Purification of α 2,6-sialyltransferase from rat liver by dye chromatography

Udo STICHER, Hans J. GROSS and Reinhard BROSSMER*

Institut für Biochemie II, Universität Heidelberg, Im Neuenheimer Feld 328, 6900 Heidelberg, Federal Republic of Germany

We describe a simple three-step purification for Gal- β 1,4-GlcNAc- α 2,6-sialyltransferase (EC 2.4.99.1) from rat liver which uses chromatography on Cibacron Blue F3GA and f.p.l.c. It gives a highly purified (11 000-fold) enzyme in 19 % yield, which is free of other sialyltransferases, CMP-NeuAc hydrolase, sialidases and proteinases.

INTRODUCTION

Sialic acids in oligosaccharide chains of glycoconjugates are involved in a variety of recognition phenomena. Two particular aspects are of increasing interest. Resialylation of cell surfaces provided conclusive evidence that influenza virus binding is mediated by sialic acid in specific linkage [1], and desialylated plasma glycoproteins show drastically reduced half-lives in circulation [2]. Sialyltransferases, which catalyse the transfer of sialic acids from the respective CMP-glycoside on to soluble or membrane-bound glycoproteins, are an essential tool for resialylation studies. We have already shown that various synthetic sialic acid analogues, with particular biochemical properties, can be enzymically activated to CMP-glycosides and subsequently transferred on to glycoprotein acceptors [3,4]. Work on the preparation of glycoproteins specifically modified by sialylation with sialic acid analogues requires high activities of the respective purified enzymes. Up to the present time several sialyltransferases with different acceptor specificities have been purified by affinity chromatography on CDP-hexanolamine-Sepharose [5–11]. This procedure was restricted to only a few laboratories because of the laborious chemical synthesis of the affinity ligand [5], required in large amounts. For this reason a simple large-scale method for purification of α 2,6-sialyltransferase from rat liver acting on *N*-glycosidically linked glycans was developed, using dye chromatography and f.p.l.c. The procedure yields a highly purified enzyme preparation which is free of other sialyltransferase activities, proteinases, sialidases and phosphodiesterase.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), Sigma (München, Germany) or Bio-Rad (München, Germany). α -Acid glycoprotein and anti-freeze glycoprotein were generously given by Professor Karl Schmid (Boston, MA, U.S.A.) and Professor Robert Feeney (Davis, CA, U.S.A.) respectively. CMP-NeuAc was prepared as described previously [4]. CMP-

[3 H]NeuAc (11.5 Ci/mol) was from New England Nuclear. 3' and 6' isomers of sialyl-lactose were isolated from cow colostrum. Quickszint 212 for liquid scintillation counting was obtained from Zinsser (Frankfurt, Germany).

Sialyltransferase assay

Standard assay (100 μ l) contained 10 μ g of acceptor substrate, 50 μ g of bovine serum albumin and enzyme (0–0.4 munit) in 50 mM-sodium cacodylate, pH 6.5, with 0.1 % Triton CF-54. Transfer was initiated by addition of 10 nmol of CMP-[3 H]NeuAc (7000 c.p.m./nmol). As acceptor substrates were used asialofetuin (140 nmol of galactose sites/mg) and asialo- α -glycoprotein (430 nmol of galactose sites/mg), both desialylated by acid hydrolysis (0.2 M-HCl, 1 h at 80 °C), and antifreeze glycoprotein (1100 nmol of galactose sites/mg). Assay was processed as described previously [4]. The final sediment was dissolved in 100 μ l of 1 M-NaOH, neutralized, and its radioactivity counted by liquid-scintillation spectrophotometry (Packard TriCarb spectrograph). One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μ mol of product/min under standard assay conditions.

Sialyltransferase activity was determined with asialofetuin as acceptor substrate. Asialo- α -acid glycoprotein served as substrate for *N*-glycan specificity, and antifreeze glycoprotein and fetuin for *O*-glycan specificity (for glycan structures see review [12]). All assays were performed within linear limits of time and amounts of enzyme by restricting the consumption of either substrate to less than 15 %.

For calculation of the specific activity and the purification factor, enzyme activity, measured in the standard assay, was multiplied by a factor of 2.0 to reflect maximal velocity obtained at saturating concentrations of CMP-NeuAc (1 mM) and asialo- α -acid glycoprotein (1.3 mM).

Assays for kinetics were performed in duplicate using 0.125 munit of α 2,6-sialyltransferase. Asialo- α -acid glycoprotein was varied to give five concentrations (0.15–1.6 mM in terms of galactose sites) while CMP-NeuAc was fixed at 0.8 mM; CMP-NeuAc was varied to give five concentrations (20–200 μ M) while asialo- α -acid glycoprotein was fixed at 1.3 mM in terms of galactose

* To whom correspondence should be addressed.

sites. Consumption of the limiting substrate was kept below 12%. Kinetic parameters were calculated from Hanes plots by linear-regression analysis [13].

Detection of α 2,3-sialyltransferase

Reaction mixtures (100 μ l) contained 0.13 M-lactose as substrate, 1 nmol of CMP-[3 H]NeuAc (130 000 c.p.m./nmol), and 8 munits of sialyltransferase preparation in 50 mM-sodium cacodylate, pH 6.5, with 0.1% Triton CF-54. Lactose concentration was saturating for α 2,3-sialyltransferase (K_m 9.4 mM) and was at the K_m value for α 2,6-sialyltransferase (K_m 129 mM) [14]. The reaction was allowed to proceed at 37 °C for 45 min. A mixture of pure 3'- and 6'-sialyl-lactose isomers (each at 1 mM) was added to the assay and samples (25 μ l) were analysed by h.p.l.c. operating at 200 nm as described previously [4]. Peaks of 3'- and 6'-sialyl-lactose were collected and incorporation was calculated by liquid-scintillation counting.

Determination of CMP-NeuAc hydrolase

Incubation mixture (400 μ l) contained 50 mM-sodium cacodylate, pH 6.5, 0.1% Triton CF-54, 340 nmol of CMP-NeuAc, and 4 munits of sialyltransferase preparation. After appropriate time periods (0–3 h), at 37 °C, samples (40 μ l) were analysed by h.p.l.c. at 275 nm for the amount of CMP-NeuAc and CMP as described previously [4].

Protein determination

The Bio-Rad Protein Assay was applied using bovine serum albumin as standard.

Buffers

The following buffers were used: A, 10 mM-sodium cacodylate (pH 6.5)/0.15 M-NaCl/25% glycerol/0.1% Triton CF-54; B, 10 mM-sodium cacodylate (pH 6.5)/75 mM-NaCl/25% glycerol/0.1% Triton CF-54; C, 10 mM-sodium cacodylate (pH 6.5)/115 mM-NaCl/25% glycerol/0.1% Triton CF-54; D, 10 mM-sodium cacodylate (pH 5.3)/25 mM-NaCl/10% glycerol/0.1% Triton CF-54; E, 10 mM-sodium cacodylate (pH 6.5)/25 mM-NaCl/10% glycerol/0.1% Triton CF-54.

Purification of α 2,6-sialyltransferase

Step 1: Cibacron Blue F3GA–Sephacrose 6B (NaCl gradient). α 2,6-Sialyltransferase was extracted from 450 g of rat liver by the procedure of Weinstein *et al.* [8]. The Triton extract was applied to a column (15 cm $\times$ 5 cm) of immobilized Cibacron Blue F3GA (4.3 μ mol/ml) pre-equilibrated with 1.5 litres of buffer A (flow rate 340 ml/h) in two portions, with a washing step in between. The column was extensively washed with buffer A (4 litres) to remove inert protein.

Sialyltransferase was eluted using a linear NaCl gradient with 400 ml of buffer A as starting buffer and 400 ml of buffer A/2.5 M-NaCl as limit buffer. Fractions were assayed for transferase activity in the presence of NaCl, which reversibly inhibits the enzyme; they were pooled from 320 ml to 670 ml of effluent and dialysed against buffer B.

Step 2: Affi-Gel Blue (CDP gradient). The dialysed enzyme from step 1 was applied to a column (15 cm $\times$ 1.6 cm) of Affi-Gel Blue (Bio-Rad, 2 μ mol of Cibacron Blue F3GA/ml of gel) pre-equilibrated with 300 ml of buffer

B. The column was washed with buffer C (1 litre) and eluted by using a linear CDP gradient with 50 ml of buffer C as starting buffer and 50 ml of buffer C/10 mM-CDP as limit buffer. Fractions were assayed for sialyltransferase activity in the presence of 10 mM-MnCl₂ to reverse CDP inhibition of the enzyme [14].

Step 3: f.p.l.c. on Mono S column. Pooled fractions from step 2 were titrated to pH 5.3 and applied to a Mono S (Pharmacia, Freiburg, Germany) f.p.l.c. column (2 cm $\times$ 0.5 cm) pre-equilibrated with buffer D (flow rate 0.2 ml/min, 0.5 MPa). After washing with buffer E, the column was eluted with a linear CDP gradient (2 ml of buffer E as starting buffer and 2 ml of buffer E/10 mM-CDP as limit buffer) by using the Pharmacia f.p.l.c. system. Active fractions were pooled (6 ml total), dialysed against buffer E, titrated to pH 5.3, and applied to a Mono S f.p.l.c. column (1 cm $\times$ 0.5 cm). After washing with buffer E, the column was eluted with 2.5 M-NaCl in buffer E. Following dialysis against buffer E, glycerol was added to a final concentration of 50%, and sialyltransferase was stored at –20 °C.

Detection of contaminating proteases

Protease activity at pH 7.5 was determined in accordance with Twining [15], and at pH 4.2 in accordance with Williams & Lin [16].

Determination of contaminating sialidases

Sialidase assay was performed as described by Potier *et al.* [17].

Preparation of Cibacron Blue F3GA–Sephacrose

Cibacron Blue F3GA (Serva) was immobilized on Sepharose 6B (Pharmacia) as described by Dean & Watson [18], yielding a coupling efficiency of 4.3 μ mol of dye/ml of resin.

RESULTS AND DISCUSSION

Enzyme purification

Step 1: chromatography on Cibacron Blue F3GA–Sephacrose 6B. α 2,6-Sialyltransferase was partially purified and concentrated from approx. 4 litres of Triton extract to 370 ml by adsorption on Cibacron Blue F3GA–Sephacrose 6B (4.3 μ mol/ml), and subsequent elution with a linear NaCl gradient (Fig. 1). The overall yield in this step was 85% with a 23-fold purification. By collecting only fractions with activity of above 4 munits/ml, purification can even be increased to a total of 48-fold, though the yield is then decreased to approx. 65%. Contaminating α 2,3-sialyltransferase activity was below 0.2%, and neither proteinase nor sialidase was detected. Enzyme obtained from this step could be stored in the presence of 25% glycerol for at least 4 days at 4 °C with only 10% loss of activity. The immobilized dye was obtained by triazine coupling as described in the Materials and methods section. The chemical stability of the linkage between the Sepharose matrix and the dye ligand is superior to that introduced by CNBr coupling of CDP-hexanolamine [18]. As Cibacron Blue F3GA–Sephacrose is easy and cheap to prepare, the resin was discarded after two or three purification cycles. Thus each time the yield of sialyltransferase was kept at an optimal level. In contrast, because of its expensive synthesis, CDP-hexanolamine–Sephacrose had to be used

Fig. 1. NaCl gradient elution of $\alpha 2,6$ -sialyltransferase from Cibacron Blue F3GA–Sephrose 6B

Chromatography of Triton extracts obtained from 450 g of rat liver was performed as described in the Material and methods section. ----, NaCl gradient. Fractions were assayed for sialyltransferase ($\blacktriangle$) and protein ($\circ$). Enzyme activities obtained in the standard assay, as described in the Materials and methods section, were not corrected for saturating conditions.

for many purifications. Thus the capacity decreased continuously despite sophisticated regeneration procedures, as observed in this laboratory ([4]; U. Sticher, unpublished work).

Step 2: Affi-Gel Blue (CDP gradient). After rechromatography on Affi-Gel Blue, purification was considerably enhanced by specific elution with CDP. Sialyltransferase activity (about 85%) was eluted ahead of a broad protein peak (Fig. 2). The remainder of the adsorbed enzyme (about 15%) eluted with a 2.5 M-NaCl pulse together with the bulk of the protein.

The sialyltransferase was reversibly inhibited by CDP, as well as by NaCl; therefore the actual recovery of enzyme activity in both pools mentioned above was calculated after extensive dialysis, which separated it from CDP or NaCl respectively.

Step 3: Mono S f.p.l.c. column. Further purification and separation of protein from CDP was readily achieved by applying f.p.l.c. with a Mono S column (a strong cation-exchanger). Enzyme activity and protein eluted

Fig. 2. CDP gradient elution of $\alpha 2,6$ -sialyltransferase from Affi-Gel Blue

Dialysed enzyme from Cibacron Blue chromatography was rechromatographed as described in the Materials and methods section. Elution was achieved with a linear CDP gradient (----) of up to 10 mM, followed by elution with a 2.5 M-NaCl pulse. The end of the CDP gradient (10 mM) and the start of the NaCl pulse is indicated by an arrow. Fractions were assayed for sialyltransferase ($\blacktriangle$) and protein ($\circ$). Enzyme activities obtained in the standard assay were not corrected for saturating conditions; CDP inhibition was partially reversed by $MnCl_2$ as described in the Materials and methods section.

from Affi-Gel Blue adsorbed completely on the column at pH 5.3. Developing the column with buffer E removed inert protein, and about 45% of the enzyme activity applied to the column was obtained by using a linear CDP gradient. The activity peak spread from 1 mM- to 10 mM-CDP and contained 8% of the adsorbed protein. By the following 2.5 M-NaCl pulse, only 3% of sialyltransferase activity, but all of the protein (92%), was eluted. Obviously CDP acted specifically, as an identical ADP gradient was ineffective. Rechromatography on a smaller Mono S f.p.l.c. column served to separate and concentrate the enzyme.

As summarized in Table 1, the overall yield of $\alpha 2,6$ -sialyltransferase was about 19% compared with the Triton extract, with a purification factor of 11 500. No loss of activity for at least 4 months was detected on storing the enzyme at -20°C in 50% glycerol.

Table 1. Purification of $\alpha 2,6$ -sialyltransferase from rat liver

Results are shown for an enzyme preparation starting from 450 g of rat liver. Details are described in the Material and methods section. Activities have been multiplied by a factor of 2.0 to reflect maximal velocities under saturating conditions.

	Volume (ml)	Protein (mg)	Activity (munits)	Yield (%)	Specific activity (munits/mg)	Purification
Triton extract	4000	30 200	7688	100	0.25	1
Cibacron Blue F3GA–Sephrose (NaCl gradient)	367	950	5380	70	5.64	22.6
Affi-Gel Blue (CDP gradient)	19	17.3	1916*	25*	111*	445*
Mono S f.p.l.c. column (CDP gradient)	6	1.4	806*	10.5*	567*	2403*
Mono S f.p.l.c. column (NaCl pulse)	0.7	0.5	1435	18.7	2870	11 480

* Activity measured in the presence of contaminating CDP.

Fig. 3. Scan of the purified $\alpha 2,6$ -sialyltransferase

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [19]. After staining the gels with Coomassie Blue, they were scanned at 633 nm. The M_r values of standard proteins, electrophoresed under the same conditions, are as follows: phosphorylase *b*, 97 400; bovine serum albumin, 66 200; ovalbumin, 42 700; carbonic anhydrase, 31 000; soya-bean trypsin inhibitor, 21 000; lysozyme, 14 400.

SDS/polyacrylamide-gel electrophoresis

The final enzyme preparation showed two bands with apparent M_r values of 41 000 (83 % of stained protein) and 31 000 (17 % of stained protein) (Fig. 3). The protein band in the 41 000 M_r region was the only one that increased significantly during the different steps of the purification procedure. The M_r value of 41 000 corresponds well to that described for the enzyme purified on CDP-hexanolamine-Sepharose (M_r 40 500) by the procedure of Weinstein *et al.* [8].

Properties of the $\alpha 2,6$ -sialyltransferase preparation

The enzyme was first purified by Weinstein *et al.* [8] from the same source, and its specificity was defined [14]. The purification described in the present paper gave nearly homogenous $\alpha 2,6$ -sialyltransferase in high yield by using dye chromatography and f.p.l.c. The purified enzyme is free of $\alpha 2,3$ -sialyltransferase (below 0.2 %), as it only forms 6'-sialyl-lactose. No activity was measured with fetuin or antifreeze glycoprotein as acceptor substrate (below 0.3 %). These data are in accordance with the published enzyme specificity [8,14]. Neither CMP-NeuAc hydrolase activity nor proteinase activity was detected, either at pH 7.5 or at pH 4.2. This result is especially important, since proteolytic degradation of glycoproteins during resialylation would result in loss of biological activity.

The apparent K_m value for asialo- α_1 -acid glycoprotein at saturating CMP-NeuAc concentration was 340 μ M in terms of galactose sites. An apparent K_m for CMP-NeuAc of 60 μ M was measured with asialo- α_1 -acid glycoprotein as acceptor. Identical kinetic data were obtained with the same enzyme but purified by chromatography on CDP-hexanolamine-Sepharose [8] using equivalent assay conditions.

General aspects of the new method

The immobilized dye exhibited a high capacity for $\alpha 2,6$ -sialyltransferase from rat liver, comparable with the

value for the specific affinity material CDP-hexanolamine-Sepharose. The procedure involves only three steps, which can be easily carried out (8 days), affording the sialyltransferase in high purity and good yields. A considerable advantage is the simple and economical synthesis of the dye conjugate, which allows easy scaling up of the purification procedure. Even without using an affinity ligand specific for the sialyltransferase, the enzyme could be purified to a certain extent, and in yields achieved previously only with CDP-hexanolamine-Sepharose. The enzyme preparation is suitable for many biological applications, especially resialylation of asialoglycoproteins on a large scale.

Further studies will show if the procedure can be applied to the purification of sialyltransferases from other sources differing in specificity.

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