[32] Sialate 9-*O*-Acetylesterase from Rat Liver

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Introduction

Recently, a family of esterases has been discovered that appear to be specific for removal of *O*-acetyl esters from the 9-position of naturally occurring sialic acids[1-4] (reviewed previously in this series by R. Schauer, Vol. 138, p. 611). We can detect and assay these enzymes using biosynthetically prepared [*acetyl*-^{3}H]9-*O*-acetyl-*N*-acetylneuraminic acid ([^{3}H]Neu5,9Ac$_2$). In the rat liver, the two major esterases detected with this substrate at neutral pH are a cytosolic nonglycosylated enzyme and a membrane-associated glycosylated enzyme, which are present in approximately equal amounts.[2] A similar cytosolic enzyme from equine liver has been previously described in this series.[1] Polyclonal antisera indicate that the two rat liver enzymes are distinct proteins. Both are also found in a buffer extract of commercially available rat liver acetone powder (at a specific activity similar to that found in fresh rat liver extracts). This chapter describes the purification and properties of the glycosylated enzyme from rat liver acetone powder.[5]

Biosynthetic Preparation of [*acetyl*-^{3}H]9-*O*-acetyl-*N*-acetylneuraminic Acid

Reagents

Isolated, intact rat liver Golgi vesicles (see [33], this volume)

[*acetyl*-^{3}H]AcCoA, 5–10 Ci/mmol, stored in aliquots at −70°

PK buffer: 10 m*M* potassium phosphate, 150 m*M* sodium chloride, pH 6.5 (1× and 2× stocks, stored at 4°)

Diisopropylfluorophosphate (DFP), 100 m*M* in 2-propanol, store in a desiccator at −20°

Arthrobacter ureafaciens neuraminidase (Calbiochem, San Diego, CA)

[1] R. Schauer, this series, Vol. 138, p. 611.

[2] H. Higa, S. Diaz, and A. Varki, *Biochem. Biophys. Res. Commun.* **144**, 1099 (1987).

[3] A. Varki, E. Muchmore, and S. Diaz, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 882 (1986).

[4] G. Herrler, R. Rott, H. D. Klenk, H. P. Muller, A. K. Shukla, and R. Schauer, *EMBO J.* **4**, 1503 (1985).

[5] H. Higa, A. Manzi, and A. Varki, *J. Biol. Chem.*, in press.

Dowex-50 AG 1- × 8 (H^+ form), washed extensively in water
Dowex 3- × 4a (formate form, in 10 m*M* sodium formate, pH 5.5)
Formic acid, 1 *M* passed over Dowex-50 AG 1- × 8 (H^+ form)
Acetic acid, 10 m*M*, passed over Dowex-50 AG 1- × 8 (H^+ form)

Procedure. Pretreatment of the Golgi vesicles with DFP (final 1 m*M* DFP, 1% 2-propanol, on ice for 15 min) inactivates an endogenous esterase activity and improves recovery of the product. However, reasonable amounts of product can be obtained without the DFP treatment.

The reaction mixture (1 ml) containing 1–2 μM [^{3}H]AcCoA (use undiluted stock) and 400 μl of Golgi vesicles (5–10 mg/ml) in PK buffer is incubated at 22° for 20 min. The reaction is quenched by filling the tube with ice-cold PK buffer and centrifuged at 100,000 *g* for 30 min at 4°. The supernate is discarded, and the pellet is surface-washed 3 times with ice-cold PK buffer and sonicated into 1 ml of 10 m*M* sodium acetate, pH 5.5. The sonicated membranes are recovered by centrifugation at 100,000 *g* for 30 min at 4°, and the pellet is briefly sonicated into 400 μl of 100 m*M* sodium acetate, pH 5.5. ^{3}H-Labeled mono-*O*-acetylsialic acids are released by incubation at 37° for 3 hr with 100 mU *Arthrobacter ureafaciens* neuraminidase and 0.1% Triton X-100 (final). The mixture is centrifuged at 100,000 *g* for 30 min at 4°C.

The ^{3}H-labeled *O*-acetylsialic acids in the supernate are purified by sequential ion-exchange chromatography, at room temperature. The supernate is loaded onto a 1-ml column of Dowex-50 AG 1- × 8(H^+ form, in water) and eluted with 4 ml of water. The pooled washings are loaded directly onto a 1-ml column of Dowex 3- × 4A (formate form) in 10 m*M* sodium formate, pH 5.5. The column is washed with 7 ml of 10 m*M* formic acid, and the mixture of ^{3}H-labeled 7-, 8- and 9-mono-*O*-acetyl-*N*-acetylneuraminic acids is eluted with 10 ml of 1 *M* formic acid. The eluate is dried on a Buchler shaker–evaporator, reconstituted in 1 ml of freshly made 20 m*M* ammonium hydroxide, and incubated at room temperature for 30 min, to induce migration of all the ^{3}H-labeled *O*-acetyl groups to the C-9 position. The sample is dried immediately on the shaker–evaporator, brought up to 10 m*M* acetic acid, and aliquots containing 10,000–50,000 cpm dried into individual microfuge assay tubes using a Savant Centrifuge evaporator. The tubes are capped immediately and stored in a desiccator at −20°C until use.

Recovery of radioactivity is monitored at each step by counting 0.5% aliquots. Final yields of radioactivity are somewhat variable, depending on the batches of Golgi and [*acetyl*-^{3}H]AcCoA. In a typical preparation, starting with 5 μCi of [*acetyl*-^{3}H]AcCoA (specific activity 5 Ci/mmol) and 400 μl of Golgi vesicles, about 1 μCi is found in the labeled membranes, and about 0.5 μCi is recovered in the final purified substrate. Note that

[*acetyl*-^{3}H]AcCoA breaks down on prolonged storage and that the resulting unlabeled CoA has a K_i of 0.78 μM for inhibition of uptake.

Assay Method

The principle of the assay is very similar to that previously described for acetylcholinesterase.[6] The aqueous reaction mixture is quenched with a solution that acidifies it, and it is then mixed with a toluene-based scintillation counting cocktail. The substrate ([^{3}H]Neu5,9Ac$_2$) cannot enter the toluene phase and hence cannot be counted, whereas the product ([^{3}H]acetate), which is protonated under these conditions, can. Thus, any increase in radioactivity over the background represents release of [^{3}H]acetate from the substrate. Note that because this esterase has a high K_m value (8.8 mM) for its natural substrate Neu5,9Ac$_2$, saturating assays are not practical. Thus, the first-order kinetics (%/hr released from nonsaturating radioactive substrate) is used to establish the activity.

Reagents

10× buffer, 1 M Tris-HCl, pH 8.0

Stopping mixture: 1 M chloroacetic acid, 0.5 M NaOH, 2 M NaCl

Toluene-based scintillation cocktail: 0.5% 2,5-diphenyloxazole (PPO) and 0.03% p-bis[2-(5-phenyloxazolyly)]-benzene (POPOP) in toluene and 20% isoamyl alcohol (stir overnight to dissolve)

Substrate: [^{3}H]Neu5,9Ac$_2$

Procedure. Substrate (10,000 cpm) is incubated with enzyme and Tris buffer in a final volume of 100 μl at 37° for 30–60 min. The reaction is quenched by addition of 100 μl of stopping mixture, chilled on ice for 15 min, and spun at 10,000 g for 5 min at room temperature. Fixed aliquots (170–190 μl, depending on pellet size) of the supernatant are transferred to 10 ml of the toluene-based scintillation cocktail. The mixture is shaken well, allowed to stand for 15 min, and the radioactivity released is determined by counting. Comparably incubated and processed blanks are always included in each assay. The blank values range from 100 to 300 cpm, depending on the particular batch of substrate, pH, and time of incubation. One unit of activity is defined as release of 1% of the radioactivity per hour of incubation at 37°. The 100% value is determined by complete de-O-acetylation using 0.1 N NaOH at 37° for 30 min, followed by stopping solution and scintillation cocktail. All assays are performed under conditions where the release is linear with time and added enzyme, and less than 20% of the substrate is consumed. After prolonged storage of the substrate, some breakdown will occur, and background values will increase. This can be corrected by adding a small amount of 10 mM acetic acid and evaporating the substrate once.

[6] C. D. Johnson and R. L. Russell, *Anal. Biochem.* **64**, 229–238 (1975).

Alternative Assay Methods

The assays described above are accurate and reliable even in crude extracts of tissues. With purified enzyme, the release of unlabeled free acetate from substrates can be monitored using a commercially available kit (catalog #148-261 from Boehringer Mannheim, Indianapolis, IN). The individual components for this acetate assay can also be purchased individually. In the case of synthetic chromogenic substrates [e.g., 4-methylumbelliferyl acetate (4-MU-OAc), see Ref. 1], hydrolysis can be followed by monitoring the reaction fluorometrically. It must be emphasized that in crude tissue extracts, other "nonspecific" esterases can cleave the 4-MU-OAc substrate.

Purification

All steps are performed at 4°. The following buffers are used: Buffer A, 20 m*M* KP_i, pH 8; Buffer B, 20 m*M* KP_i, pH 8, with 100 m*M* NaCl; Buffer C, 20 m*M* KP_i, pH 5.5, with 50 m*M* NaCl; Buffer D, 20 m*M* KP_i, pH 5.5; Buffer E, 20 m*M* KP_i, pH 5.5, with 370 m*M* NaCl; Buffer F, 20 m*M* KP_i, pH 5.5, with 900 m*M* NaCl; Buffer G, 30 m*M* K_2HPO_4, 2 m*M* EDTA.

Step 1: Extraction. Rat liver acetone powder (100 g) is homogenized gently into 500 ml of Buffer A with a Polytron homogenizer for approximately 1 min, until all clumps are dispersed. The homogenate is stirred for 1 hr, and the mixture is centrifuged at 100,000 *g* for 30 min (33,000 rpm, 50.2 Ti Beckman rotor). The supernatant is passed through a plug of glass wool. The pellet is resuspended and extracted again, exactly as above.

Step 2: DEAE-Cellulose Chromatography. The supernatants are pooled and loaded onto a column of DE-52 (Whatman) (1130 ml packed volume, 7.8 × 23.8 cm, in Buffer A). The column is washed with 600 ml of the same buffer, and 20-ml fractions are collected. The glycoprotein enzyme runs through the column, whereas the cytosolic enzyme is bound. If desired, a partially purified preparation of the latter can be obtained by elution with a 1700 ml linear gradient of 0–300 m*M* NaCl in Buffer A.

Step 3: Concanavalin A-Sepharose Chromatography. The pooled run-through from the DE-52 column is adjusted to 0.1 *M* NaCl with a stock of 4 *M* NaCl and loaded at about 1 ml/min onto a 1.5 × 31 cm (55 ml) Con A-Sepharose column equilibrated in Buffer B. The column is washed with 200–250 ml of Buffer B. One column volume of 100 m*M* α-methylmannoside in Buffer B (55 ml) is loaded onto the column, and the flow stopped for 8–15 hr. The enzyme binds to the column and requires prolonged exposure to the glycoside for elution. Column flow is resumed and elution with the α-methyl mannoside continued for 4 additional column volumes. Fractions (40 ml) are collected and monitored for activity. A small portion

of the activity (5–20%) may run through the column. If optimal yield is desired, a second smaller column of ConA-Sepharose (25 ml) can be run to bind and elute this remaining activity.

Step 4: Ammonium Sulfate Precipitation. The active fractions eluted from the ConA-Sepharose column are pooled and adjusted to 80% saturation with ammonium sulfate (56.1 g/100 ml) by gradual addition of the solid powder with continuous stirring. After stirring for 3–6 hr, the precipitate is collected by centrifugation at 30,000 *g* (SS-34 Sorvall rotor, 16,000 rpm) for 30 min.

Step 5: Sephacryl S-200 Chromatography. The ammonium sulfate precipitate is dissolved in 3 ml of Buffer C, and loaded onto a Sephacryl S-200 column (1.5 × 47 cm, 83 ml) equilibrated in the same buffer, and eluted at 20 ml/hr. Fractions (1 ml) are collected and monitored for activity. A single, symmetrical peak of activity is found between 40 and 60 ml of the elution volume.

Step 6: Procion Red-Agarose Chromatography. The active fractions are pooled and applied to a column of Procion Red-Agarose (1.5 × 17 cm, 30 ml) equilibrated in Buffer D, at a flow rate of 20 ml/hr. The column is washed with 300 ml of the same buffer and eluted with 400 ml of Buffer E. Fractions (8.5 ml) are collected and monitored for activity. This final step separates the sialic acid esterase from another glycoprotein acetylesterase that can subsequently be eluted from the column with 300 ml of Buffer F. The latter enzyme hydrolyzes 4-MU-OAc, but not the sialic acid substrate. Its natural substrate remains unknown.

Step 7: Concentration. The active fractions eluted with Buffer E are pooled and diluted with an equal volume of Buffer G, to give a final concentration of 25 m*M* KP_i, 185 m*M* NaCl, pH 7.5. The volume is then reduced to less than 0.5 ml (5–10 mg/ml), using several Centricon concentrators (Amicon, catalog #4301). The purified, concentrated enzyme is divided into aliquots that are stored at −20°. A typical purification is summarized in Table I.

Properties

Physical Properties. The purified esterase is a heterodimeric protein of M_r 61K whose serine active site can be labeled with [^{3}H]DFP. On reduction, two subunits of M_r 36K and 30K are generated, and the subunit of M_r 30K carries the [^{3}H]DFP label. The protein has N-linked oligosaccharides that are partly resistant to endo-β-*N*-acetylglycosaminidase H, but are sensitive to peptide *N*-glycosidase F, indicating that they are mostly complex-type glycans. A polyclonal antibody raised against the purified enzyme does not cross-react significantly with the cytosolic, nonglycosylated esterase.

Stability. The activity of the concentrated purified enzyme is stable at

TABLE I
PURIFICATION OF RAT LIVER GLYCOPROTEIN SIALATE 9-*O*-ACETYLESTERASE

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (-fold)	Yield (%)
Total homogenate	700	99	69,272	6.6	458,500	1	100
Supernate	780	17.4	13,541	26.8	362,895	4.0	79.2
DEAE-cellulose[a]	904	5.2	4,710	30.9	145,544	4.7	31.7
Con A-Sepharose	338	0.3	102	973	99,597	147	21.7
Ammonium sulfate/Sephacryl S-200	21	2.8	59	1,638	97,020	248	21.2
Procion Red-agarose and final concentration	0.33	9.0	3	20,311	60,706	3,068[a]	13.2[a]

[a] At the DEAE step, the glycoprotein enzyme is separated from the cytosolic enzyme, which represents about one-half of the total activity in the supernatant. Thus the final purification (-fold) and yield of the glycoprotein are approximately twice those listed.

4° for up to 1 month, and at 37° 40% of activity is lost in 20 hr. The enzyme is stable for prolonged periods (>6 months) in the frozen state. A single cycle of freeze–thaw results in little loss of activity. However, repeated freeze–thaw results in gradual loss of activity. Lyophilization and reconstitution result in loss of 30% of activity. The activity becomes unstable on excessive dilution (> 100-fold).

Inhibitors and Activators. Diisopropylfluorophosphate (DFP) and diethyl-*p*-nitrophenyl phosphate (paraoxan) irreversibly inactivate the enzyme, indicating a serine active site mechanism. Arginine-modifying reagents (phenylglyoxal and butanedione) also inhibit the activity.[7] Bis-paranitrophenyl-phosphate, Hg^{2+}, fluoride, *p*-chloromercuribenzoate, and 9-acetamido-Neu5Ac at 5 m*M* concentration had little effect on the activity.

Effects of Detergents. Detergents do not activate the enzyme. At 0.5–2.0% concentration, Triton X-100, Triton CF-54, and saponin have no significant effect, while CHAPS lowered the activity by 40%. Deoxycholate resulted in significant loss of activity (by 80% at 2% concentration).

Catalytic Properties. The enzyme activity has a broad pH optimum range between 6.0 and 8.0, has no divalent cation requirement, and is unaffected by reduction. Kinetic studies against various substrates indicate several interesting properties. In particular, it cleaves acetyl groups at the 9-position of sialic acids, but not those at the 7-position. The apparent K_m for Neu5,9Ac_2 is 8.8 m*M*, with a V_{max} of 48 nmol/min/mg protein. It also

[7] B. K. Hayes and A. Varki, *J. Biol. Chem.* in press.

works on di-*O*-acetyl- and tri-*O*-acetyl-*N*-acetylneuraminic acids by first cleaving the residue at the 9-position. The 7-*O*-acetyl-Neu5Ac thus produced undergoes spontaneous migration of the ester to the 9-position, where it can then be cleaved, resulting in production of *N*-acetylneuraminic acid. Since *O*-acetyl groups at the 8-position undergo spontaneous migration to the 9-position very rapidly, activity against the substrate cannot be studied. There is a low rate of cleavage of 4-*O*-acetyl groups (< 10% of the rate against 9-*O*-acetyl groups). However, 9- and 7-*O*-acetyl groups are not cleaved from O-acetylated polysialic acids. The enzyme also does not show significant activity against acetyl groups from acetylcholine, acetyl-PAF, and *O*-acetylserine. A low but significant rate of hydrolysis occurred with triacetin (tri-O-acetylated glycerol). Addition of a methyl ester to the carboxyl group of sialic acid results in loss of activity. Likewise, the β-methyl glycoside of Neu5,9Ac$_2$ is not a substrate. Neu5,9Ac$_2$ α-glycosidically bound to N-linked oligosaccharides is cleaved at a rate 32% that of the free sugar. The 9-*O*-acetyl ester of 2,3-dehydro-2,6-anhydro-Neu5Ac is cleaved at a rate about 50% that of Neu5,9Ac$_2$. These results suggest that the enzyme recognizes several features of the sialic acid molecule, including the carboxyl group and possibly the glycerol side chain.

Synthetic substrates carrying *O*-acetyl esters were studied at 37° and pH 6.5, using 100 μM concentrations of each (saturating assays cannot be performed for these substrates because some fall out of solution above 150 μM). Under these conditions, hydrolysis of 4-MU-OAc occurred at a rate of 0.84 μmol/min/mg protein, at 11.6 μmol/min/mg for α-naphthyl acetate, and 12.8 μmol/min/mg for *p*-nitrophenyl acetate. Hydrolysis of 4-methylumbelliferyl butyrate occurs at a rate too slow for accurate measurement.

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