Affinity Chromatography of Soluble Galactosyltransferases Using an Easily Synthesized N-Acetylglucosamine-Agarose Bead[1]

E. G. BERGER[2], M. M. WEISER and K. J. ISSELBACHER

Department of Medicine, Harvard Medical School and Gastrointestinal Unit, Massachusetts General Hospital, Boston (Massachusetts 02114, USA), 11 December 1975.

Summary. Agarose derivatized with *p*-nitrophenyl-N-acetyl-β-D-glucosamine was used for affinity chromatography for soluble galactosyltransferase from various sources. Moreover, this adsorbant acts as an acceptor for galactose when incubated with prepurified galactosyltransferase.

Work by BARKER et al.[3] has previously shown that N-acetylglucosamine linked to agarose by a hexanolamine spacer specifically adsorbs galactosyltransferase. The linkage of this spacer to the adsorbing sugar and then to CNBr-activated agarose involved complicated chemical synthetic steps. The adsorbing beads, e.g., N-acetylglucosamine-agarose, had also been shown to serve as an acceptor for galactose using their purified galactosyltransferase from whey[3].

Recently BLOCH and BURGER[4] described a new method for covalently attaching sugars to agarose beads which is rapid and simple. In a reduction step, *p*-nitrophenyl-derivatives of various sugars were used to produce an agarose bead linked to a sugar by an aminophenyl group. We have found the N-acetylglucosamine:agarose beads made by this method were useful in the purification of various soluble galactosyltransferases. These same beads have also been shown to act as galactose acceptors for purified galactosyltransferases.

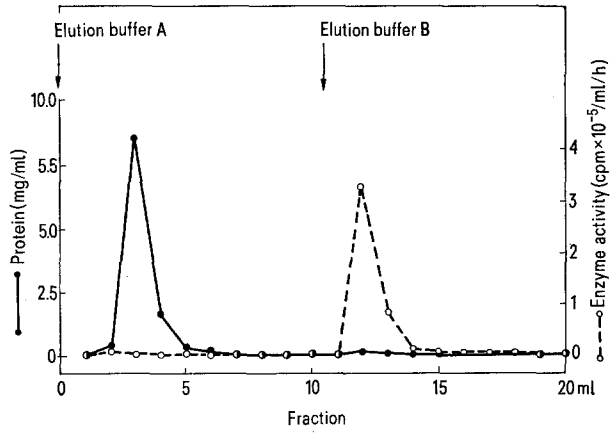

Fig. 1. Affinity chromatography of human serum galactosyltransferase on *p*-aminophenyl-N-acetyl-β-glucosamine: agarose. Elution buffer A: Na-cacodylate 0.1 *M*; $MnCl_2$ 0.025 *M*; UMP 0.0005 *M*. Elution buffer B: Na-cacodylate 0.1 *M*; NaCl 0.04 *M*; urea 1.5 *M*; N-acetylglucosamine 0.005 *M*; EDTA 0.025 *M*; β-mercaptoethanol 0.005 *M*.

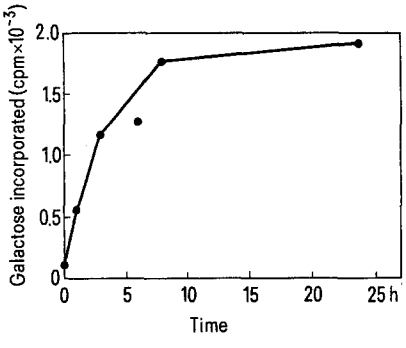

Fig. 2. Time dependency of galactosylation of *p*-aminophenyl-N-acetyl-β-D-glucosamine: agarose beads by partially purified fetal calf serum galactosyltransferase. The assay mixture contained in a final volume of 0.12 ml; 50 µl partially purified fetal calf serum galactosyltransferase, stabilized with BSA (1 mg/ml); 0.154 *M* NaCl; 0.1 *M* Na-cacodylate pH 7.4; 0.025 *M* $MnCl_2$; 4.8 µ*M* UDP-[1-^{3}H]galactose (1.17 Ci/mmole); 25 µl adsorbant acceptor.

Materials and methods. CNBr-activated agarose was purchased from Pharmacia Fine Chemicals, *p*-nitrophenyl derivatives of N-acetylglucosamine and galactose were from Sigma Chemical Company and UDP-D-[1-^{3}H] galactose (1.17 Ci/mmole) and UDP-D-[U-^{14}C] galactose (258 mCi/mmole) were from New England Nuclear Company. Fetuin (Spiro method) was obtained from Gibco.

The specific affinity adsorbents were made according to BLOCH and BURGER[4]. Estimation of the amount of sugar bound to agarose was performed by measuring the difference in adsorbance at 290 nm in the supernatant before and after coupling. The molar extinction for the free *p*-aminophenyl-N-acetyl-β-D-glucosamine was determined to be 0.282×10^3. The final concentration of ligand was usually around 1 µmole/ml of wet agarose.

Galactosyltransferase assay. The standard assay mixture with a total volume of 0.1 ml contained 0.1 *M* Na cacodylate, pH 7.4, 25 m*M* $MnCl_2$, 6.8 µ*M* UDP-D-[1-^{3}H] galactose and 0.2 mg of the glycoprotein acceptor fetuin from which the terminal sialic acid and penultimate galactose had been removed (SGF-fetuin) as previously described[5]. Incubation was performed for 1 h at 37 °C and the reaction stopped by adding 1 ml of ice-cold 5% phosphotungstic acid in 2 *N* HCl. The precipitate was filtered and washed with ice-cold ethanol and the activity counted on filters in scintillation vials as previously described[6].

In experiments using adsorbent as acceptor for galactose, the packed adsorbent was diluted 1:1 with 0.154 *N* NaCl, suspended evenly and pipetted into the assay tubes. The assay was carried out in a shaking water bath and stopped by adding ice-cold 0.1 *M* NaCl, centrifuged, decanted, washed twice and then poured over glass fibre filters.

Product identification. The adsorbing beads were labeled by adding 8.7 µ*M* UDP-D-[U-^{14}C] galactose to the same assay as described above, and the beads were eluted from the glass fibre filters into water by vigorous

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The use of N-acetylglucosamine: agarose as an acceptor in the galactosyltransferase reaction

	Galactose incorporated (cpm/3 h)
Complete system [a]	1154
Minus enzyme	42
Minus Mn^{2+}	111
Minus adsorbent + CNBr activated ethanolamine inactivated agarose	62
Minus adsorbent + *p*-aminophenyl galactose-agarose	87

[a] As described in Figure 2.

stirring overnight. The beads were subjected to hydrolysis with 2 *N* HCl at 100 °C for 4 h. The supernatant was deionized by passing over a mixed ion exchange resin (Biorad No. AG501-X8), the eluate lyophilized, redissolved in H_2O and chromatographed on Whatman No. 1 paper in 2 solvent systems: 1. butanol-pyridine-H_2O, 6:4:3, for 33 h, and 2. pyridine-ethylacetate-H_2O-acetic acid, 5:5:3:1, for 24 h. The paper strip containing the radioactive sample was cut into 2.5 cm wide pieces and put into scintillation vials for counting. The sugar standards, including N-acetylglucosamine, D-galactose, D-glucose and lactose were detected on the chromatograms by the alkaline-$AgNO_3$ reaction[7]. The label co-chromatographed in both systems with D-galactose. Protein was determined according to LOWRY et al.[8].

Result and discussion. Figure 1 shows the purification of serum galactosyltransferase from 0.2 ml of normal human serum by 1 ml of packed adsorbent. The elution buffers A and B (for composition cf. legend to Figure 1) are made according to BARKER. Fractions of 1 ml were collected and dialyzed against 0.1 *M* Na-cacodylate buffer, pH 7.4, before assaying for activity and protein concentration. The increase in specific activity varied between 50- and 150-fold by this single purification step. The recovery of activity was close to 100% when measured immediately after dialysis, in agreement with the almost complete depletion of galactosyltransferase activity from serum. The affinity column proved suitable for the purification of soluble galactosyltransferases from different sources such as fetal calf serum, calf serum and human amniotic fluid.

Labeling of the adsorbent. These experiments were carried out with partially purified galactosyltransferase from fetal calf serum (150-fold purified) suspended in bovine serum albumin (1 mg/ml). As demonstrated by TRAYER and HILL[9], the purified galactosyltransferase required bovine serum albumin (1 mg/ml) for stabilization of activity. As shown in Figure 2, the reaction with the beads as acceptor was linear up to 3 h. The Table demonstrates that the reaction, as with other acceptors, required Mn^{2+} and that agarose without the N-acetylglucosamine arm could not act as acceptor.

These data demonstrate that soluble galactosyltransferase can be purified by affinity chromatography with a system similar to that described by BARKER et al.[3] but using beads prepared by a simple and rapid method. BLOCH and BURGER[4] originally made these agarose beads for the purification of various lectins. The main advantage of this procedure lies in the rapidity of coupling the adsorbent to the activated agarose, taking advantage of the *p*-nitrophenyl group as a spacer group already attached to different sugars which are commercially obtainable and inexpensive.

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Abnormally Soluble Collagen Produced in Fibroblasts Cultures[1]

G. FRANCIS, PATRICIA V. DONNELLY and N. DI FERRANTE

Laboratories of Connective Tissue Research, Department of Biochemistry, Baylor College of Medicine, Houston (Texas 77030, USA), 17 December 1975.

Summary. Abnormally soluble collagen is synthesized in vitro not only by skin fibroblasts of Marfan patients but also by those of patients with Ehlers-Danlos type V and cutis laxa. The excessive solubility of collagen is corrected by the addition to the culture medium of a synthetic flavonoid, (+)-catechin.

In 1973 PRIEST, MOINUDDIN and PRIEST[2] demonstrated that cultured skin fibroblasts derived from patients affected by Marfan syndrome produced a collagen which was more soluble than normal. Although the molecular defect responsible for this excessive solubility was not identified, the authors concluded that the findings were important because they might lead to the clarification of the basic defect of the disease and might be useful for early and possibly prenatal diagnosis.

We report here that the synthesis of excessively soluble collagen is not a peculiarity of Marfan cultured fibroblasts because it occurs also in cultures of fibroblasts from patients affected respectively by the sex-linked form of Ehlers-Danlos (Type V)[3] and by the autosomal recessive form of cutis laxa. Moreover, the addition of a synthetic flavonoid capable of stabilizing collagen[4] to the culture medium of the various types of mutant fibroblasts studied decreased the abnormal solubility of their collagen, while it did not affect the solubility of the collagen synthesized by normal, control fibroblasts.

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