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Hen Ovomucoid-Agarose: A New Conjugate for the Isolation by Affinity Chromatography of UDP-Galactose:Glycoprotein Galactosyl-Transferase

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Abstract

A new conjugate for the affinity chromatography of UDP-galactose:glycoprotein galactosyltransferase has been synthesized by coupling hen ovomucoid, a ligand similar to the acceptor substrate, to agarose.

The hen ovomucoid–Sepharose conjugate binds galactosyl transferase more tightly that other acceptor–Sepharose conjugates.

The new adsorbent gives comparable yields and purifications with those obtained by ligands similar to the nucleotide moiety of the substrate and to the "specifier" protein, α-lactalbumin.

The soluble galactosyltransferase from rat ventral prostate is effectively removed from the high speed supernatant by an ovomucoid–Sepharose column. The enzyme can be eluted with buffer containing EDTA and *N*-acetylglucosamine in a high yield (75–80%) and in a purified form (4000-fold purification). The stability of ovomucoid to heat and to high concentrations of urea and its inhibition of some proteases makes the conjugate easy to operate with and quite useful even with rather crude preparations.

Index Entries: Hen ovomucoid–agarose; ovomucoid, hen; agarose, conjugate with hen ovomucoid; affinity chromatography, of UDP–galactose:glycoprotein galactosytransferase; galactosyltransferase, binding to ovomucoid–Sepharose.

Introduction

Galactosyltransferase catalyzes the β 1-4 transfer of galactose from UDP-galactose to *N*-acetylglucosamine (either in free form or present terminally in the

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oligosaccharide moieties of glycoproteins) or to glucose. The latter reaction is favored when galactosyltransferase interacts with α-lactalbumin (*1*).

Purification of galactosyltransferase has been achieved by affinity chromatography on column of α-lactalbumin–Sepharose conjugate (*2,3*).

Specific adsorbents with functional groups resembling the nucleotide moiety of the substrate have been prepared and successfully used for the isolation of the enzyme (*4*).

Ligands structurally related to the acceptor substrate have also been used (*4,5*).

However, the enzyme shows a low affinity for *N*-acetylglucosamine–Sepharose probably because of its high apparent K_m for this acceptor.

The K_m values for glycoprotein acceptors are considerably lower, thus the enzyme should be tightly bound to an agarose column to which the specific macromolecular acceptors are coupled. Ovalbumin–Sepharose has been proposed (*5*), but has not proved particularly advantageous on other methods; furthermore, the instability of ovalbumin to heat, ultraviolet irradiation, shaking, and to the action of urea (*6*) discouraged its wide-spread utilization.

These considerations prompted us to select another glycoprotein acceptor as ligand for the isolation by affinity chromatography of galactosyltransferase.

Materials and Methods

Male Wistar strain rats obtained from the departmental animal house were used. The ventral prostates, dissected free of connective tissue and fat, were blotted, finely minced, and then homogenized at 4°C in 25 m*M* sodium cacodylate buffer, pH 7.4, containing 25 m*M* $MnCl_2$, 10 m*M* mercaptoethanol, and 0.5 m*M* UMP. The homogenate was centrifuged at 105,000*g* for 60 min; the galactosyltransferase in the supernatant fluid is considered soluble.

UDP–galactose:glycoprotein galactosyltransferase was assayed with minor modifications of a previously described method (*7*). The standard assay contained in a final volume of 0.1 mL:50m*M* Mops,† pH 6.8; 10m*M* $MnCl_2$; 1 mg hen ovomucoid (trypsin inhibitor type II-0, Sigma Chemical Co.); 0.1m*M* UDP-(^{3}H)galactose (5×10^4dpm) (New England Nuclear Co.); 0.1% (v/v) Triton X-100.

N-acetylglucosamine–Sepharose was prepared according to Vretblad (*8*) using epoxy-activated Sepharose 6B from Pharmacia. The conjugate of Sepharose and hen ovomucoid was prepared following the general method of Porath et al. (*9*) and Axén et al. (*10*) using CNBr-activated Sepharose from Pharmacia. The ligand con centrations were 3.5 μmol *N*-acetylglucosamine and 2.5 mg ovomucoid mL/wet gel.

Proteins were precipitated by additon of TCA in a final concentration of 10% (w/v). Protein concentration was determined by the method of Lowry et al. (*11*) using serum albumin as the standard.

†Abbreviations used: Mops, morpholinopropanesulfonic acid; TCA, trichloroacetic acid.

Results and Discussion

Properties of Crude Soluble UDP–Galactose:Glycoprotein Galactosyltransferase from Rat Ventral Prostate

It has been shown that, among the large number of adult rat tissues examined the ventral prostate exhibited a very high glycoprotein galactosyltransferase activity, which was second only to that of lactating mammary gland (*7*). Although most of the intracellular galactosyltransferase in the prostate appears to be firmly associated with cytoplasmic membranous structures, a large amount of the enzyme is also present in a soluble and non-membrane-bound form (*12*).

This crude soluble galactosyltransferase shows an absolute requirement for Mn^{2+} that cannot be replaced by Mg^{2+}, Ca^{2+}, Zn^{2+}, or Co^{2+} (Table 1).

The enzyme activity is maximum at 10 m*M* Mn^{2+}; 10 m*M* EDTA inhibits the enzyme in the presence of Mn^{2+}. The addition of CDP–choline does not have any effect on the reaction suggesting the absence in the high speed supernatant of rat ventral prostate of appreciable amounts of UDP–galactose pyrophosphatase (*13*). The stimulation by Triton X-100 is very low, and it becomes negligible if compared with the manifold stimulation exerted by the detergent on the membrane-bound enzyme (*14,15*).

The galactosyltransferase activity is proportional with time up to 1 h and with enzyme concentration up to 30 ng of protein in 0.1 mL with or without Triton X–100 in the reaction mixture.

The apparent K_m for ovomucoid was $8.9 \times 10^{-6} M$. Addition of *N*-acetylglucosamine to the assay system results in a strong inhibition of transfer of galactose to ovomucoid owing to competition of these two acceptors for the active site of the enzyme (Table 1). Hen ovomucoid exhibits then a specific and reversible

TABLE 1
Properties of Crude Soluble UDP–Galactose:Glycoprotein Galactosyltransferase for Rat Ventral Postate

Requirements	Enzyme activity[a], pmol galactose transferred/min/mg
Complete	66.7
$-Mn^{2+}$	1.4
$-Mn^{2+} + Mg^{2+}$ (10m*M*)	5
$-Mn^{2+} + Ca^{2+}$ (10m*M*)	7
$-Mn^{2+} + Zn^{2+}$ (10m*M*)	2
$-Mn^{2+} + Co^{2+}$ (10m*M*)	1.1
+EDTA (10m*M*)	0
+CDP–choline (1m*M*)	68.2
−Triton X-100	61.7
+*N*-acetylglucosamine (2m*M*)	52.7
+*N*-acetylglucosamine (8m*M*)	33.4

[a]Enzyme activity was measured by the method described under Materials and Methods.

binding affinity for the galactosyltransferase and can be used as a good biospecific ligand for affinity chromatography of the enzyme.

Chromatography on Hen Ovomucoid–Sepharose of Soluble Galactosyltransferase

The proposal of hen ovomucoid as a ligand for the isolation of galactosyltransferase by affinity chromatography has been tested by linking the commercially available ovomucoid to CNBr-activated Sepharose 4B

The choice of this glycoprotein has been dictated by, besides the high affinity of the enzyme for it, the convenience of having a commercially available acceptor that can be used without any need for prior chemical and/or enzymic treatments (*15,16*) to uncover sufficient amounts of appropriate terminal sugar moieties to serve as acceptors.

Hen ovomucoid–Sepharose is very stable and no detectable ovomucoid is lost from the column on repeated use. Moreover, because of the unusual stability of ovomucoid to denaturation by heat and high concentrations of urea in neutral and acidic solutions (*16*), ovomucoid–Sepharose can withstand accidents in operation and storage and under drastic conditions of regeneration without apparent loss in binding capacity.

Lipids and other contaminants adhere to ovomucoid–Sepharose limiting its use (*17*). To minimize this interaction, Triton X-100 was added to the high speed supernatant in a final concentration of 0.2% (v/v) before being applied to the column. This concentration of detergent has no inhibitory effect on the enzyme and ensures a constant performance of the adsorbent.

The soluble galactosyltransferase obtained from 1.5 g of rat ventral prostate was applied to a column (1.6 × 7 cm) of ovomucoid–Sepharose equilibrated at 4°C with 25 m*M* sodium cacodylate, pH 7.4, containing 25 m*M* $MnCl_2$, 10 m*M* mercaptoethanol, 0.5 UMP, and 0.02% (v/v) Triton X-100.

The column was washed with the equilibration buffer until the absorbance at 280 nm indicated that essentially all the inert protein had passed through. The galactosyltransferase was eluted as a sharp peak at the front of the developing buffer (decrease in the absorbance at 254 nm and increase in the drop size) containing sodium cacodylate, pH 7.6, 10 m*M* mercaptoethanol, 25 m*M* EDTA and 5 m*M* *N*-acetylglucosamine.

For comparison, an equal amount of galactosyltransferase was applied to a column (1.6 × 7 cm) of *N*-acetylglucosamine–Sepharose that was equilibrated, washed, and eluted in a similar manner. The results obtained by these two procedures are given in Table 2 and Fig. 1.

The hen ovomucoid–Sepharose affinity column effectively adsorbs galactosyltransferase, showing a large capacity for the enzyme, comparable with that obtained by α-lactalbumin or UDP–hexanolamine affinity columns (70–80% yield) (*2–5,18*). On the contrary, the *N*-acetylglucosamine affinity column, which is functionally equivalent and directly comparable, binds the enzyme poorly (Table 2) as already reported (*4*).

TABLE 2
Isolation of Soluble UDP–Galactose:Glycoprotein Galactosyltransferase by Affinity Chromatography on Hen Ovomucoid–Sepharose or *N*-acetylglucosamine–Sepharose[a]

Stage	Volume, mL	Protein, mg/g tissue	Total activity, nmol galactose transferred/min/ g tissue	Yield %	Specific activity, nmol galactose transferred/min/ mg protein	Purification
Homogenate	10	62.3	73	—	1.172	—
High speed supernatant	9.5	46.7	3.66	100	0.078	1
Hen ovomucoid–Sepharose	7	0.0092	2.93	80	318.5	4083
N-acetylglucosamine–Sepharose	21	0.1785	1.54	42	8.61	110

[a]The results are taken from one typical preparation. The procedure used to obtain each fraction is described under Materials and Methods. Protein determinations and galactosyltransferase assays were conducted as described under Materials and Methods.

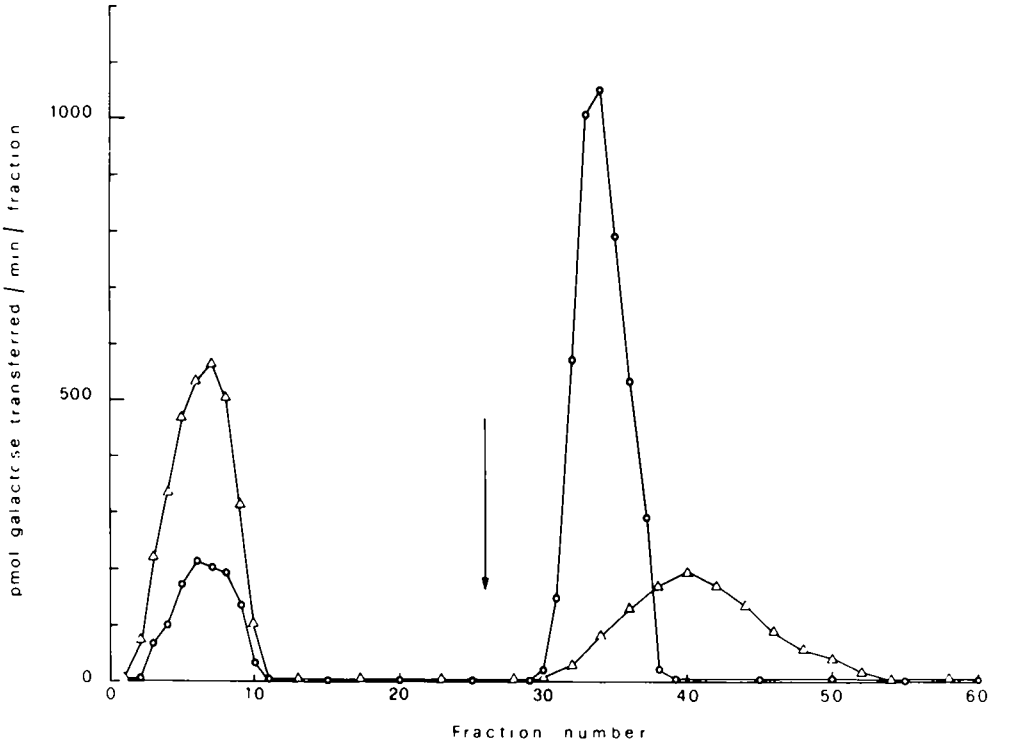

Fig. 1. Chromatography of soluble UDP–galactose:glycoprotein galactosyltransferase on hen ovomucoid–Sepharose or *N*-acetylgluocosamine–Sepharose. An equal amount of soluble galactosyltransferase was applied to each column. The columns were washed with equilibration buffer and eluted (arrow) with elution buffer containing 5 m*M* *N*-acetylglucosamine and 25 mM EDTA. The enzyme activity was measured as described under Materials and Methods: ○—○, chromatography of galactosyltransferase on ovomucoid–Sepharose; △—△, chromatography of galactosyltransferase on *N*-acetylglucosamine.

The enzyme can be eluted in a reasonably concentrated eluate with a buffer containing *N*-acetylglucosamine and EDTA and it is not necessary to add urea to the desorbing agents or to change buffer (sodium tetraborate, pH 8.5) to overcome the extreme dilution of the enzyme resulting from the affinity chromatography on UDP–hexanolamine and *N*-acetylglucosamine–Sepharose columns (*4*).

The degree of purification obtained by this new affinity column is very high (4000-fold purification compared with the high speed supernatant).

Differences in the enzyme sources and in the preliminary treatments used by the various authors prior to the chromatography on other conjugates (*2,4,5,18,19*) make it difficult to compare our results quantitatively with those in the literature. However, direct comparison with *N*-acetylglucosamine column clearly shows how more effective the new specific adsorbent is. The consideration that one pass through the ovomucoid column yields an enzyme with a specific activity only one order of magnitude lower than that of pure bovine and human milk

galactosyltransferase (*2,3*) even more emphasizes the usefulness of this adsorbent for the purification of UDP–galactose:glycoprotein galactosyltransferase.

The choice of ovomucoid as the specific ligand for the purification of galactosyltransferase is of interest in another aspect. Hen ovomucoid is an inhibitor of trypsin and, when contaminated by ovoinhibitor, of chymotrypsin (*16*); thus it is not attacked by proteolytic enzymes of these two families. This also allows the use of affinity chromatography at early stages of enzyme purification, the danger for the ligand being minimal.

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