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Immobilization of Galactosyltransferase and Continuous Galactosylation of Glycoproteins in a Reactor

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We immobilized human milk galactosyltransferase covalently to CNBr- and tresylchloride-activated Sepharose. The enzyme was also immobilized non-covalently to Concanavalin A-Sepharose and to monoclonal anti-galactosyltransferase antibodies which were bound *via* their Fc-fragment to Protein G-Sepharose. With the covalent methods, up to 72% of the enzyme could be bound to the carrier, but more than 90% of the specific activity was lost. In contrast, non-covalent immobilization yielded only about 50% immobilization efficiency, but 21% and 25% of specific activity, respectively, could be recovered.The stability of immobilized galactosyltransferase as compared to native enzyme was considerably increased: at room temperature, 55% of initial immobilized activity was lost after 65 hours compared to 95% of loss of soluble enzyme activity. Immobilized galactosyltransferase was then used for continuous galactosylation of the glycoproteins ovalbumin, endo H-treated yeast invertase and bovine serum albumin-*N*-acetylglucosamine in a "slurry" reactor. 55%, 35% and 25%, respectively, of all acceptor sites on these glycoproteins could be galactosylated by this method.

During the last decade, great efforts have been made to produce recombinant proteins for therapeutic use. Many of them carry N-linked sugar chains which may contribute to their behavior *in vivo* [1-3]. Progress in glycan structure determination has revealed that these recombinant glycoproteins show considerable diversity concerning the number and/or structure of their attached oligosaccharides. Glycosylation is normally cell type-, tissue- and species-specific [4, 5] and depends also on the physiological [6] and developmental status of the expressing cell. Recombinant glycoproteins often have altered glycosylation patterns compared to their "natural" counterparts although the peptide backbone is the same. As a consequence, pharmacokinetics of recombinant glycoproteins may depend on the sugar make-up in analogy to different glycoforms of serum glycoproteins [7]. There must be ways, therefore, to ensure that drugs produced in heterologous systems be properly glycosylated before application *in vivo*.

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At least two strategies may prove useful. First, a careful choice of the expressing cell type could prevent many problems arising from the species-specificity of glycosylation. Second, inaccurately processed carbohydrate chains could be modified *in vitro*. Glycosidases and glycosyltransferases, enzymes of the glycosylation pathways of the cell [8, 9], may provide tools which allow to sequentially remove inadequate glycosyl residues and to add the correct ones [10-12].

The aim of our work was to demonstrate the applicability of this second approach. We used human milk galactosyltransferase to modify the three acceptor substrates ovalbumin, endoglycosidase H-treated invertase from yeast and the neoglycoprotein BSA-GlcNAc (*N*-acetylglucosamine covalently attached to bovine serum albumin). The galactosylation was run in a "slurry" reactor for several hours with the enzyme immobilized to a Sepharose carrier. We first compared four different methods of immobilization, two with covalent attachment of the enzyme to the carrier and two with non-covalent binding to a ligand. Each method was characterized [13] by determining the efficiency of immobilization and retention of specific activity of the bound enzyme. Tresylchloride-activated Sepharose and Concanavalin A-Sepharose were finally chosen as carriers for the enzyme, in order to compare the suitability of these rather distinct methods of immobilization for operation in a reactor.

Experimental Procedures

Materials

UDP-Gal:*N*-acetylglucosamine β(1-4)galactosyltransferase (EC 2.4.1.22) was purified from pooled human milk as previously described [14]. A solution of monoclonal anti-galactosyltransferase antibodies of the IgG_3 subtype, designated 36/118/2, was obtained as truly monoclonal by limiting dilution from a 10x concentrated hybridoma cell supernatant [15]. Yeast β-D-fructofuranoside fructohydrolase (invertase, EC 3.2.1.26) and UDP-Gal were from Boehringer (Mannheim, Germany). Uridine diphospho-D-[U-^{14}C]galactose, batch A 256 mCi/mmol, batch B 319 mCi/mmol, was from Amersham (Oakville, Canada). Egg white albumin (ovalbumin), 5x cryst., salt-free, lyophilized,was from Fluka (Buchs, Switzerland). (GlcNAc-β-*O*-CETE)$_n$-BSA (BSA-GlcNAc) was from Janssen (Beerse, Belgium). CNBr-activated Sepharose 6MB, tresylchloride-activated Sepharose 4B, Concanavalin A-Sepharose 4B and Protein G-Sepharose 4FF were obtained from Pharmacia (Uppsala, Sweden). A liquid scintillation cocktail, Irgascint A 300, (Ciba-Geigy, Basle, Switzerland) was used. Glass microfibre filters GF/A were from Whatman (Maidstone, England). Ultrafiltration cells, 10 ml, with omega series membranes were obtained from Filtron (Clinton, USA). Protein concentrations were determined by the Bio-Rad Microassay procedure (Bio-Rad, Richmond, USA) using bovine serum albumin as a standard.

Assay Procedures

Activity and specific activity of the enzyme are expressed in international units (U) and units/mg, respectively. Specific activity of native and immobilized enzyme was determined by initial velocity measurement of the reaction: UDP-Gal + ovalbumin → UDP + ovalbumin-Gal at 37°C. Aliquots were taken at time intervals of 5 min and the reaction was stopped by

acidic precipitation in 15% trichloroacetic acid + 5% phosphotungstic acid. The precipitate was filtered on Whatman GF/A glass microfibre filters and incorporation of radiolabeled galactose was determined by heterogeneous liquid scintillation counting.

For both soluble and immobilized enzyme, the following assay procedure was used (all concentrations are final concentrations): 0.09% NaCl, 10 mM $MnCl_2$, 0.33 mM ovalbumin as acceptor substrate, 0.3 mM UDP-Gal and 5.84 µM UDP-[^{14}C]galactose (batch A) as donors. The reaction was buffered in 0.1 M sodium cacodylate.

Assay Procedure for Soluble Enzyme: The reaction mixture without enzyme was pre-incubated at 37°C. An aliquot for zero-point determination was taken and the reaction was started by adding the enzyme.

Assay Procedure for Immobilized Enzyme: The immobilized enzyme was suspended in the reaction mixture, containing all components except ovalbumin, and pre-incubated at 37°C. After brief centrifugation, an aliquot was taken from the supernatant for zero-point determination and the reaction was started by adding the acceptor substrate. In order to minimize diffusion limitations, the gel was hold in suspension during the reaction by intermittent shaking.

General Procedure for Immobilization

All immobilizations were carried out at room temperature. The gel was suspended in the required coupling buffer (see specific procedures) containing the enzyme, and immobilization occurred during the required time by end-over-end rotation. In order to prevent nonspecific binding at the active center, the enzyme was immobilized in presence of its substrate UDP-Gal and Mn^{2+}. All supernatants and washing solutions produced during the preparation of the immobilized enzyme were collected and protein concentration and enzymatic activity were determined as described.

Efficiency of immobilization, defined as the amount of bound enzyme, was determined by subtracting the enzymatic activity in the supernatant and washing solutions after coupling from that added before coupling. *Retention (recovery) of specific activity* was determined by assaying the immobilized enzyme and expressing the measured specific activity in percent of the specific activity of the native enzyme. The actual activity in its immobilized state, expressed in percent of the total amount added, gave the *immobilization yield*.

Immobilized enzyme was stored at 4°C in the coupling buffer, containing 0.01% NaN_3 for preservation.

Immobilization to CNBr-Activated Sepharose

CNBr-activated Sepharose 6MB (58 mg, which give a final gel volume of 200 µl) was swollen for 15 min in 1 mM HCl. The gel was then washed three times in the same buffer and twice in coupling buffer (0.1 M H_3BO_3, pH 8.0, containing 0.5 M NaCl). Human galactosyltransferase (180 µl, 0.6 mg/ml) was diluted in 1 ml coupling buffer, containing 10 mM $MnCl_2$ and 0.2 mM UDP-Gal. The gel was suspended in 1 ml of this solution, and immobilization occurred

by end-over-end rotation for 2 h. Remaining active groups were blocked by incubation for 2.5 h in 1 M ethanolamine, pH 8.0, containing 0.5 M NaCl. Unbound protein was removed by washing the gel several times in coupling buffer.

Immobilization to Tresylchloride-activated Sepharose.

Tresylchloride-activated Sepharose 4B (58 mg) was rapidly washed twice in coupling buffer (0.1 M H_3BO_3, pH 8.0, containing 0.5 M NaCl). Human galactosyltransferase (180 µl, 0.6 mg/ml) was diluted in 1 ml of coupling buffer, containing 10 mM $MnCl_2$ and 0.2 mM UDP-Gal. The gel was suspended in 1 ml of this solution and incubated 2 h by end-over-end rotation. The gel was then washed first in coupling buffer and then in 0.2 M Tris-HCl, pH 8.0. Remaining active groups were blocked by incubating the gel in the Tris-buffer for 30 min by end-over-end rotation.

Immobilization to Concanavalin A-Sepharose

Suspended Concanavalin A-Sepharose 4B (320 µl), which give a final gel volume of 200 µl, was washed five times in coupling buffer (0.1 M H_3BO_3, pH 7.35, containing 0.1 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$). The divalent metal ions are required by the lectin for stable binding of substrate [16]. Human galactosyltransferase (180 µl, 0.6 mg/ml) was diluted in 1 ml coupling buffer, containing in addition 10 mM $MnCl_2$ and 0.2 mM UDP-Gal, and the gel was then suspended in 1 ml of this solution. Immobilization occurred for 2 h by end-over-end rotation. The gel was finally washed several times in coupling buffer.

Immobilization to Protein G-Sepharose

Suspended Protein G-Sepharose 4 FF (160 µl), which give a final gel volume of 100 µl, was washed three times in deionized water, pH 7.0, and twice in 0.07 M sodium phosphate buffer, pH 7.0. Monoclonal antibodies (mAb) were immobilized to the gel according to the following procedure: Each 250 µl of the antibody solution were diluted in 750 µl 0.07 M sodium phosphate buffer, pH 7.0. The gel was then suspended in this solution and incubated for 1 h by end-over-end rotation. This procedure was repeated five times, up to a total volume of 1500 µl mAb solution. The gel was washed three times in 0.07 M sodium phosphate buffer, pH 7.0, and twice in coupling buffer (0.1 M H_3BO_3, pH 7.35, containing 0.5 M NaCl). Human galactosyltransferase (70 µl, 0.38 mg/ml) was diluted in 1330 µl of coupling buffer, containing in addition 10 mM $MnCl_2$ and 0.2 mM UDP-Gal. The gel was suspended in 1 ml of this solution and incubated 2 h by end-over-end rotation. It was then finally washed several times in coupling buffer.

Continuous Galactosylation in a Reactor

Stirred ultrafiltration cells of a volume of 10 ml with membranes of 100 K NMWL for experiment A and 300 K for experiments B and C were used as reactors. Substrate was continuously fed to the reactor by a peristaltic pump. Product was withdrawn by a second pump and collected by an automatic sample collector. In each experiment, immobilized enzyme was equilibrated in the reactor in a solution containing all components except the donor UDP-Gal. The reaction was then started by the addition of the required amount of donor. All reactor experiments were run at room temperature.

Table 1. Immobilization efficiency, retention of specific activity and immobilization yield obtained by the four different immobilization methods.
Human milk galactosyltransferase was immobilized as described in the experimental protocol. CNBr: Immobilization to CNBr-activated Sepharose. Tresyl-Cl: Immobilization to tresylchloride activated Sepharose. Con A: Immobilization to Concanavalin A- Sepharose. mAb-Protein G: Immobilization to Protein G-Sepharose through binding to monoclonal antibodies.

Method	Immobilization efficiency[a] (%)	Specific activity[b] (%)	Immobilization yield[c] (%)
CNBr	72	6	4.3
Tresyl-Cl	56	9	5.0
ConA	48	21	10.1
mAb-Protein G	44	25	11.0

[a] Amount of bound protein in percent of the amount added.
[b] Specific activity of the bound enzyme in percent of the specific activity of the native enzyme.
[c] Actual enzymatic activity bound in percent of the activity added.

During the run, aliquots of product solutions were removed out of the flow after pump 1. Incorporation of radiolabeled galactose was determined by acidic precipitation and scintillation counting as described above. The products were characterized by sodium dodecylsulphate-polyacrylamide gel electrophoresis and autoradiography .

In experiment A, 45 ml of a substrate solution of the following composition was fed through the reactor: 0.1 M sodium cacodylic acid, pH 7.35, 0.09% NaCl, 10 mM $MnCl_2$, 0.3 mM UDP-Gal together with 2.0 nM UDP-[^{14}C]galactose (batch B) as donor and the acceptor ovalbumin in a concentration of 10 mg/ml (0.22 mM). Human milk galactosyltransferase was immobilized to 400 µl Concanavalin A-Sepharose 4B yielding an activity of 74 mU. The reaction volume in the tank was held constant at 4 ml by a flow rate of 1.5 ml/h.

In experiment B yeast invertase was partially deglycosylated as previously described [10]. The invertase was then diluted to a concentration of 1 mg/ml (16.4 µM) in a total volume of 22 ml of the following solution: 0.1 M sodium cacodylic acid, pH 7.0, containing 0.5 mM $MnCl_2$, 850 µM UDP-Gal and 2.0 µM UDP-[^{14}C]galactose (batch B). Human milk galactosyltransferase was immobilized to 1.0 ml tresylchloride-activated Sepharose yielding 80 mU activity. The reaction solution was held constant at a volume of 8 ml by a flow rate of 0.75 ml/h.

In experiment C BSA-GlcNAc was used as acceptor at a concentration of 1.6 mg/ml (22.9 µM) in a total volume of 17 ml. This artificial glycoprotein contains on the average 35 mol acceptor chains per mol BSA molecules. Human milk galactosyltransferase was bound to 400 µl Concanavalin A-Sepharose 4B yielding 36 mU activity. A substrate solution of the same composition as in experiment A, except the acceptor, was pumped through the reactor with a flow rate of 0.75 ml/h. The reaction volume was held constant at 4 ml.

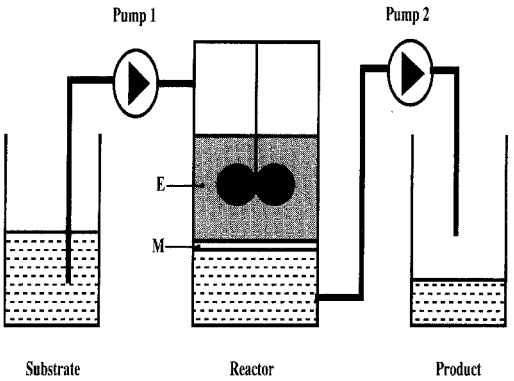

Figure 1. Apparatus for the continuous galactosylation of different substrates. An ultrafiltration cell with a volume of 10 ml was used as reactor. The immobilized enzyme (E) was retained in the reactor by the membrane (M); continuous stirring ensured that the gel was always held in suspension.

Results

Immobilization of Galactosyltransferase by Four Different Methods

Human milk galactosyltransferase was immobilized by four different methods. *Immobilization efficiency, retention of specific activity* and *immobilization yield* of the immobilized enzyme were determined for each of the methods (see experimental protocol and Table 1).

One of the carriers most frequently used for covalent immobilization of proteins is CNBr-activated Sepharose. We immobilized galactosyltransferase to this carrier according to the coupling procedure recommended by the manufacturer. Because covalent coupling of protein to the support involves mainly the epsilon amino group of lysine [17] and therefore is not specific, the active center of the enzyme had to be protected during the immobilization procedure. This was achieved by immobilizing the enzyme in the presence of its donor substrate UDP-Gal and the required cofactor Mn^{2+}. As can be seen from Table 1, efficiency of immobilization was rather high as expected, but almost 95% of the specific activity of the native enzyme had been lost by the immobilization.

Another carrier material frequently used for covalent coupling is tresylchloride-activated Sepharose [18]. In a preliminary test, enzyme was immobilized together with UDP-Gal but without Mn^{2+}. The immobilization efficiency was 58%, but only 1% of the specific activity of the native enzyme could be preserved by the bound enzyme. Galactosyltransferase (580 mU) was then used for immobilization on 200 µl gel, together with 0.2 mM UDP-Gal and 10 mM Mn^{2+}. Table 1 shows that the retention of specific activity was slightly higher than that obtained by the CNBr method.
The same amount of galactosyltransferase (580 mU) was then also used for immobilization on 200 µl Concanavalin A-Sepharose gel. Concanavalin A (ConA) binds with high affinity to oligosaccharides containing the trimannose structure Manα1-6(Manα1-3)Man [19]. Human

galactosyltransferase is glycosylated with bi- and tri-antennary complex-type oligosaccharides with this core structure [20] and is thus bound by ConA [21]. Table 1 shows that the retention of specific activity was more than twice as high compared to the two covalent methods; the immobilization efficiency was only slightly decreased.

The immobilization of an enzyme based on the binding *via* monoclonal antibodies to a Protein A-Sepharose carrier was described a few years ago [22]. We used monoclonal antibodies of the IgG_3 subclass against human milk galactosyltransferase as mediator for the immobilization. Protein A, however, binds subclass 3 IgG molecules only weakly. Protein G, a recombinant Streptococcal Fc receptor protein, is an alternative since it binds all subclasses with high affinity [23]. This protein, immobilized to Sepharose, is also commercially available. Table 1 shows that the retention of specific activity was the highest of the four methods examined. Specificity of binding was examined in a parallel test by incubating the enzyme with a Protein G-Sepharose gel without prior immobilization of monoclonal antibodies. All enzymatic activity added was recovered in the supernatant which clearly shows that nonspecific binding of the enzyme to the carrier did not occur.

Continuous Galactosylation of Different Acceptor Substrates.

The "slurry" reactor, introduced by Bossow *et al.* [24] for enzymatic formation of C-C bonds, is basically a stirred ultrafiltration cell. The ultrafiltration membrane with a defined nominal molecular weight limit of 100 or 300 kDa serves as a barrier. Compounds of enzymatic galactosylation can pass this barrier whereas the immobilized galactosyltransferase with a molecular weight of >300 kDa is retained in the reactor. Another advantage of immobilization is that it prevents the enzyme from accumulation at the membrane (concentration polarization) or adsorption to the walls of the reactor. The arrangement of our experimental apparatus is schematically shown in Fig. 1. Substrate was fed through the reactor by the two pumps which were adjusted to operate with the same flow rates. In order to determine the galactosylation efficiencies, aliquots were removed directly out of the product flow after pump 2.

In experiment A, a total amount of 450 mg ovalbumin was used as acceptor at a concentration of 0.22 mM acceptor sites, assuming one mol acceptor sites per mol of protein. Galactosyltransferase was immobilized to 400 µl Concanavalin A-Sepharose yielding an activity of 74 mU. The mean residence time of the substrates in the reactor was about 2.7 h. In order to determine the galactosylation efficiency (concentration of galactosylated acceptor in percent of total concentration) under steady-state conditions, the reaction was run for 27 h. Fig. 2 shows that the galactosylation efficiency remained stable at approximately 50% after 5 h.

In experiment B, yeast invertase was deglycosylated with endoglycosidase H (endo H) in order to serve as substrate, yielding seven moles of acceptor sites per mol of invertase. A total amount of 22 mg invertase was used, giving an acceptor concentration in the reaction solution of 0.12 mM. In contrast to the other reactor experiments, tresylchloride-activated Sepharose was used as support because under non-denaturing conditions not all mannan chains of the invertase can be split off by the endo H treatment [25] and partially deglycosylated invertase is bound by Concanavalin A. Galactosyltransferase was immobilized to the support yielding 80 mU activity. The mean residence time of the substrates in the

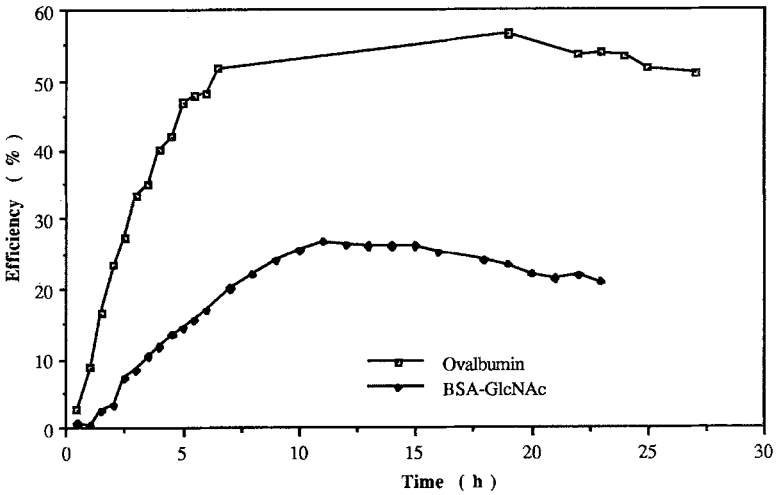

Figure 2. Galactosylation of ovalbumin and BSA-GlcNAc in the reactor. Galactosyltransferase was immobilized to each 400 µl Concanavalin A-Sepharose yielding 74 mU and 36 mU activity, respectively. 450 mg Ovalbumin in 45 ml and 25 mg BSA-GlcNAc in 17 ml reaction solution were totally used, giving acceptor concentrations of 0.22 mM for ovalbumin and 0.8 mM for BSA-GlcNAc. The reaction volume was 4 ml by flow rates of 1.5 ml/h and 0.75 ml/h, respectively. Efficiency is defined as concentration of galactosylated acceptor in percent of the total acceptor concentration.

reactor was 10.6 h. The reaction was first run for 22 h. Fig.3 shows that the galactosylation efficiency was almost constant for several hours. The pooled product fractions were then pumped through the reactor for a second time. The galactosylation efficiency could thus be raised more than twice as compared to the first run.

In experiment C, a neoglycoprotein was used as acceptor substrate. BSA-GlcNAc is a bovine serum albumin with spacer arms which carry terminal *N*-acetylglucosamine residues. A total amount of 25 mg BSA-GlcNAc was used. The acceptor concentration in the solution was 0.8 mM. Galactosyltransferase was immobilized to Concanavalin A-Sepharose yielding 36 mU activity. Reaction conditions were the same as in experiment A except for the flow rate, which was adjusted to give a mean residence time for the substrates of 5.3 h. The reaction was run for 23 h. Fig. 2 shows that the galactosylation efficiency reached a maximum after about 11 h and then slightly decreased during the steady-state period. An aliquot of this immobilized enzyme was assayed after the run and activity compared to that measured before the reaction. No decrease of activity could be detected.

Stability of Immobilized Galactosyltransferase

The stabilities of galactosyltransferase immobilized to tresylchloride-activated Sepharose and Concanavalin A-Sepharose were compared to the stability of the soluble enzyme. Each enzyme preparation was stored at room temperature (22°C) in the respective buffer used for

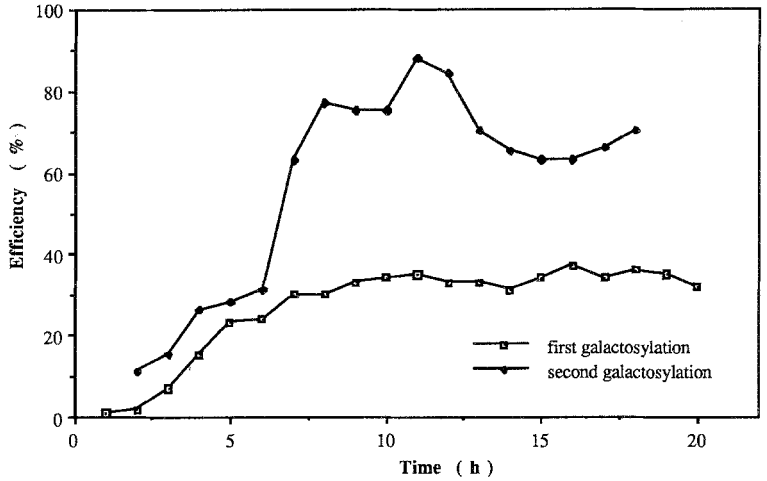

Figure 3. Galactosylation of endo H-treated invertase. Galactosyltransferase was immobilized to 1.0 ml tresylchloride-activated Sepharose yielding an activity of 80 mU. A total amount of 22 mg invertase in 22 ml reaction solution was used, giving an acceptor concentration of 0.12 mM. The reaction volume was 8 ml at a flow rate of 0.75 ml/h. The product of the first run was galactosylated a second time under similar conditions

galactosylation in the reactor. Aliquots were taken at several times and assayed for enzymatic activity. Fig. 4 shows that both immobilization procedures stabilized the enzyme considerably. Whereas almost all of the activity of the soluble enzyme was lost after 65 h, up to 50% of the initial immobilized activity could be recovered with both methods.

Characterization of Products

Aliquots of the product solutions were electrophoresed under denaturing conditions on a polyacrylamide gel and incorporation of labeled galactose was detected by autoradiography. Fig. 5 confirms that galactose was incorporated in the acceptor substrates ovalbumin, yeast invertase and BSA-GlcNAc (lines A, B and C).

Discussion

Since the first applications in the 1970's, immobilization of enzymes has now become an established technology. Many methods for immobilization have since been developed, but none of them can considered to be the method of choice for all purposes.

We immobilized human milk galactosyltransferase by two covalent methods and two affinity methods. Both covalent and affinity-based immobilization methods showed good reproducibility. Table 1 shows immobilization efficiency, retention of specific activity and immobilization yield determined with the four methods. As would be expected, covalent immobilization with CNBr- and tresylchloride-activated carriers show the best binding efficiencies, but the specific activity of the enzyme was considerably lowered after coupling.

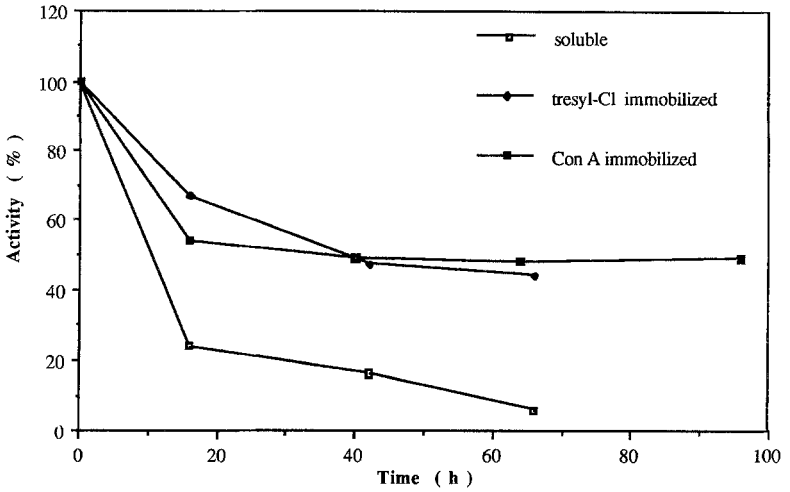

Figure 4. Stability of soluble, tresylchloride-immobilized and Concanavalin A-immobilized galactosyltransferase. Soluble and immobilized enzyme were incubated at 22°C in 0.1 M sodium cacodylic acid, containing 0.09% NaCl and 10 mM $MnCl_2$. At indicated times, the activity was determined in aliquots and expressed in % of initial activity.

Affinity chromatography with lectins is nowadays widely used for screening [26] and fractionation [27] of glycoproteins. Because of its selective binding, it should also provide a good method for immobilization of glycoprotein enzymes, taking advantage of the fact that sugar chains are normally excluded from the active center. Furthermore, the chains may act as long spacer arms which should allow for a better accessibility of the active center for the substrates.The separation of human galactosyltransferase into approximately equal groups of ConA binding and non-binding forms was confirmed by the binding efficiency. Retention of specific activity was twice as high as with the covalent methods. The coupling to the carrier was specific and only at one point of the molecule, thus the enzyme is expected to sustain little steric constraints.

The second method of non-covalent immobilization makes use of two highly selective affinity bindings simultaneously. Intuitively, this should provide a good method for eliminating substrate access limitations if one considers that the Protein G together with the bound antibody molecule would build a long spacer arm extending orthogonally from the support. The activity recovery of 25% indicates that also for this mild and selective method constraints must exist which cause a drop of specific activity after immobilization. Carboxypeptidase A has been previously immobilized by the same method [21] with up to 100% retention of specific activity. By comparing these data, however, it must be considered that galactosyltransferase requires the access to the active center of three substrates - donor, a high molecular weight acceptor and Mn^{2+} as cofactor - in an ordered sequence of binding [28] and loss of activity by substrate limitations may thus be amplified.

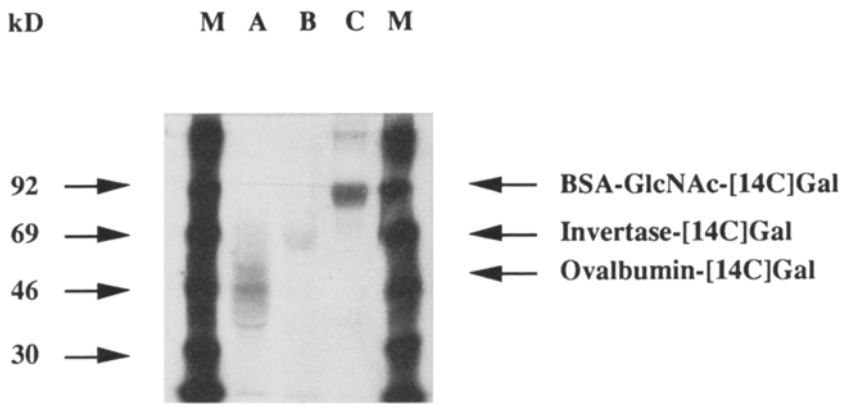

Figure 5. Incorporation of galactose into the acceptor substrates ovalbumin, yeast-invertase and BSA-GlcNAc in experiments A, B and C, respectively, determined by SDS-polyacrylamide gel electrophoresis and autoradiography.

The increased stability favors the use of immobilized enzyme for continuous galactosylation of a substrate, e.g. in a "slurry" reactor. Another advantage of immobilization is that the enzyme is retained in the reactor by the ultrafiltration membrane, whose pore size can be chosen large enough for the product to pass. Continuous stirring ensures that the immobilized enzyme is always held in suspension; the reaction can thus be considered as almost homogeneous and substrate limitations should not be as limiting as e.g. in a column reactor. The experiments show that by this method good glycosylation efficiencies can be obtained even on a single continuous run. Improvements may be achieved by feeding the substrate multiple times through the reactor, as shown in the experiment with invertase, or by using a recirculation reactor.

The galactosylation efficiency obviously also depends on the amount of enzyme used for the reaction, so the affinity methods with their relatively high immobilization yield may be the methods of choice for immobilization and application in a reactor. For large scale operation, however, also cost/benefit considerations must be taken into account.

We believe, the methods described for *in vitro* glycosylation may contribute to solve one of the problems which emerge with the heterologous expression of pharmacologically active glycoproteins, i.e. the possibly altered pharmacokinetics of glycoproteins with inadequate sugar moieties. The labeled products may be used without extensive further purification for *in vivo* studies. Tissue distribution and half-life time can thus be determined by virtue of their incorporated radioactive label. We anticipate that this approach will gain importance at the pace of the development of recombinant and actively expressed mammalian glycosyltransferases. Some of them have been cloned to full length, as recently reviewed [29]. These enzymes are expected to be available as recombinant glycoproteins soon.

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