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SOLUBILIZATION AND STABILIZATION OF HUMAN LIVER GLYCOPROTEIN SIALYLTRANSFERASE

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Summary

Triton X-100 is increasingly effective in solubilizing human liver glycoprotein (asialofetuin) sialyltransferase (CMP-*N*-acetylneuraminate:D-galactosylglycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) activity as its concentration is increased in the homogenizing buffer. At the optimal concentration of 1.5% (v/v), essentially all of the homogenate sialyltransferase activity was solubilized into the supernatant fluid. Higher concentrations of Triton X-100 inhibited sialyltransferase activity.

Several kinetic properties of the solubilized asialofetuin-sialyltransferase activity were compared to those of the membrane-bound enzyme(s) (in homogenates made without Triton X-100 or in resuspended pellets). No major difference was apparent, suggesting that solubilization has not significantly altered the properties of sialyltransferase.

The solubilized sialyltransferase activity is quite unstable, losing approximately 50% of its activity after one week of storage at 4° C. Various detergents (Zwittergent, sodium taurocholate and sodium deoxycholate) are differentially effective in stabilizing the solubilized activity. Sodium taurocholate (1.5%, w/v) was most effective with no loss in activity after 40 days and minimal loss (14%) after 60 days storage at 4° C. The solubilized sialyltransferase preparation retains full activity after storage in the frozen state (—20° C) for at least 159 days.

Introduction

Glycoprotein sialyltransferases (CMP-*N*-acetylneuraminate:D-galactosylglycoprotein *N*-acetylneuraminyltransferase, EC 2.4.99.1) catalyze the transfer

of sialic acid from its nucleotide derivative cytidine monophosphate *N*-acetylneuraminic acid to the terminal portion of the oligosaccharide moiety of sialoglycoproteins. Several mammalian sialyltransferases exist with differing substrate specificities: sialic acid can be transferred to form α2-3, α2-4 and α2-6 linkages to galactose, α2-6 linkages to *N*-acetylgalactosamine and α2-8 linkages to other sialic acid residues [1—3]. Studies on these mammalian sialyltransferases are important since sialylation of molecules and cells has been shown to be important for their biological function: sialic acid residues appear to be involved in the half-life, transport and binding of molecules, the masking of cellular antigens, and in membrane-related cellular phenomena such as charge, shape, recognition, cellular migration, aggregation, contact inhibition and malignant transformation [3—4]. Previous studies on the membrane-bound sialyltransferases have been limited due to the insolubility and instability of these enzymes.

In the present investigation we have found conditions for solubilizing essentially all of human liver asialofetuin-sialyltransferase activity, compared the kinetic properties to the membrane-bound form(s) of the enzyme and found conditions for fully stabilizing the solubilized activity at 4° C for at least 40 days. Thus, conditions are now available for attempting to purify and characterize human liver glycoprotein sialyltransferase(s).

Methods

General

Protein was determined by the method of Lowry et al. [5] using human serum albumin as standard. All procedures were carried out at 0—4° C unless otherwise stated. Cytidine 5′-monophosphate [G-^{3}H]sialic acid, specific activity 0.15 Ci/g, was purchased from New England Nuclear. Nonradioactive cytidine monophosphate-*N*-acetylneuraminic acid was prepared as described by Kean and Roseman [6] and was used to increase the concentration of the radioactive substrate in the sialyltransferase assay.

Preparation of asialofetuin

Fetuin was purchased from Sigma Chemical Co. and asialofetuin was prepared by hydrolyzing 1 g fetuin in 400 ml 0.125 M H_2SO_4 at 80° C for 1 h [7] followed by extensive dialysis in the cold room against distilled H_2O. Assay of the lyophilized retentate for sialic acid by the method of Warren [8] indicated 95% removal of sialic acid. This asialofetuin was used for the sialyltransferase assays.

Sialyltransferase assay

Asialofetuin-sialyltransferase activity was determined essentially as previously described [9]. In the standard assay, enzyme preparations were incubated in a total volume of 300 μl containing 75 μM asialofetuin, 0.08 μM cytidine monophosphate *N*-[^{3}H]acetylneuraminic acid, 30 μM nonradioactive cytidine monophosphate *N*-acetylneuraminic acid, 16 mM $MgCl_2$ and 0.8—0.9% (v/v) Triton X-100 (Sigma Chemical Co.) in 100 mM Tris-HCl buffer (pH 7.0). Incubations were performed at 37° C for 45 min. The reaction was terminated

with 0.5 ml 1% (w/v) phosphotungstic acid in 0.5 M HCl. The precipitate was collected on Whatman glass fiber filter paper (GF/A) and washed with 2 ml phosphotungstic acid solution. The precipitate was dried, solubilized with 2 ml 2.5 M NH_4OH and counted in 10 ml Riafluor in a Beckman scintillation counter. Control incubations without asialofetuin were carried out for all assays and this endogenous incorporation was subtracted from the incorporation in its presence. Enzyme activity was linear with respect to enzyme amount (5—50 μl tissue preparation) and time (15—45 min). Product identification (incorporation of radioactive sialic acid into asialofetuin to yield radiolabelled fetuin) has been demonstrated previously by us using polyacrylamide gel electrophoresis [10].

Tissue preparation

Human livers which appeared normal on gross pathological inspection were obtained from autopsied individuals and stored at —20° C until used. The procedures which involved human tissues were approved by the Committee on Investigations/Activities Involving Human Subjects of the School of Medicine (University of California, San Diego). Liver homogenates were prepared as follows: 0.2—1.0 g tissue was homogenized (1 : 5, w/v) in 0.1 M, Tris-HCl buffer (pH 7.0) containing 5 mM $MgCl_2$ using a motor-driven glass-Teflon Potter-Elvehjem homogenizer. In the solubilization study, varying amounts of Triton X-100 (0—2.5%, v/v) were put in the homogenizing buffer. These homogenates were assayed for sialyltransferase activity and centrifuged for 10 min at 10 000 $\times g$. The resulting pellets and supernatants contained the membrane-bound and solubilized sialyltransferases, respectively.

Kinetic studies

Various kinetic studies were performed on homogenate, resuspended pellet and Triton X-100-solubilized sialyltransferase preparations. Apparent Michaelis constants (K_m values) were determined by the Lineweaver-Burk method [11] for asialofetuin as previously described [9,10] by adding increasing amounts of asialofetuin to the incubation mixture while maintaining the final concentration of cytidine monophosphate *N*-acetylneuraminic acid at 30 μM. To obtain the desired concentration of cytidine monophosphate *N*-acetylneuraminic acid, both radiolabelled and non-radioactive substrate were used. pH optimum curves were determined as previously described [10] in 0.1 M Tris-HCl buffers (pH 6.4—8.8). The reaction was initiated with cytidine monophosphate *N*-[^{3}H]-acetylneuraminic acid and incubated for 45 min at 37° C. Thermal stability studies were performed as previously described [10] on sialyltransferase preparations preincubated at 37° C at 30 min intervals to 150 min prior to the addition of substrates and subsequent assay for 45 min. Curves of asialofetuin-sialyltransferase activity versus Triton X-100 concentration (0—1.5%, v/v) were determined for resuspended pellet and Triton X-100-solubilized sialyltransferase preparations.

Stability studies

Solubilized sialyltransferase activity was assayed regularly in liver supernatants (10 000 $\times g$ for 10 min) after storage at 2—4° C for various amounts of

time (0—60 days) in the presence of the following: (a) nothing added (control); (b) sodium deoxycholate (0.01—0.25%, w/v); (c) Zwittergent (0.2—1.5%, w/v); (d) sodium taurocholate (0.2—1.5%, w/v); (e) cytidine diphosphate (1—3 mM); (f) cholesterol (5—50 μg/ml); (g) DL-α-lecithin (5—50 μg/ml), (h) β-mercaptoethanol (10^{-2}—10^{-6} M), (i) lubrol PX (0.1—1.0%, v/v) and (j) spermine (0.01—0.2 mM). All of the above additives were from Sigma Chemical Co. except Zwittergent which was from Calbiochem. The additives were chosen for the stabilization study at the concentrations indicated above after preliminary studies on the effects of these addivites (at various concentrations) on asialofetuin-sialyltransferase activity. The stability of solubilized sialyltransferase activity was also determined after storage at −20° C for various amounts of time up to 159 days.

Results

Table I lists data on the solubilizing effects of Triton X-100 when it is used in the homogenizing buffer at varying concentrations (0.5—2%, v/v). As the concentration of Triton X-100 increases, a greater percentage of the asialofetuin-sialyltransferase activity is solubilized and found in the supernatant. Without Triton X-100 in the homogenizing buffer, 88% of the sialyltransferase activity is found in the resuspended pellet (and is presumably membrane-bound). When 1.5—2.0% (v/v) Triton X-100 concentrations are used, little activity (6—10%) is found in the resuspended pellet and the bulk of the activity (90—94%) is found in the supernatant and appears to be solubilized.

Several comparative kinetic studies were performed on homogenate, resuspended pellet and Triton X-100 solubilized sialyltransferase activity to determine if solubilization had altered the catalytic properties. Fig. 1 indicates that no major difference exists in pH optimum curves for the solubilized and membrane-bound (resuspended pellet) sialyltransferases. Both enzyme preparations had an optimum near pH 7.0 with the suggestion of a second optimum near pH 8.0. Apparent Michaelis constants (K_m values) for asialofetuin were determined (by analysis of the Lineweaver-Burk double reciprocal plots) to be 100 and 50 μM for homogenate and solubilized sialyltransferase, respectively. The resuspended pellet liver sialyltranfersase K_m for asialofetuin has previously been shown by us to be 60 μM [10].

TABLE I

SOLUBILIZATION OF HUMAN LIVER GLYCOPROTEIN SIALYLTRANSFERASE WITH VARYING AMOUNTS OF TRITON X-100

Triton X-100 in homogenizing buffer (% v/v)	Recovered sialyltransferase activity in (%):	
	Supernatant	Resuspended pellet
0	12	88
0.5	54	46
1.0	66	34
1.5	94	6
2.0	90	10

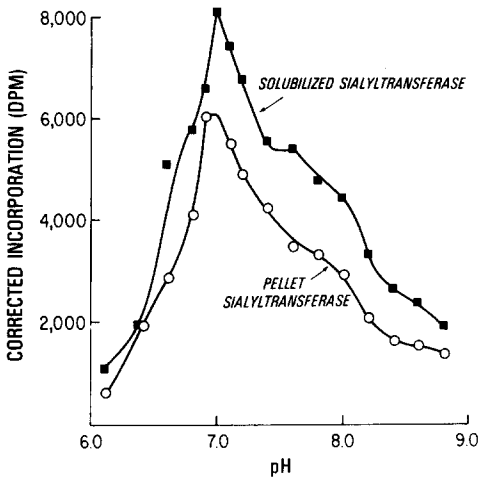

Fig. 1. pH optimum curves for human liver resuspended pellet and solubilized asialofetuin-sialyltransferase activities.

Heat denaturation curves for homogenate, resuspended pellet and solubilized sialyltransferases are shown in Fig. 2. The curves for homogenate and solubilized preparations are very similar. The initial portion of the resuspended pellet curve is somewhat different from the other two for short preincubation times (30—90 min). However, for longer preincubation times (120—150 min) all three curves are very similar, with approximately 60% of the initial sialyltransferase activity remaining after 150 min preincubation at 37° C.

Fig. 3 depicts curves of resuspended pellet and Triton X-100 solubilized sialyltransferase activity versus the concentration of Triton X-100 in the assay medium. For both enzyme preparations, Triton X-100 stimulated activity with maximal activity near 0.8% (v/v). Enzymatic activity of the solubilized preparation was stimulated slightly more than the activity of the pellet preparation. Higher concentrations of Triton X-100 (greater than 1.5%, v/v) inhibit asialofetuin-sialyltransferase activity [10].

Most of the additives used in an attempt to stabilize the solubilized sialyltransferase activity were ineffective. However, three additives (sodium deoxy-

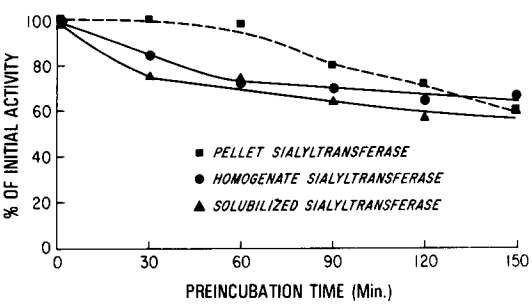

Fig. 2. Heat denaturation curves for human liver resuspended pellet, homogenate and solubilized asialofetuin-sialyltransferase activity.

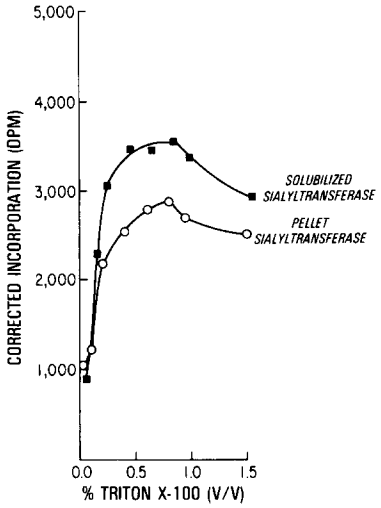

Fig. 3. Human liver resuspended pellet and solubilized asialofetuin-sialyltransferase activities versus Triton X-100 concentrations.

cholate, sodium taurocholate and Zwittergent) were differentially effective in stabilizing sialyltransferase activity while stored at 2—4° C (Table II). Sodium taurocholate (1.5%, w/v) was most effective with no loss in activity up to 40 days and minimal loss (14%) after 60 days of storage. A lower concentration of sodium taurocholate (1.0%, w/v) was not as effective in stabilizing enzymatic activity. The control preparation (no additives) lost 60% and 95% of its activity after storage for 10 and 60 days, respectively. It is noteworthy that the solubilized sialyltransferase preparation retained full activity after storage in the frozen state (—20° C) for at least 159 days without any additives (other than the 1.5/, v/v, Triton X-100 used in the solubilization).

Discussion

Several mammalian sialyltransferases exist with varying properties and substrate specificities. Some are primarily membrane-bound [12] and other are

TABLE II

ACTIVITY OF SOLUBILIZED SIALYLTRANSFERASE (STORED AT 2—4°C IN THE PRESENCE OF VARIOUS ADDITIVES) VERSUS TIME

Additive	Original activity (%) remaining after						
	10	15	20	30 (days)	40	50	60
Control *	40	33	24	14	10	7	5
Sodium deoxycholate (0.25%, w/v)	98	92	77	62	64	47	40
Zwittergent (1.5%, w/v)	100	90	67	51	46	30	20
Sodium taurocholate (1%, w/v)	>100	>100	84	70	62	47	37
Sodium taurocholate (1.5%, w/v)	>100	>100	>100	>100	>100	82	86

* This control contains 1.5% (v/v) Triton X-100 used in the homogenizing buffer for solubilizing sialyltransferase.

soluble [1]. The soluble sialyltransferases have been partially purified by conventional procedures from goat colostrum and sheep submaxillary gland [1]. Recently, a soluble sialyltransferase from bovine colostrum has been purified to apparent homogeneity by affinity chromatography on cytidine diphosphate-hexanolamine agarose [13]. This soluble sialyltransferase transfers sialic acid to form *N*-acetylneuraminic acid α2-6 galactose β1-4 *N*-acetylglucosamine type oligosaccharides [14,15].

Previous studies on membrane-bound sialyltransferases have been limited by the insolubility and instability of these enzymes. They have been partially purified from rat liver [16,17], calf thyroid [18] and human liver [19] after partial solubilization with sodium deoxycholate and/or Triton X-100. These enzymes are particularly labile after solubilization [12,16]. A prerequisite for purifying and characterizing these important liver sialyltransferases is a means for more fully solubilizing and stabilizing their enzymatic activity. It is particularly important to stabilize their activity at temperatures above freezing (greater than 0° C) so that further studies can be performed on them.

In the present investigation we have studied human liver asialofetuin-sialyltransferase activity, an activity primarily involved with transfering sialic acid to form *N*-acetylneuraminic acid α2-3 galactose β1-4 *N*-acetylglucosamine type oligosaccharides, since this is the major sialic acid linkage found in fetuin [20]. However, the crude preparations may contain several different asialofetuin-sialyltransferases since sialic acid is also found in fetuin linked α2-6 to galactose [21] and α2-6 to *N*-acetylgalactosamine [22]. In a previous study [10] we found that human liver appears to contain at least two different asialofetuin-sialyltransferases.

Increasing concentrations of Triton X-100 in the homogenizing buffer are very effective in increasing the amount of asialofetuin-sialyltransferase activity found in the supernatant relative to that found in the resuspended pellet. This is presumably due to release of membrane-bound sialyltransferase(s) into the supernatant by the nonionic detergent Triton X-100. Since higher Triton X-100 concentrations (greater than 1.0%, v/v) inhibit asialofetuin-sialyltransferase activity [10], a concentration of 1.5% was chosen for solubilizing sialyltransferase activity as a compromise between maximum solubilization with minimum inhibition of enzymatic activity. At this concentration essentially all of the homogenate sialyltransferase activity can be released into the supernatant with only a trace amount of activity remaining in the resuspended pellet.

Several kinetic properties of the solubilized asialofetuin-sialyltransferase activity have been studied and compared to those of the membrane-bound enzyme(s) in resuspended pellets and/or in homogenates (made without Triton X-100). In these homogenates made without Triton X-100, the majority of the enzymatic activity appears, as previously shown in the rat [12], to be membrane-bound (Table I). For all properties investigated (pH optima, K_m values, activity vs. Triton X-100 concentration and thermostability), no major difference could be seen between the solubilized and membrane-bound sialyltransferases. The pH optmum of 7.0 found in the present study is similar to that found previously for human and porcine liver [10,23] and rat liver [17,24] sialyltransferase activity. A slightly higher K_m exhibited by the membrane-bound sialyltransferase for asialofetuin may indicate a greater accessibility of

this macromolecular substrate to the solubilized form(s) of the enzyme. The slightly greater stimulation of the solubilized sialyltransferase activity by Triton X-100 suggests that this detergent may be more important to the activity of the enzyme(s) when removed from a membraneous milieu. Previous investigators have also found that Triton X-100 stimulates porcine [23] and rat [17,25] liver sialyltransferase activities. The residual enzymatic activity after preincubation for 120—150 min at 37°C was very similar for pellet, homogenate and solubilized sialyltransferase preparations despite an apparent increased thermostability of the pellet enzyme for shorter preincubation times. The overall similarity of kinetic properties suggests that solubilization has not significantly altered the properties of sialyltransferase. The kinetic properties found in the present study are essentially identical to those previously reported for human liver asialofetuin-sialyltransferase activity [10].

The solubilized sialyltransferase activity is quite unstable, losing approximately 50% of its initial activity after one week of storage at 4°C. This instability has previously been shown for human liver homogenate sialyltransferase(s) [10,19] as well as for the solubilized rat liver enzyme(s) [16,17]. In the present study conditions have been found for stabilizing the solubilized sialyltransferase activity without freezing. Various detergents are differentially effective (Table II), with sodium taurocholate (1.5%, w/v) fully preserving activity for 40 days with only small losses (approximately 15%) of activity after 60 days. To our knowledge, this is the first report of the long-term stabilization of solubilized sialyltransferase activity in an unfrozen state. The solubilized preparation can also be stored frozen for at least 159 days without any loss of enzymatic activity.

The conditions described in this study for solubilizing and stabilizing human liver glycoprotein sialyltransferase(s) should be useful for future attempts to purify and characterize these important enzymes. Only then can the complete substrate specificity of these enzymes and their role in glycoprotein biosynthesis be determined.

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