almost universally in all cases, where ligands determined for immobilization will be substances having a carbohydrate moiety — glycoproteins, glycolipids, polysaccharides, etc.

2. Experimental

2.1. Chemicals

Galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* (450 I.U., 500–1500 I.U./mg, partially purified), catalase (E.C.1.11.1.6) from bovine liver (2800 I.U./mg), peroxidase (E.C.1.11.1.7) from horseradish (5000 U), D-galactose, *o*-phenylendiamine (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). Bio-Spin-6 desalting columns (0.3 ml gel volume) were obtained from Bio-Rad Labs (Richmond, CA, USA) and 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) [21]. Hydrazide derivatives of poly(HEMA-co-EDMA) microspheres (2 μmol hydrazide/g of sorbent) and of magnetic poly(HEMA-co-EDMA) microspheres (4.8 μmol hydrazide/g of sorbent) were also prepared at IMC Prague [15]. Macroporous bead cellulose Perloza MT 200 with particle diameter 80–100 μm was supplied by Lovochemie a.s. (Lovosice, Czech Republic). Magnetic macroporous bead cellulose (particle diameter 125–250 μm) was a laboratory product of IMC Prague [22]. 2-Hydroxyethyl methacrylate (HEMA; Röhm, Germany) and ethylene dimethacrylate (EDMA; Ugilor, France) were purified by distillation under reduced

pressure in a nitrogen atmosphere. 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 (according to Ref. [8], slightly modified)

Galactose oxidase (350 I.U.) was dissolved in 2.5 ml of 0.1 *M* acetate buffer (pH 5.5) containing 2 m*M* $CuSO_4$ and 1 m*M* 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 of 0.01 *M* $NaIO_4$ was added and the reaction mixture stirred at 4°C for 30 min. The reaction was stopped with the addition of 30 μl of ethylene glycol and the mixture was further stirred for 10 min. Low molecular-mass components were removed by dialysis for 24 h or by gel filtration on a Sephadex G-25 column. Oxidized galactose oxidase was stirred with 1.5 ml of activated support for 24 h at 4°C. The support conjugated with galactose oxidase was then washed with 0.1 *M* acetate buffer containing 0.5 *M* NaCl (pH 4) to remove free enzyme. The washing procedure was continued with 0.1 *M* phosphate buffer containing 2 m*M* $CuSO_4$ (pH 6) until zero enzyme activity in the supplement was reached. After coupling 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* phosphate buffer containing 2 m*M* $CuSO_4$ (pH 6) and sodium merthiolate. The approximate amount of enzyme immobilized on the carrier was determined by measuring the respective enzymatic activities in the reaction mixture supernatants before and after the reaction.

2.3. Direct immobilization of galactose oxidase to macroporous bead cellulose (according to Ref. [23], slightly modified)

Macroporous bead cellulose (1.5 ml of sedimented carrier) was resuspended in 10 ml 0.1 *M* $NaIO_4$ and stirred for 1 h at room temperature. The activated carrier was washed in redistilled water and separated by centrifugation. Galactose oxidase (350 I.U.) was dissolved in 1 ml of 0.1 *M* $NaHCO_3$, pH 9, and added to the activated carrier. The mixture was gently stirred for 2 h at room temperature. Support conjugated with galactose oxidase was washed five times with 0.1 *M* $NaHCO_3$, pH 9 to remove the unbound enzyme. After coupling the resulting sorbents were treated with 0.1 *M* $NaHCO_3$ and 0.08 *M* sodium borohydride for 20 min to block the residual reactive groups. Then the sorbent beads were separated and repeatedly washed with 0.1 *M* $NaHCO_3$ and a mixture of 0.1 *M* $NaHCO_3$ in 1 *M* NaCl until the enzyme activity in the eluent was zero. Carrier with the immobilized enzyme was equilibrated with 0.1 *M* phosphate buffer containing 2 m*M* $CuSO_4$ (pH 6) and sodium merthiolate. The approximate amount of the immobilized enzyme was determined by measuring the respective enzymatic activities in the reaction mixture supernatants before and after the reaction.

2.4. Determination of galactose oxidase activity

The activity of free galactose oxidase was measured spectrophotometrically by determination of the peroxidase-chromogen (OPD) assay based on oxidation of D-galactose as a substrate [24]. The reaction was performed under the following experimental conditions: 0.1 *M* phosphate buffer, pH 6, with 2 m*M* $CuSO_4$, 1.5 *M* D-galactose, 0.8 mg peroxidase, 3.75 mg OPD. Activity of the immobilized galactose oxidase was measured using the following procedure: 50 μl of 3 *M* D-galactose and 100 μl of 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 of the sedimented carrier. The reaction mixture was gently stirred for 15 or 30 min at 37°C and one drop of HCl (36%) was added to the separated supernatant to stop the reaction, and the absorbance at 450 nm was measured.

2.5. Reactivation of support with the immobilized enzyme

Immobilized enzyme (1 ml of sedimented carrier) was reactivated introducing pulses of 1 ml of 1 m*M* potassium ferricyanide for 1 min repeatedly four times. The activity of the immobilized enzyme was

measured immediately after each two pulses of potassium ferricyanide. The auto-activation was also performed by repeated addition of D-galactose (3 *M*, 15 min) and the activity of immobilized enzyme was measured immediately after each pulse of D-galactose.

2.6. Oxidation of pig IgG with sodium periodate

Pig IgG (5 mg) was dissolved in 1 ml of 0.02 *M* sodium acetate buffer (pH 5) with 0.15 *M* NaCl; 100 μl of 0.1 *M* $NaIO_4$ was added to begin the oxidation. After 10 or 40 min incubation in the dark at 4 or 25°C, the reaction was stopped by addition of 50 μl of ethylene glycol and 15 min incubation. The low molecular-mass components were removed by 24-h dialysis or by gel filtration on a Sephadex G-25 column.

2.7. Oxidation of pig IgG with enzyme reactor

Pig IgG (3 mg in 1 ml of 0.1 *M* sodium acetate buffer, pH 5) was incubated with soluble neuraminidase (0.5 I.U.) at 37°C for 24 h under gentle shaking. IgG was then dialyzed against 0.1 *M* phosphate buffer (pH 6) with 2 m*M* $CuSO_4$; 1 ml of bead cellulose with galactose oxidase (55 I.U.) equilibrated in the same buffer was added. After incubation for 36 h at 37°C, oxidation of IgG was stopped by separation of the carrier from the reaction mixture.

2.8. Determination of aldehyde moieties after oxidation of glycoprotein

Aldehyde groups on oxidized pig IgG were determined using dye containing hydrazide functionalities [25]. A mixture of 10 μl of the Lucifer Yellow solution (5 mg/ml of 0.02 *M* acetate buffer with 0.15 m NaCl, pH 5) and 100 μl of the oxidized enzyme solution was incubated for 2 h at room temperature. After adding 10 μl of 1 *M* Tris buffer (pH 8.0) a 75-μl aliquot was desalted by centrifugation with a Bio-Spin 6 column equilibrated in 0.1 *M* Tris buffer with 0.15 *M* NaCl (pH 8.0). Fluorescence at 435 nm and absorbance at 280 nm determined the amount of the dye and the protein in the adequately diluted eluate. The extent of labelling was calculated as the molar ratio of dye to protein after subtracting the absorbance at 280 nm due to the dye presence.

2.9. Oxidation of D-galactose with immobilized galactose oxidase for NMR analysis

A 1-ml aliquot of D_2O-washed sedimented carrier with immobilized galactose oxidase (55 I.U.) was added to the reaction mixture prepared from D_2O and containing 2 *M* D-galactose in 0.1 *M* phosphate buffer (pH 6) with 2 m*M* $CuSO_4$. After incubation for 36 h at 37°C the reaction was stopped by separation of the support from the mixture. The ^1H and ^{13}C NMR spectra were measured in deuterium oxide on a Bruker AMX 360 spectrometer at 360.13 and 90.56 MHz, respectively. The ^1H–^{13}C HMQC spectra (with and without proton decoupling) were measured using a microprogram provided by the producer of the NMR spectrometer. The ^1H and ^{13}C chemical shifts were referred to internal DSS and converted to δ scale.

3. Results and discussion

Galactose oxidase, an extracellular enzyme secreted by the fungus *Dactylium dendroides*, is a monomeric substance with a relative molecular mass of 68 000 and its carbohydrate content amounting to about 1.7% [26]. The enzyme catalyses a two-electron transfer reaction during stereospecific oxidation of a broad range of primary alcohol substrates to corresponding aldehydes due to its unique mononuclear copper site essential for catalysis.

The main attraction of our immobilization strategy was the orientation of enzyme molecules with their active sites oriented into the reaction solution. As the active site of the enzyme is situated in the protein part only, we used the carbohydrate chain for the immobilization. Glycoprotein galactose oxidase was oxidized under mild conditions with sodium periodate [8]. Although a partial decrease in the enzyme activity during oxidation with sodium periodate is usual [27], the activity of the oxidized galactose oxidase dropped to 15–20%. The copper site localized on the surface of the protein, which participated in the catalysis, is extremely rich in aromatic side chains (Tyr 272, His 496, His 581,Tyr 495) [28].

This enzyme showed high sensitivity to the oxidation agent.

During oxidation with sodium periodate we looked for the reaction conditions minimizing the decrease in enzyme activity. The decrease in periodate concentration in the reaction mixture from 0.01 *M* usually used to 0.001 *M* had a positive effect on the activity. The total amount of the immobilized enzyme on the carrier remained unchanged. Hamilton et al. [29] already described a negative influence of superoxide formed during the oxidation on galactose oxidase activity. Inactivation of the enzyme can be partly evaded by adding catalase to the reaction mixture according to Petkov et al. [9]. After oxidation, the enzyme activity has dropped to 40%. Further we tried to protect the active site of the enzyme by specific substrate D-galactose, although we knew the specific inhibition effect of the substrate [30]. The protective effect of D-galactose was not proved even in the presence of catalase. We have discovered the influence of various types of supplements to the reaction e.g. Cu^{2+} ions, D-fucose, $K_3Fe(CN)_6$ (Table 1).

D-Fucose is not metabolized but galactose oxidase has an affinity to it [30]. The qualitatively different influence of ferricyanide depending on its concentration was described [29]. The presence of $CuSO_4$ in the enzyme solution during the whole time of oxidation and immobilization is necessary to prevent formation of the inactive copper-free apoenzyme [31]. The highest activity was reached after oxidation. Galactose oxidase was oxidized under the following conditions: 0.1 *M* acetate buffer, pH 5.5 containing 2 m*M* $CuSO_4$ 1 m*M* D-fucose, catalase (100 U) and 0.001 *M* $NaIO_4$ for 30 min at 4°C. After removing the low molecular-mass components, the oxidized enzyme was incubated with corresponding volume of the support.

In order to obtain an oxidation reactor with a high active and stable enzyme for daily use in a biochemical laboratory, we tested four types of hydrazide derivative supports. Mostly, macroporous materials with a highly specific surface are used for immobilization of glycoproteins [4]. Of course they have to have sufficiently high limits of elimination for the macromolecular afinant and high molecular-mass substrates [32]. To immobilize galactose oxidase, we used hydrazide derivatives of both non-magnetic (15 μmol hydrazide/ml of support) and magnetic (20 μmol hydrazide/ml of support) macroporous bead cellulose. If reaction time is one of the limiting factors, it would be more advantageous to use a nonporous material. It has to be taken into consideration that the binding capacity of this support is lower in comparison to the porous one. We used hydrazide-derivatized nonporous poly(HEMA-co-EDMA) microspheres (particle size 1.2 μm) with 2 μmol hydrazide/g of support and also hydrazide-derivatized magnetic nonporous poly(HEMA-co-EDMA) microspheres with 4.8 μmol hydrazide/g of support for galactose oxidase immobilization. Higher non-specific sorption of glycoprotein molecules to the carrier has been proved. The washing procedure was then performed with 0.1 *M* acetate buffer containing 0.5 *M* NaCl and 5 *M* NaCl, and finally with 0.1 *M* phosphate buffer containing 2 m*M* $CuSO_4$. This procedure was performed until zero enzyme activity in the supernatant was reached. Perloza MT 200 macroporous bead cellulose was used for direct immobilization of the enzyme.

All types of carriers were used in the batch-wise arrangement. Positive properties of both magnetic carriers, i.e. macroporous bead cellulose and nonporous poly(HEMA-co-EDMA) microspheres were entirely confirmed. The advantages, i.e. extremely high speed of the carrier separation from the reaction mixture without centrifugation, no dilution of the sample, no loss of the carrier during washing and

Table 1
Effect of supplements added to the reaction mixture during periodate oxidation on the resulting activity of the immobilized enzyme

Supplement	Activity of the immobilized enzyme (U/ml of sed. carrier)	Activity of the immobilized enzyme (U/g of dry carrier)
0.1 m*M* $K_3Fe(CN)_6$	2.24	24
2 m*M* $CuSO_4$	4.1	44
1 *M* D-fucose	17.7	190
No supplement	4.4	48

Table 2
Enzyme activities of immobilized galactose oxidase

Support	Activity of immobilized enzyme (U/ml of sed. carrier)	Activity of immobilized enzyme (U/g of dry carrier)
Poly(HEMA-co-EDMA microspheres	15.3	138
Magnetic poly(HEMA-co-EDMA microspheres	21.2	112
Macroporous bead cellulose	56.8	611
Magnetic macroporous bead cellulose	85.4	585

delicacy to the biologically active protein and immobilized enzyme make the materials useful in daily laboratory practice. The resulted activities of the oxidation reactors are given in Table 2.

Supports with immobilized enzymes showed the catalase activity too. Average activity was 350–400 I.U./g of dry carrier. Another task of our research was to compare two of the above mentioned ways of obtaining reactive aldehyde groups on glycosidic chains of the glycoprotein. Pig IgG used as a model glycoprotein was oxidized with sodium periodate under various conditions (reaction time, temperature), as well as with galactose oxidase oxidation reactor, or with the same soluble enzyme. Before the enzymatic oxidation IgG was modified with neuraminidase to release terminal *N*-acetyl neuraminic acid. After oxidation, glycoproteins were incubated with fluorescent dye Lucifer Yellow CH. The fluorescent marker containing hydrazide functionalities reacted with aldehyde groups generated by oxidation. The extent of the labelling was determined according to Morehead et al. [25]. The efficiencies of both oxidation methods are summarised in Table 3. Using the enzyme reactor amount of the generated aldehyde groups (1.2 mol) in 1 mol IgG is sufficient and optimal for coupling to a hydrazide support.

In order to understand more about the reaction products arising during the chemical oxidation, we used a model reaction of D-galactose with sodium periodate. The products of the reaction were followed by NMR analysis. NMR analysis of oxidation products of galactose is a relatively difficult task because galactose exists in water solution as a mixture of α- and β-anomers derived from appropriate furanose and pyranose [33], four components being in dynamic equilibrium, and the composition of reaction products is even more complicated. Oxidation of galactose by $NaIO_4$ was carried out in deuterium oxide solution and ethylene glycol was added after 20 min to stop oxidation.

The role of ethylene glycol was studied first. In ^{1}H and ^{13}C NMR spectra of ethylene glycol (16 mg in 0.6 ml of D_2O), there are singlets resonating at 3.72 ppm (^{1}H) and 66.7 ppm (^{13}C), respectively, while

Table 3
The amount of reactive aldehyde groups in the glycoprotein glycosidic chains after the oxidation

Oxidation	Time, temperature	IgG (nmol)	LY-CH (nmol)	Aldehyde groups (nmol)
Chemical	10 min, 4°C	1.48	4.53	3.06
Chemical	40 min, 4°C	1.66	5.98	3.59
Chemical	10 min, 25°C	1.54	6.14	3.98
Enzyme reactor A (55 U)	36 h, 37°C	0.731	0.88	1.20
Soluble enzyme (35 U)	36 h, 37°C	0.725	0.45	0.62

after addition of 105 mg of $NaIO_4$ the reaction product gives singlets at 4.87 ppm (^1H) and 85.9 ppm (^{13}C). No oxidation was observed because the reaction product corresponds very likely to ethylene glycol ester of periodic acid. Analogous structures are reported in literature as an intermediate in oxidation of glycol to aldehydes and ketones and its structure is formulated as $[cycloOCH_2CH_2O]I(O)(OH)_3$ [34]. The signal of ester resonating at 4.81 ppm was observed in ^1H NMR spectrum even in the case when the reaction was carried out using a 20-fold molar surplus of $NaIO_4$ which means that no further oxidation of ethylene glycol takes place under the reaction conditions used.

Galacturonic acid is a potential oxidation product of galactose. Similarly as galactose, galacturonic acid also provides α- and β-anomers derived from furanose and pyranose. Except for the region of anomeric proton resonances, there is a strong overlap of other signals (we measured NMR spectra of a sample prepared by mixing of commercially available galactose and galacturonic acid). The situation is ever more complicated when there are also signals of appropriate aldehydes present in reaction mixture since a spectrum of 12 components (and possibly further degradation products) in a mixture is measured.

The galactose/$NaIO_4$ ratio was changed systematically. Use of ca. 10 equivalents of $NaIO_4$ to one equivalent of galactose dissolved in water at 20°C led to the degradation of galactose to formic acid (singlet in ^1H NMR spectrum, $\delta(^1H)=8.40$). For the galactose/$NaIO_4$ ratio equal to 2–3, galacturonic acid was the main oxidation product. Optimal results from the viewpoint of the presence of aldehydes were reached when the galactose/$NaIO_4$ ratio was 1. The ^{13}C NMR spectra show signals in the region of 160–175 ppm (Fig. 1). Resonances corresponding to –CHO groups were split into doublets in ^1H–^{13}C HMQC spectra (measured without proton decoupling) having coupling constants $^1J(^{13}C, ^1H)$ ca. 230 Hz typical of aldehydes [35]. The number of –CHO group resonances is higher than four (i.e. the expected number of two α- and two β-anomers of aldehydes derived from appropriate furanose and pyranose) which can be explained by the presence of ring degradation products. The galacturonic acid content is low under these conditions.

These results are in an agreement with the conclu-

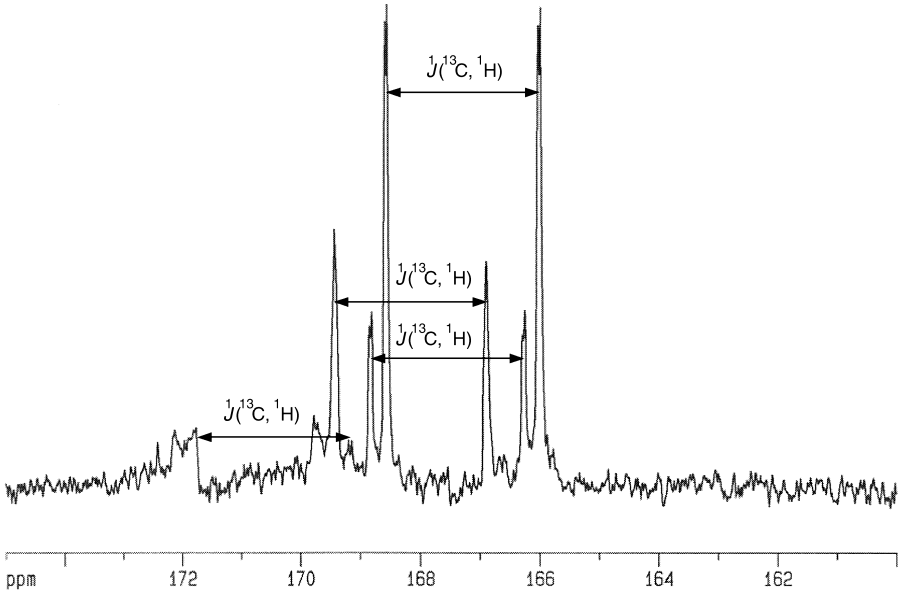

Fig. 1. ^{13}C NMR spectrum of reaction products of galactose with $NaIO_4$ in D_2O (coupling constants $^1J(^{13}C, ^1H)$ noted).

sions of Wolfe and Hage [14]. If exactly defined conditions of chemical oxidation of a certain glycoprotein are not ensured, it can result in formation of other reaction products. They cannot react with active hydrazide moieties causing a low binding efficiency. This is another reason why we have decided to use fine and more specific enzyme for the glycoprotein oxidation.

For oxidation of D-galactose as a model solution, we have used our own sorbent composed of orientedly immobilized galactose oxidase on hydrazide bead cellulose (55 I.U./1 ml of sedimented carrier). All types of carriers were used in the batch-wise arrangement. The activity and stability of the reactor with orientedly immobilized enzyme were compared with enzyme directly immobilized under the inappropriate reaction conditions. Both enzyme reactors were prepared in acetate buffer (0.1 *M*, pH 5.5) with all supplements, except Cu^{2+} ions, and were stored for 5 days in phosphate buffer (0.1 *M*, pH 6), except Cu^{2+} ions too. The enzyme activity of both reactors rapidly decreased during 5 days (Fig. 4).

Dahodwalla et al. [36] described that $K_3Fe(CN)_6$ can prevent an auto-inactivation of immobilized galactose oxidase. To a limited degree, immobilized enzyme could also be auto-activated by the repeated application of D-galactose. Conclusive positive influence of repeated pulses of the $K_3Fe(CN)_6$ or D-galactose to the reactivation of the enzyme is demonstrated in Figs. 2 and 3.

After this finding, we tried to restore the activity of the immobilized enzyme by three pulses of D-galactose and the enzyme reactors were then kept in 0.1 *M* phosphate buffer pH 6.0 containing 2 m*M* $CuSO_4$. Relative activity of about 40% was reached by the reactivation. Contrary to orientedly immobilized enzyme, the enzyme activity of the directly immobilized enzyme was reduced during the following days (Fig. 4). Lower susceptibility of the orientedly immobilized enzyme due to inappropriate microenvironmental conditions was confirmed.

Freshly immobilized galactose oxidase oxidized in the presence of all supplements was found to be very stable during 6 months when stored at 4°C in 0.1 *M* phosphate buffer, pH 6, containing 2 m*M* $CuSO_4$ (Fig. 5). Prior to the use of the immobilized enzyme reactor for kinetic characterization or analytical application, three pulses of D-galactose reactivated

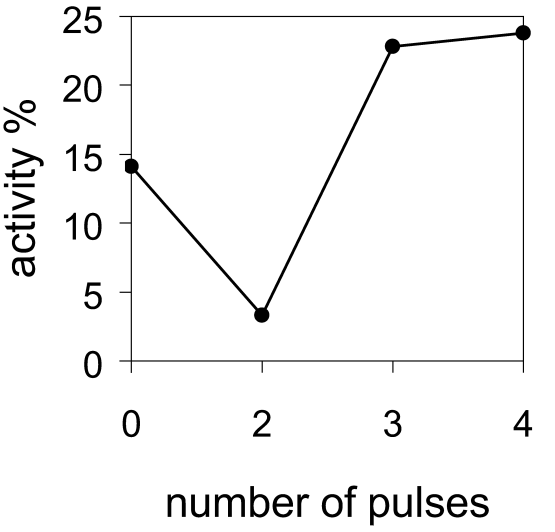

Fig. 2. The effect of potassium ferricyanide pulses on the activity of immobilized galactose oxidase. Reactivation was carried out by four pulses of 100 μl of 1 m*M* potassium ferricyanide. Activity of immobilized enzyme was measured after each pulse.

the enzyme. The negative effect of hydrogen peroxide during repeated pulses of D-galactose [30] was not confirmed, probably due to coimmobilized catalase.

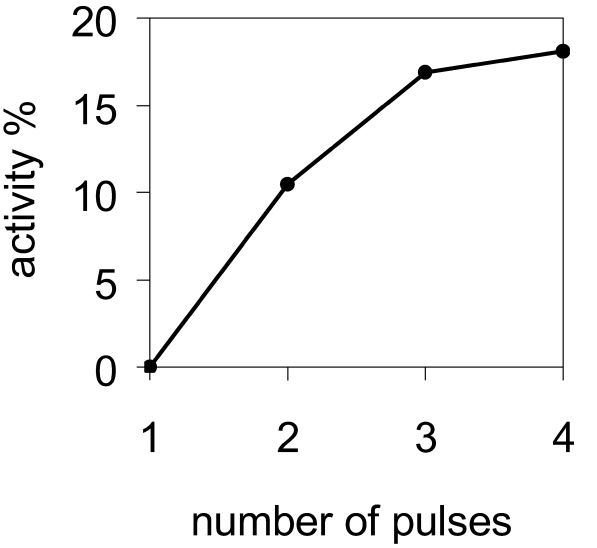

Fig. 3. Effect of D-galactose pulses on the activity of immobilized galactose oxidase. Reactivation was carried out by four pulses of 100 μl of 3 *M* D-galactose for 15 min. Activity of immobilized enzyme was measured immediately after each pulse.

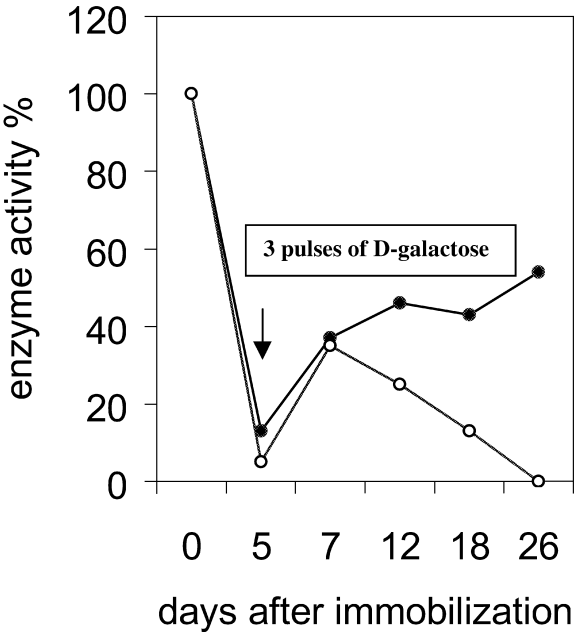

Fig. 4. Stability of orientedly immobilized (GaOX — OI ●) and directly immobilized galactose oxidase (GaOX — DI ○) prepared in the solution without Cu^{2+} ions. Reactivation by three pulses of 3 *M* D-galactose and following storage was performed in phosphate buffer with 2 m*M* $CuSO_4$.

4. Conclusion

Oriented immobilization of galactose oxidase through the carbohydrate part resulted in a highly active and stable oxidation reactor with very good sterical accessibility of its active site. We used hydrazide derivatives of non-magnetic and magnetic bead cellulose and of magnetic and non-magnetic poly(HEMA-co-EDMA) microspheres. Activation of the enzyme molecules was done by sodium periodate in the presence of supplements (fucose, $CuSO_4$, catalase). A reactor prepared from macroporous bead cellulose had very good stability. Nearly origin activity could be reached by reactivation by three pulses of D-galactose after 6 months of storage. The magnetic form of macroporous bead cellulose provided the best working comfort from four types of hydrazide-derivatized supports used. Orientedly immobilized galactose oxidase has high adaptability after incubation even under inappropriate microenvironmental conditions.

This enzyme reactor provides an economical, efficient and selective system for enzymatic oxidation of glycoproteins suitable for immobilization to hydrazide derivatized supports (e.g. monoclonal anti-

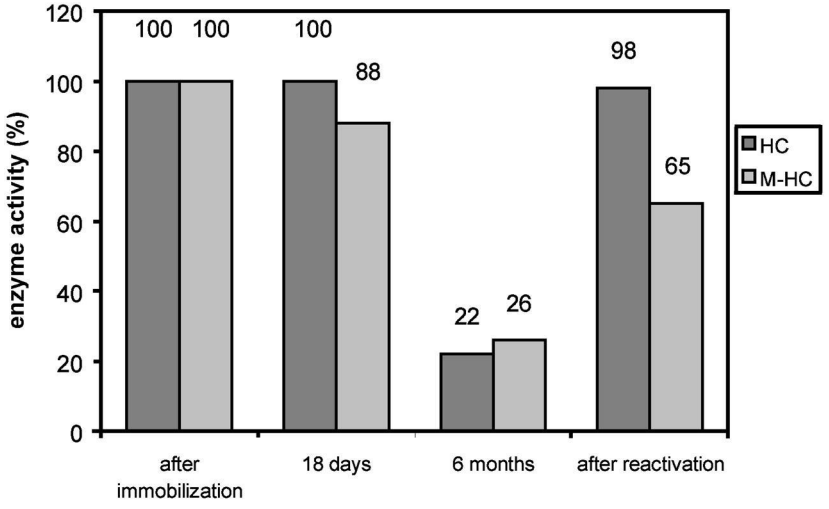

Fig. 5. Storage stability of galactose oxidase immobilized orientedly to hydrazide derivative of bead cellulose (HC ■) and to magnetic form of this (M-HC ▨). Enzyme reactors were kept for 6 months in phosphate buffer containing 2 m*M* $CuSO_4$ (pH 6) at 4°C. Three pulses of 3 *M* D-galactose performed reactivation.

bodies without impairing immunological activity of the antibodies). It can also be widely used in glycoconjugate research, e.g. in labelling cell surface glycoproteins, for pre- or post-column derivatization of saccharides for detection in a liquid chromatographic system, determination of galactose or lactose concentration in complex biological fluids, etc.

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