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Solubilization and Purification of Galactosyltransferase from Golgi Membranes of Rat Ventral Prostate

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Abstract

UDP-galactose ovomucoid galactosyltransferase, a membrane-bound enzyme involved in the biosynthetic pathways for formation of the nonreducing terminal oligosaccharide sequences on glycoproteins, has been solubilized and purified from rat ventral prostate Golgi membranes.

Solubilization was effected by treatment of the particulate fraction with Triton X-100 (0.5% v/v) and $MnCl_2$ (25 m*M*). The solubilized enzyme was purified by affinity chromatography on hen ovomucoid–sepharose column.

The purified galactosyltransferase showed three protein bands of approx. 74,000, 60,000, and 54,000 daltons on sodium dodecyl sulfate gel electrophoresis. On gel filtration, enzyme activity eluted at approx. 70,000 daltons with a broad shoulder between 60,000 and 50,000 daltons.

Isoelectric focusing of the purified enzyme resolved at least five active bands with pHi of 9, 7.4, 6.75, 6.1, and 4.8.

Index Entries: Galactosyltransferase, solubilization and purification of; Golgi membranes, galactosyltransferase from; rat ventral prostate, galactosyltransferase from; ovomucoid-sepharose; isoelectric focusing, of galactosyltransferase; prostate, rat, galactosyltransferase from.

Introduction

Galactosyltransferases (UDP-galactose glycoprotein, β-1–4 galactosyltransferase, EC 2.4.1.22) catalyse the transfer of galactose from UDP-galactose to glycopro-

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tein with terminal *N*-acetylglucosamine residues, free *N*-acetylglucosamine, and glycosyl derivatives of *N*-acetylglucosamine. These transferases are usually bound to membranes in the particulate fractions of most tissues (*1–5*), but a considerable amount of the enzymes are also found in (a) soluble fractions of tissue homogenates (*5–7*), (b) various extracellular fluids such as blood serum (*8–10*), amniotic fluid (*11, 12*), and the secretions of certain glands (*5, 13–16*). In this connection it is of course conceivable that there exist isoenzymes of galactosyltransferase. Indeed it has been shown on the basis of differences in size, composition, and kinetic properties that there are isoenzymes of galactosyltransferase in human serum (*8*). On the other hand, the separable forms of soluble galactosyltransferase from other sources are apparently derived from the same single protein that has either undergone restricted proteolytic cleavage (*17, 18*), or has had some of the attached carbohydrate removed (*19*). Conversely, the question of the significance of soluble forms and their relatedness to membranous galactosyltransferases remains unanswered. It is likely that the secretion enzymes are simply derived from membrane-bound ones and may indeed represent cleavage products (*3*). On the other hand it has not yet been possible to clarify whether the soluble enzymes found intracellularly are released from their membrane attachment by the preparative procedure or whether they represent a functionally separate pool and/or precursor enzymes moving to Golgi membrane for final glycosylation prior to insertion. The question of the relationship between the membrane-bound and soluble forms of these enzymes could be resolved by a study of the purified enzymes. A close examination of the differences and similarities between the membrane-bound and soluble forms of galactosyltransferases might prove useful in determining whether the soluble enzymes represent a derivative of the supposed functionally active membrane-bound enzymes or are structurally and functionally different.

In this report we describe the solubilization and purification by hen ovomucoid affinity chromatography of membrane-bound galactosyltransferase from rat ventral prostate. In addition some properties of the enzyme are determined.

Materials and Methods

Materials

UDP-^{3}H galactose (14.6 Ci/mmol) was obtained from New England Nuclear Corporation. Mops (Morpholinepropanesulfonic acid), sodium cacodylate, Triton X-100, unlabeled UDP-galactose, hen ovalbumin, hen ovomucoid (Trypsin inhibitor type II-O) were purchased from Sigma Chemical Company; lactate dehydrogenase, malate dehydrogenase, bovine serum albumin from Boehringer GmbH; Sephadex G 100 and CNBr-activated Sepharose 4B from Pharmacia; ampholine PAG plate from LKB; acrylamide gel reagents and sodium dodecyl sulfate from Bio-Rad Laboratories; Soluene-350 from Packard-Instrument Company.

Methods

Golgi membranes from homogenates of rat ventral prostate were prepared by centrifugation through two discontinuous sucrose density gradients and the Golgi

fraction obtained was concentrated by centrifugation at $105{,}000g_{av}$ for 60 min and the pellet was resuspended in 25 m*M* sodium cacodylate buffer, pH 7.4, to give stock solutions of approx. 10 mg Golgi protein/mL of suspension.

Purification of Galactosyltransferase from Golgi Membranes

Three to five milligrams or less of Golgi membrane protein was resuspended in 3 mL of 25 m*M* sodium cacodylate buffer containing 25 m*M* $MnCl_2$, 0.1 m*M* UMP, 0.5% (v/v) Triton X-100, pH 7.4. After 30 min agitation at 4°C the suspension was centrifuged at $105{,}000g_{av}$ for 60 min. The supernatant was loaded on a column of hen ovomucoid-sepharose (1.6 × 7cm) prepared as previously described (*20*) and equilibrated at 4°C with 25 m*M* sodium cacodylate buffer containing 25 m*M* $MnCl_2$, 0.1 m*M* UMP, 0.05% (v/v) Triton X-100, pH 7.4. The column was washed with the same buffer at a flow rate of 5 mL/h until the absorbance at 280 nm of the column effluent indicated that all the inert protein had passed through. The galactosyltransferase was eluted as a sharp peak at the front of the developing buffer containing 25 m*M* sodium cacodylate, 5 m*M* *N*-acetylglucosamine and 25 m*M* EDTA, pH 7.6. The effluent was collected in 2 mL fractions. The fractions were assayed for galactosyltransferase activity. Fractions containing the enzyme were pooled, concentrated by ultrafiltration through a PM-10 Amicon membrane, and then diafiltrated with 25 m*M* sodium cacodylate buffer containing 0.02% (v/v) Triton X-100, 0.1 *M* NaCl, pH 7.4.

Measurement of Galactosyltransferase Activity

Galactosyltransferase assays were performed by a radiochemical procedure as described previously (*21*). The standard 0.1 mL incubation mixture contained 50 m*M* Mops, pH 6.8, 20 m*M* $MnCl_2$, 1 mg of hen ovomucoid, 0.1 m*M* UDP-^{3}H galactose (5×10^4 dpm) and 0.1% (v/v) Triton X-100. Incubations were conducted at 37° C for 30 min. The reaction was terminated by the addition of 2 mL of trichloroacetic acid (TCA) (5%, w/v)/phosphotungstic acid (1%, w/v) mixture. The precipitated protein was collected by centrifugation, washed with 2×2 mL of TCA/phosphotungstic acid mixture and 1×2 mL of ethanol/ether (3: 1, v/v). The washed precipitate was dissolved in 0.5 mL of Soluene-350 and radioactivity was determined by scintillation counting. The enzyme unit is defined as nanomoles of galactose transferred per minute per milligram of protein.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed by the method of Fairbanks et al. (*22*) using 5.6% polyacrylamide containing 0.1% SDS. About 10 μg of protein were applied to the gel, which was stained for protein with Coomassie brilliant blue.

Analytical Gel Filtration

Determination of molecular weights was performed using a calibrated column of Sephadex G-100 (1.6 × 80 cm) equilibrated at 4°C in 25 m*M* sodium cacodylate buffer containing 0.05% (v/v) Triton X-100, 0.1 *M* NaCl, pH 7.4. Molecular

weight markers were dissolved in 1 mL of equilibrating buffer and applied to the column. The column was developed with the same buffer at a flow rate of 3.5 mL/h. The markers for calibration were: lactate dehydrogenase, malate dehydrogenase, bovine serum albumin, and hen ovalbumin.

Isoelectric Focusing

Premanufactured polyacrylamide slab gels, pH 3.5–9.5, from LKB were used according to manufacturers' instructions and focusing took place in a LKB multiphor 2117. After the run, the pH gradient of the gel was measured with a surface electrode. The gel was sliced into fractions 2.5 mm wide and each fraction, after quartering, was incubated overnight at 4°C in 0.2 mL of 0.2 *M* Mops, pH 6.8. Determination of the galactosyltransferase from gel fraction eluents was carried out using a modification of the procedure described above: assay mixture contained 0.1 m*M* UDP-^{3}H galactose (5×10^5 dpm).

Analytical Procedure

Proteins were precipitated from the collected fractions by addition of trichloroacetic acid to a final concentration of 10% (w/v). Protein was determined by the method of Lowry et al. (*23*) with bovine serum albumin as standard.

Results

Solubilization

A range of procedures were investigated for effectiveness in solubilizing galactosyltransferase, the criterion for enzyme solubilization being failure of enzyme activity to appear in sediment from samples centrifuged at $105{,}000g_{av}$ for 60 min at 4°C, conditions under which vesicles derived from Golgi apparatus membranes are otherwise quantitatively sedimented. Ultrasonic vibrations at 4°C for 1–4 min solubilized only 16% of the enzyme. Alteration in ionic conditions either by increasing the ionic strength (addition of NaCl up to 0.5 *M*) or by removing electrolytes (addition of EDTA 1 m*M*) produced negligible solubilization of galactosyltransferase (2–4%). Addition of Triton X-100 to the suspension of Golgi vesicles releases the enzyme into the supernatant, but the solubilization of the enzyme is associated with a decreased and erratic recovery. Table 1 (expt. 1) shows the effect of Triton X-100 concentration on the solubilization of galactosyltransferase. When the solubilization of the enzyme by Triton X-100 is performed in the presence of 60 m*M* NaCl, the recovery is quantitative and the concentration of detergent required to initiate the solubilization ranges between 0.02 and 0.5% (v/v) (Table 1 expt. 2). In the presence of 0.1% Triton X-100, increasing the concentration of NaCl results in an almost complete solubilization of the enzyme (Table 1 expt. 3). This effect is not limited to NaCl, but is shared by other salts and the degree of solubilization is dependent not only on the ionic strength,

TABLE 1
Solubilization of Galactosyltransferase with Triton X-100 and Salts[a]

Expt. No.	Solubilization conditions	nmol/min, Pellet	Supernatant	Total
1	—	—	—	46
	Distilled water	46	0	46
	Tritron X-100 0.01%	45	0	45
	Tritron X-100 0.02%	46	0	46
	Tritron X-100 0.03%	32	2	34
	Tritron X-100 0.05%	20	10	30
	Tritron X-100 0.1 %	16	14	30
	Tritron X-100 2 %	10	12	22
2	—	—	—	39
	Distilled water	39	0	39
	NaCl 60 m*M*	40	0	40
	NaCl 60 m*M* + Triton X-100 0.01%	31	2	33
	NaCl 60 m*M* + Triton X-100 0.03%	20	10	30
	NaCl 60 m*M* + Triton X-100 0.05%	12	26	38
	NaCl 60 m*M* + Triton X-100 0.1 %	11	27	38
3	—	—	—	46
	Distilled water	47	0	47
	NaCl 500 m*M*	46	0	46
	Triton X-100 0.1%	17	11	28
	Triton X-100 0.1% + NaCl 30 m*M*	17	11	28
	Triton X-100 0.1% + NaCl 60 m*M*	14	31	45
	Triton X-100 0.1% + NaCl 100 m*M*	14	33	47
	Triton X-100 0.1% + NaCl 500 m*M*	13	34	47
4	—	—	—	40
	Distilled water	39	0	39
	Triton X-100 0.1%	13	10	23
	Triton X-100 0.1% + NaCl 30 m*M*	14	10	24
	Triton X-100 0.1% + KCl 30 m*M*	14	10	24
	Triton X-100 0.1% + $MnCl_2$ 10 m*M*	7	32	39
	Triton X-100 0.1% + $MgCl_2$ 10 m*M*	6	31	37

[a]Golgi membranes of rat ventral prostate were centrifuged at 105,000g_{av} for 60 min at 4°C and the pellet was resuspended in 25 m*M* sodium cacodylate buffer, pH 7.4, at a final protein concentration of about 1 mg/mL; 0.5 mL aliquots were mixed with Triton X-100, NaCl, KCl, $MnCl_2$, and $MgCl_2$ to give the final concentration stated. The change in volume (15% max.) was compensated by suitable amount of distilled water. After an incubation of 30 min at 4°C, the tubes centrifuged at 105,000g_{av} for 1 h. The supernatants were collected and the pellets were resuspended in 0.5 mL of buffer. These fractions were assayed in the standard assay system taking into account the Triton X-100 and $MnCl_2$ concentration of the samples. NaCl, KCl, and $MgCl_2$ at the final concentration to which the enzyme was diluted in the assay do not significantly effect the reaction.

but also on the net charge of each salt: NaCl = KCl < $MgCl_2$ = $MnCl_2$. (Table 1, expt. 4).

Purification by Chromatography on Hen Ovomucoid Sepharose

Because of a limited supply of Golgi membrane material, the procedure was used only on small batches of material. All steps in the purification procedure were performed at 4°C. Golgi membrane protein (3 to 5 mg) was suspended in 5 mL of 25 m*M* sodium cacodylate buffer containing 0.5% Triton X-100, 25 m*M* $MnCl_2$, and 0.1 m*M* UMP, pH 7.4. After 30 min agitation at 4°C the suspension was centrifuged at 105,000g_{av} for 60 min. Ninety percent of the galactosyltransferase activity was recovered in the supernatant. The solubilized enzyme obtained by this detergent salt extraction was used. The purification was achieved by affinity chromatography on hen ovomucoid sepharose. This ligand has been successfully used to purify soluble galactosyltransferase from rat ventral prostate (*20*). The supernatant was loaded on the column and the chromatography carried out as described in the Materials and Methods section. The results of a typical experiment are shown in Fig. 1 and Table 2. The degree of purification of the membrane-

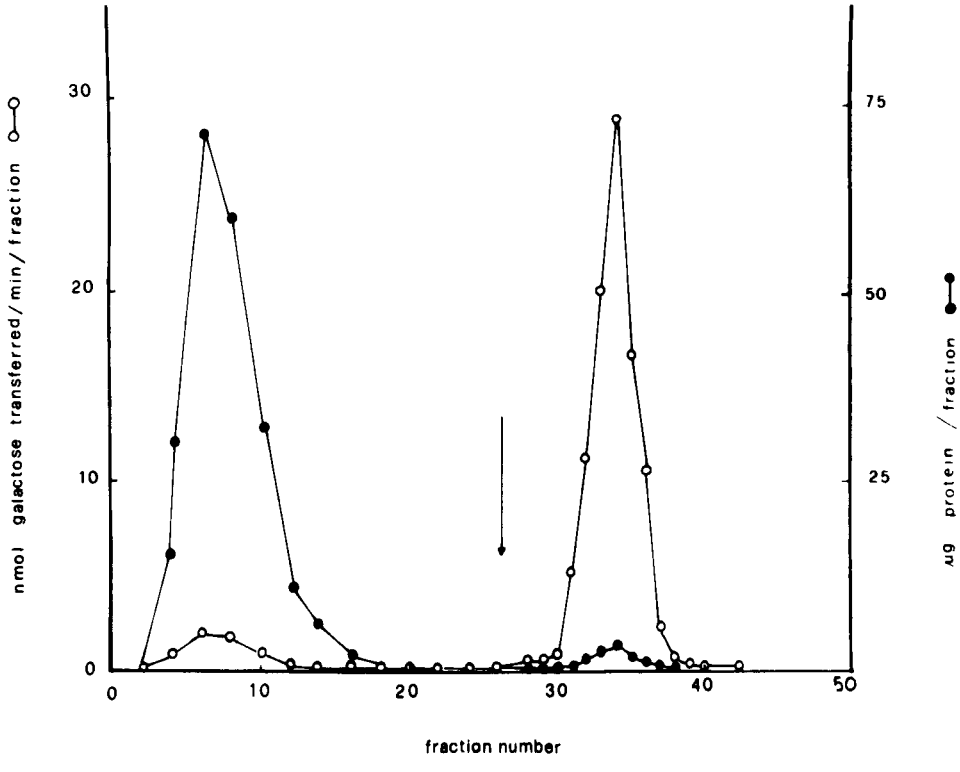

Fig. 1. Purification of galactosyltransferase on hen ovomucoid–sepharose. The solubilized enzyme was applied to a column of hen ovomucoid–sepharose (1.6 × 7 cm). The column was washed with equilibration buffer and eluted (arrow) with elution buffer containing 5 m*M* *N*-acetylglucosamine, 25 m*M* EDTA. Details of the column conditions are given in the Materials and Methods section. Enzyme activity (◯) and protein (;m$DL) were measured as described in the Materials and Methods section.

TABLE 2
Purification of Galactosyltransferase from Golgi Membranes of Rat Ventral Prostate

Step	Protein mg	Total units, nmol/min	Spec. act., nmol/min/mg	Purification From homogenate	Purification From Golgi	Yield, %
Homogenate	1253	1437	1.15	1	—	—
Golgi	4.3	345	80.2	69.7	1	100
Triton X-100	2.57	312	121.4	105.6	1.52	90.4
Ovomucoid-column	0.024	240	10000	8696	125	69.5

derived enzyme obtained by the hen-ovomucoid sepharose column is very high (8000-fold purification compared to the homogenate) and the specific activity (10,000 U/mg) is similar to that of enzyme purified to homogenity from other sources (*3*).

Molecular Properties of the Purified Galactosyltransferase

Analytical Gel Filtration. The molecular size of the purified enzyme was investigated by analytical gel filtration. When the enzyme was run on a column of Sephadex G-100 in the absence of Triton X-100 in the equilibration and elution buffer, it was found to have high tendency to aggregate in a specie that was excluded from the column. Gel filtration of the enzyme in the presence of 0.05% v/v Triton X-100 on calibrated column gave a major activity peak centrated on approx. 70,000 daltons with a broad shoulder between 60,000 and 50,000 daltons (Fig. 2). Since both peak and shoulder had about equal specific activity, these results suggest that the galactosyltransferase consists of different active molecular weight forms.

SDS Polyacrylamide Gel Electrophoresis. The purified enzyme is resolved by the SDS polyacrylamide gel electrophoresis into three components. Comparison of the mobilities of these components with those of standard proteins of known sizes gives apparent molecular weights of about 74,000, 60,000, and 54,000 daltons.

Isoelectric Focusing. Isoelectric focusing of the purified galactosyltransferase was carried out with a pH range from 3.5 to 9.5. The results are shown in Fig. 3. Five distinct peaks of activity were resolved with pHi of 9, 7.4, 6.75, 6.1, and 4.8.

Properties of the Purified Galactosyltransferase. The enzyme in 25 m*M* sodium cacodylate buffer containing 0.02% (v/v) Trition X-100, 0.1 *M* NaCl, pH 7.4 was stable for at least several months when stored at −20°C.

The activity in the standard assay was proportional with time up to 2 h and with enzyme concentration.

Addition of ATP, CDP-choline, and GDP-fucose to the assay mixture did not cause any increase in transferase activity; a direct measurement also showed that the purified preparation was totally free of contaminating UDP-galactose pyrophosphatase (*24*).

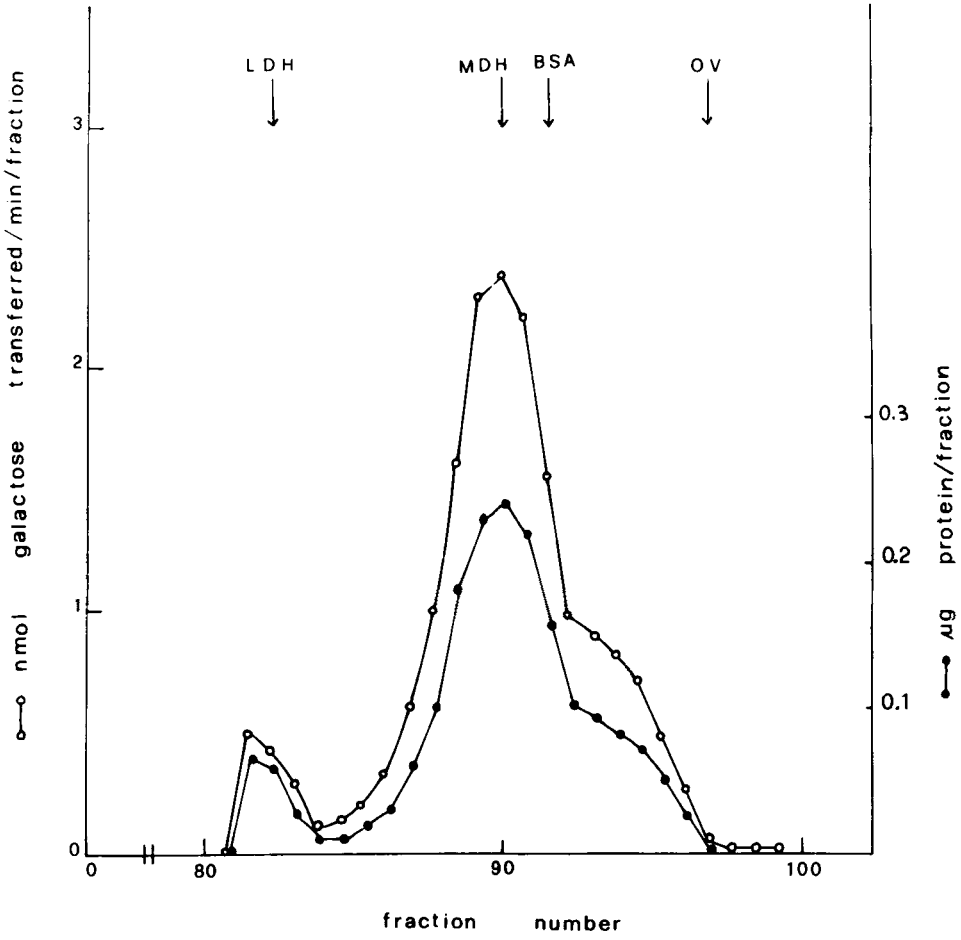

Fig. 2. Gel filtration of purified galactosyltransferase on Sephadex G-100. The concentrated enzyme from hen ovomucoid–sepharose column was chromatographed as described in the Materials and Methods section. The arrows indicate elution positions corresponding to molecular weight markers (lactate dehydrogenase, malate dehydrogenase, bovine serum albumin, ovalbumin). Fractions were collected and assayed for galactosyltransferase (○) and protein (●) as indicated in the Materials and Methods section.

The enzyme showed a relative broad pH optimum with a maximum at pH 6.8. With hen ovomucoid as galactose acceptor, the apparent K_m for UDP-galactose was 11 μM. The reaction is dependent on the presence of an acceptor. The K_m of galactosyltransferase was 7.6×10^{-5} *M* for ovalbumin, 2.9×10^{-5} *M* for desialized and degalactosylated fetuin (25) and 8.9×10^{-6} *M* for hen ovomucoid. The K_m for free *N*-acetyl glucosamine was 6×10^{-3}. Dialysis of the purified enzyme added with 10 m*M* EDTA rendered the enzyme totally dependent of $MnCl_2$ addition. The enzyme activity is maximum at 10 m*M* Mn^{2+}.

Discussion

These results show that a simple and rapid method enables us to isolate a highly purified galactosyltransferase solubilized from rat ventral prostate Golgi mem-

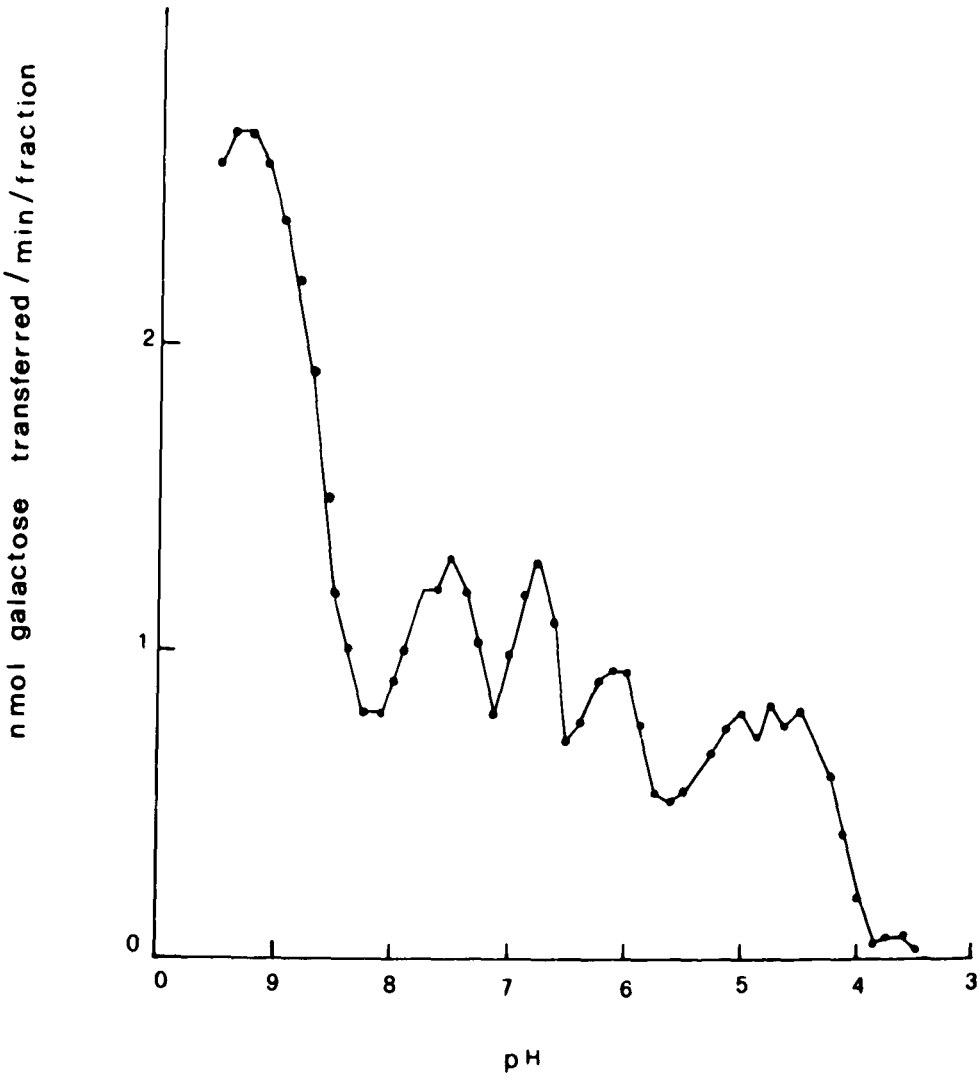

Fig. 3. Analytical isoelectric focusing of purified galactosyltransferase. The concentrated enzyme from hen ovomucoid–sepharose column was applied to a polyacrylamide gel slab, pH 3.5–9.5 gradient. After the run the gel was sliced and the determination of galactosyltransferase activity from gel fractions was carried out as described in the Materials and Methods section.

branes. This purification relies upon solubilization of the membrane-bound enzyme by a detergent salt procedure (*26, 27*). The enzyme, which appears to be an intrinsic membrane component, not being solubilized by extraction with salt or with EDTA, is apparently released into the supernatant by treatment of Golgi membranes by various concentration of Triton X-100. Unfortunately the solubilization of the enzyme is associated with a decreased recovery of activity. Ionic strength seems to be an important factor in the solubilization of the membrane-bound enzyme by Triton X-100. The presence of the salt results in a very good recovery. The requirement of salt for the efficient extraction of the enzyme may indicate that this galactosyltransferase is bound to the membrane by

both hydrophobic and ionic interactions. High concentration of salt probably weakens the aspecific binding of detergent-solubilized enzyme with lipid–detergent micelles. This hypothesis is supported by the basic nature of the most prominent isoelectric form of galactosyltransferase solubilized from rat ventral prostate membrane, which may be expected to interact with the charged head group of acidic phospholipids. The presence of salt furthermore may counteract the tendency of solubilized and partially purified preparations of galactosyltransferases to aggregate with apparent loss of activity (*27*).

The purification of the solubilized enzyme is achieved by an affinity chromatography procedure we have recently developed for the purification of soluble form of the enzyme from rat ventral prostate (*20*). The chromatography on hen ovomucoid sepharose is very effective in the purification of the membrane derived enzyme. The specific activity of the purified enzyme (10,000 U/mg) is closely similar to that previously observed for pure galactosyltransferase solubilized from Golgi membrane of lactating mammary glands (16,000 U/mg) (*3*). The high purification of the enzyme obtained with only one pass on the column probably results from the type of ligand used. Many galactosyltransferases have been described that are distinguished by their specificities towards different acceptors (*28, 29*). The choice of this macromolecular acceptor as a ligand allows the specific adsorption of the UDP-galactose ovomucoid galactosyltransferase. Hen ovomucoid is substrate also of fucosyltransferases of rat ventral prostate (*21*), but these enzymes do not adsorb to the column when $MnCl_2$ is present in the medium (B. Tadolini and G. Hakim, unpublished observation). The purified enzyme appears heterogeneous in size as shown by the electrophoretic analysis in SDS. The gel shows that the enzyme contains at least three different species with apparent molecular weights of 74,000, 60,000, and 54,000 daltons. The identification of these three protein components as forms of galactosyltransferase is supported by the high specific activity of this preparation, as well as the observation of the distribution of the galactosyltransferase activity on gel filtration on Sephadex G 100. This conclusion is in agreement also with the heterogeneous isoelectric distribution of the purified enzyme. Charge heterogeneity of purified soluble galactosyltransferase from milk, amniotic fluid, and malignant ascites has been described (*19*), but the different forms were acidic in nature. Most of the activity of the purified membrane-derived galactosyltransferases was, on the contrary, associated with the basic forms. The structural basis of this gross charge difference between the membrane-derived and the secretion-derived enzyme could be ascribed to different causes: (a) deamination of glutamine and asparagine is likely to take place while the enzyme resides in secretions and body fluids; (b) Golgi membrane-derived enzyme has been shown to be considerably larger than the most intact soluble form of enzyme (*2–4*). It has been reported that the use of different ligands in the affinity chromatography of galactosyltransferase has some effect on the extent of concomitant proteolytic breakdown of bovine enzyme (*29*). The specific ligand used for this purification of membrane-derived galactosyltransferase is hen ovomucoid, a well known trypsin inhibitor, which might protect the enzyme from proteolytic attack at the level of basic amino acids.

It is interesting to note the similarities between this membrane-bound enzyme and the cancer-associated galactosyltransferase studied by Podolsky (*8*). The cancer enzyme in fact not only has a high molecular weight, but also a basic electrophoretic mobility. It is tempting to speculate that a relationship between these enzymes exists. It is possible that the cancer enzyme may be either an incomplete cleavage product of the membrane-bound enzyme or a precursor that could not enter the membrane because of a failed/saturated insertion/anchor machinery.

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