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SPECIAL CONSIDERATIONS IN THE PURIFICATION OF THE GM_3 GANGLIOSIDE FORMING ENZYME, CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1): SOLUBILIZATION OF SAT-1 WITH LAURYLDIMETHYLAMINE OXIDE*

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SUMMARY: Lauryldimethylamine oxide (LDAO) was employed in the purification of the GM_3 ganglioside forming enzyme, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) (4). This detergent has advantages over the typically employed Triton detergents in the solubilization and stabilization of this sialyltransferase. Crude protein fractions solubilized from rat liver Golgi by several such detergents are very similar in composition as determined by two-dimensional gel electrophoresis. However, LDAO appears to activate and stabilize SAT-1 activity. It is possible that SAT-1 activation involves the structurally similar hydrophobic moieties and quaternary amino groups of LDAO and phosphatidylcholine.

Detergent solubilization is essential for the purification of integral membrane proteins. Detergents serve to replace the native lipids of the membrane bilayer about the hydrophobic domain(s) of the protein so that routine biochemical and chromatographic methods can be employed for purification. Many times the choice of detergents is by trial and error, optimized to stabilize a particular enzyme activity. Although the stability of a protein in detergent in not clearly understood, it is likely related to the artificial conformation imposed on the protein by a particular detergent environment. These properties of detergents have been reviewed (1,2) and recently summarized (3). We report here the application of a nonionic/cationic detergent, lauryldimethylamine oxide (LDAO) in the purification of a sialyltransferase, the GM_3-forming Golgi enzyme, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1).

METHODS

Materials: Lauryldimethylamine oxide (LDAO), also called Ammonyx LO, was obtained from the Stepan Chemical Co, (Northfield, IL). Triton CF-54, β-octylglucoside and myosin were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100, Gelcode molecular weight markers and Extracti-Gel D were purchased from Pierce Chemical Co. (Rockford, IL). CMP [$^{14}C_{4,5,6,7,8,9}$] sialic acid was obtained from New England Nuclear (Boston, MA). Sep Pak C_{18} cartridges were purchased from Waters (Milford, MA). Carbamylated pI standard markers were from Pharmacia (Piscataway, NJ). The reverse-phase RP-

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Abbreviations used: SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase; LDAO, lauryl dimethylamine oxide, also known as Ammonyx LO; GM_3, NeuAcα2-3Galβ1-4Glcβ1-1Cer; PC, phosphatidylcholine; 2D, two-dimensional; SDS, sodium dodecyl sulfate; PAGE, polyacrylaminde gel electrophoresis; LD_{50}, lethal dose with 50% survival; HPLC, high performance liquid chromatography; Kd, kilodalton.

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300, C_8 HPLC column was obtained from Applied Biosystems (Foster City, CA). All other reagents and chemicals were of reagent grade.

Rat Liver Golgi: Rat liver Golgi was prepared as described previously (4) using well-established procedures (5-7).

CMP-sialic acid:Lactosylceramide α2-3 Sialyltransferase (SAT-1) Activity Assay: SAT-1 activity was assayed as previously described (8,9).

Two-Dimensional Gel Electrophoresis: Rat liver Golgi, 0.3% Triton X-100 extracted Golgi, 1% Triton CF-54 extracted Golgi, and 15% LDAO extracted Golgi proteins were isoelectrofocussed in the first dimension in tube gels according to the O'Farrell method (25) and in the second dimension on 10-20% SDS-PAGE according to the Laemmli system as described by Dunbar (10). The carrier ampholytes were a mixture of 2 parts Pharmacia Pharmalytes pH 5-8 and 1 part (LKB Ampholines pH 3.5-10/Pharmacia Pharmalytes pH 3-10, 1:1). The 2D SDS-PAGE patterns were analyzed by the Bio Image Visage 110 computerized digital image analysis system (Millipore, Milford, MA)

RESULTS AND DISCUSSION

LDAO was employed in the purification to homogeneity of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) from rat liver Golgi (4). LDAO is a nonionic/cationic detergent which protonates under acidic conditions (pH < 7) conferring a net positive charge to its hydrophilic head group (3). It's use has been primarily for the solubilization of photoreactive centers from chloroplasts (11,12). This is the first report of the use of LDAO for the solubilization of a glycosyltransferase.

Typically, Triton X-100 (13-19) or Triton CF-54 (20) have been the surfactants employed in sialyltransferase solubilizations from Golgi vesicles. Two-dimensional computerized digital image analyses of the proteins solubilized from rat liver Golgi vesicles by either 0.3% Triton X-100, 1% (w/v) Triton CF-54 or 15% (w/v) LDAO gave very similar protein patterns under the conditions employed relative to the reference image obtained with unextracted rat liver Golgi. Each detergent resolved about 300 silver-stained polypeptides within the pI range of 4.7 to 8.3. LDAO resolved 284 polypeptides, Triton CF-54 318, and Triton X-100 295. The reference pattern of rat liver Golgi protein (no detergent digestion) contained 315 proteins. The Bio Image Visage 110 match-paired 156, 175 and 191 (for LDAO, Triton X-100 and Triton CF-54, respectively) of these proteins to the reference image. The two-dimensional map of the LDAO-soluble Golgi proteins is shown in Figure 1.

LDAO offers several advantages over the Triton surfactants. First, LDAO enhances SAT-1 activity from rat liver Golgi 12 to 15-fold higher than either Triton CF-54, Triton X-100, or β-octylglucoside (Figure 2). This activation of SAT-1 may reflect a stabilization of the solubilized enzyme in a structural motif similar to its native conformation in a phosphatidylcholine (PC) membrane. PC, a major phospholipid of the Golgi, has been shown previously to give a 16-fold activation of SAT-1 (21).

A second advantage of LDAO is that it does not absorb at A_{280nm}, as do the Tritons, permitting the monitoring of protein elution at A_{280nm} during purification steps involving column chromatography. LDAO exhibits end-group absorption below 215 nm.

The hydrophobic nature and positive charge of LDAO below pH 7.0 confers considerable stability on SAT-1 for purification and storage. SAT-1 has been stored in 25 mM sodium cacodylate (pH 6.5) containing 15% (w/v) LDAO at -80°C for periods of 6-12 months without appreciable loss of activity (Table 1). We attribute this to the hydrophobicity of the detergent and the structural similarity between LDAO and PC. Glew and coworkers (*private communication*) have observed a similar stabilization by charge with glucocerebrosidase, a membrane-bound lysosomal protein, and phosphatidylserine (PS). Acyl CoA, a negatively charged amphipatic molecule, confers the same effect as PS.

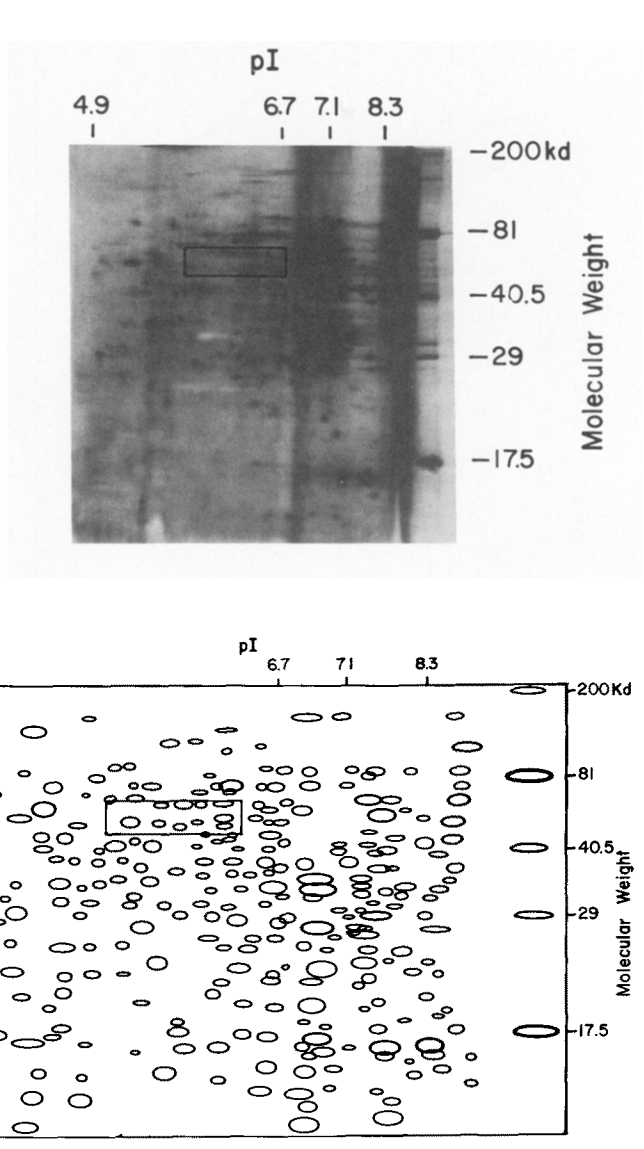

Figure 1. Two-Dimensional SDS-PAGE of Detergent-Solubilized Rat Liver Golgi Proteins. Protein from rat liver Golgi was extracted in the presence of 3.0% (w/v) Triton X-100, 1.0% (w/v) Triton CF-54 or 15% (v/v) lauryldimethylamine oxide. The proteins solubilized from the Golgi membranes under these conditions were analyzed by two-dimensional gel electrophoresis. First-dimension tube gels were performed according to the method of O'Farrell (25) with an ampholyte mixture of 1 part pH 3-10 and 2 parts 5-8 on sixty micrograms of total protein. The second-dimension slab gels were standard 10-20% Laemmli SDS-polyacrylamide gels. The two-dimensional patterns were analyzed following silver staining (26) by computerized digital imaging using the Bio Image Visage 110 (Millipore/Bio Image, Ann Arbor, MI). Bio Image parameter settings were filter width 15, spot threshold 6, minimum spot width 4, minimum filter width volume 15, and minimum spot size 60. A 2D SDS-PAGE reference image was from sixty micrograms of unextracted Golgi membrane. Panel A shows the 2D pattern from 13.1 µg 15% LDAO soluble Golgi proteins. A composite image of the analysis of unextracted rat liver Golgi proteins, LDAO extracted protein, Triton X-100 extracted proteins and Triton CF-54 extracted proteins gave very similar 2D patterns (for discussion see text). Panel B illustrates a typical diagrammatic representation plotted for these samples obtained from the digital image analysis on the Bio Image Visage 110. This pattern is from the LDAO extracted Golgi proteins. The boxed area on the plot indicates the pI and MW range of the purified SAT-1, a 60,000 dalton glycoprotein in the pI range of approximately 5.7 to 6.2 (4). The molecular weight markers 200Kd, myosin; 81Kd transketolase; 40.5Kd, creatinine phosphokinase; 29Kd, phosphoglucomutase; and 17.5Kd myoglobin. The carbamylation standards for determining pI were creatinine phosphoinase (pI4.9-7.1); glyceraldehyde-3-Phosphatedehydrogenase (pI 4.8-8.3) and carbonic anhydrase (pI 4.7-6.7).

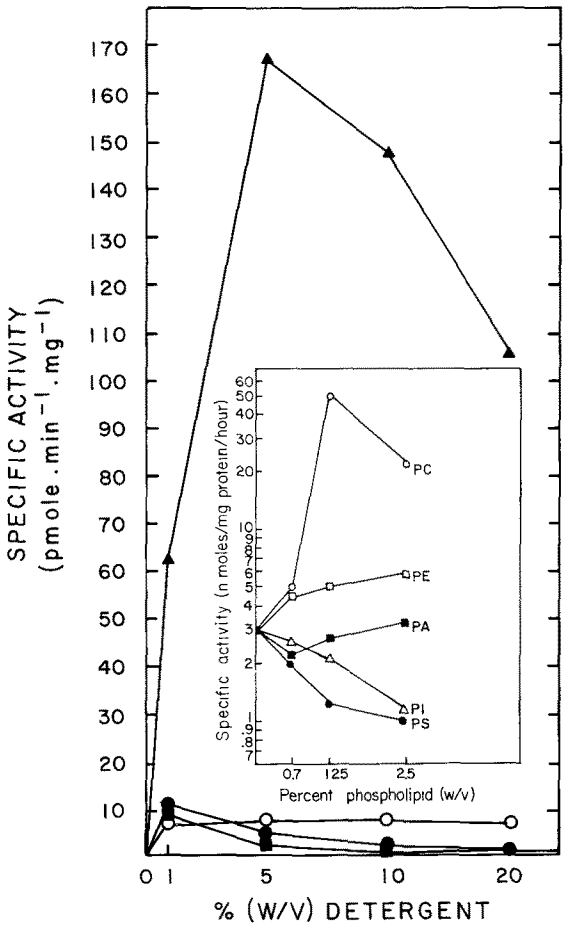

Figure 2. Effects of Various Surfactants on SAT-1 Activity in Rat Liver Golgi. Golgi vesicles were prepared from rat liver by well-established procedures (5-7) and is described in detail elsewhere (4). Increasing concentrations of LDAO, Triton CF-54, Triton X-100 and β-octylglucoside were added to standard reaction mixtures (4,8-9) with Golgi-encriched microsomes as the enzyme source and assayed immediately. The GM_3 product was recovered as described (8) and the SAT-1 activity determined. The inserted panel serves for comparison of SAT-1 activated by LDAO and PC and is reproduced with permission from Journal Lipid Research 25, 1541-1547 and the authors (21).

LDAO remains tightly associated with the enzyme, a factor which may complicate some protein analyses. Several methods have been explored for the removal of LDAO. LDAO will dialyze, but the process is slow. Extracti-Gel D (Pierce Chemical Co.) is not a viable alternative for SAT-1 purification because both LDAO and the hydrophobic SAT-1 sialytransferase are adsorbed. TCA precipitation is ineffective. Further, analysis of the glycan residues on SAT-1 (4) indicate SAT-1 is a glycoprotein containing sialic acid. The use of TCA to precipitate sialylated proteins for carbohydrate analysis is not recommended as sialic acid hydrolysis occurs under the acidic conditions. Combined ethanol and acetone precipitations, which serve to precipitate proteins by changing their solvation properties, removes sufficient LDAO from SAT-1 sialyltransferase to allow SDS-PAGE gel electrophoresis. For electrophoretic analysis of SAT-1, 5-20 μg of the sialyltransferase is precipitated in 90% (v/v) ethanol at -20°C for 36-48 hrs followed by centrifugation at 14,000 x g for 30 min. The ethanol is carefully removed and the pellet taken to dryness in a Speedvac. The sialyltransferase pellet is resuspended in HPLC-grade acetone to desalt. Precipitation

TABLE 1

STABILITY OF LDAO SOLUBILIZED SAT-1 IN 15%(V/V) LDAO AT -20°C FOR 6 MONTHS

Fraction	Specific Activity t_0	Specific Activity t_6	% Activity Remaining after 6 mos. at -20°C
CH	0.43	0.07	16 5%
PMS	0.74	0.23	31.3%
DE	0.52	0.56	106.3%
CMPI(NaCl)	1036	1170	113%
CMPII(CMP)	5110	4980	97.5%
LacCer(CMP)	15350	15640	102%

of SAT-1 in acetone is allowed to proceed at -20°C for 18 hrs. Good resolution of SAT-1 by 2D SDS-PAGE is achieved (4). However, the hydrophobic nature of LDAO allows some of it to co-precipitate with SAT-1 even in ethanol/acetone.

LDAO at high concentration is toxic. The LD_{50} of LDAO is 3.6 g/kg (value supplied by the manufacturer). For immunological work, LDAO can be exchanged with PC at a minimum of a 1:10 ratio of detergent to phospholipid and dialyzed. The exchange of PC for LDAO decreases the toxicity of the immunogen. Further, incorporating SAT-1 antigen into PC liposomes has the advantage that the half-life of the antigen in circulation is increased. This methodology permitted us to raise a monoclonal antibody to rat liver SAT-1 (4).

The most effective means of LDAO removal is reverse-phase HPLC. Resolution of purified SAT-1 sialyltransferase from LDAO is achieved on a C_8 microbore HPLC system (Applied BioSystems). SAT-1 in 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO is applied onto an Aquapore C_8, RP300 (250 x 1.0 mm x 7 μm) reverse-phase microbore column and eluted on a 90 min linear gradient established between 0.1% (v/v) trifluoroacetic acid and 90% (v/v) acetonitrile. The differences in hydrophobicity permit the separation of SAT-1 from LDAO (Figures 3A and 3B). SAT-1 sialyltransferase exhibits greater hydrophobicity than LDAO and elutes at 76% and 82 % of the B solvent (i.e., 68.4% and 73.8% acetonitrile). LDAO resolves into several peaks, with the majority of the detergent eluting from 47% to 54% of the B solvent (i.e., 42.3 to 48.6% acetonitrile). The two SAT-1 peaks, 56Kd and 60Kd by SDS-PAGE, respectively, were verified to be immunologically reactive on Western Blots with M12CG7, a specific anti-SAT-1 monoclonal antibody (4). The 56Kd polypeptide may be a proteolytic product of the 60Kd SAT-1. SAT-1, like other purified sialyltransferases, is subject to degradation by an associated endogenous proteolytic activity (for review, 22 and references therein). Further, this activity is present even after one week of storage at -80°C following SAT-1 purification. One possible explanation is that a brush border protease associates with SAT-1 via the LDAO detergent, enhancing its stability and activity. Preliminary investigation by limited digestion of the HPLC 56Kd and 60Kd, M12GC7 positive, SAT-1 polypeptides with 0.2 units cathepsin D and 2 units N-glycanase for 30 minutes at 37°C prior to SDS gel electrophoresis resulted in formation of 56Kd proteolytic and 43Kd deglycosylated polypeptide/products from both protein

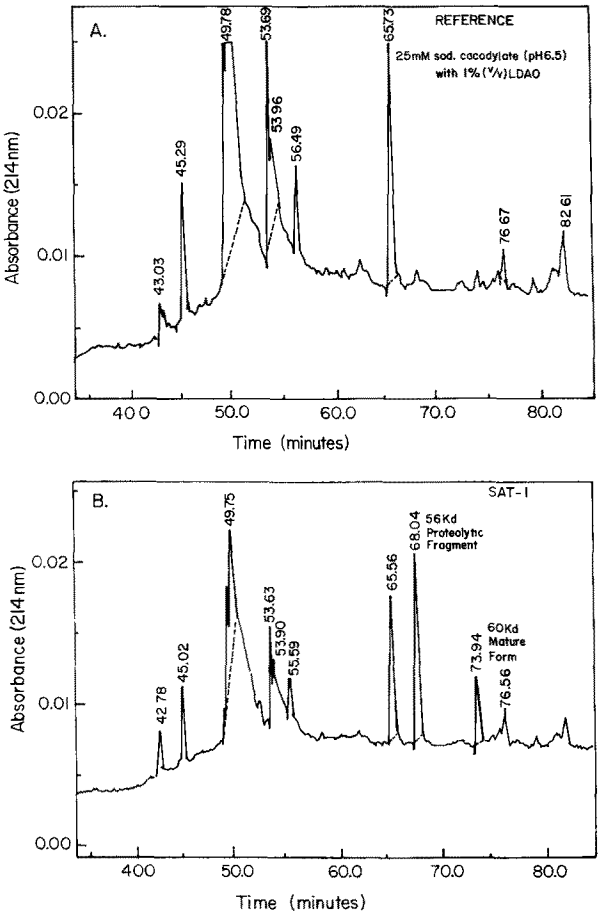

Figure 3A and 3B. Reverse-phase C_8 HPLC of SAT-1 vs. Reference Buffer Containing 5% (v/v) Lauryldimethylamine Oxide (LDAO). Resolution of purified SAT-1 sialyltransferase from LDAO is achieved on a C_8 microbore HPLC system (Applied Biosystems). SAT-1 in 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO is applied onto an Aquapore C_8, RP300 (250 x 1.0 mm x 7 μm) reverse-phase microbore column and eluted on a 90 min linear gradient established between 0.1% (v/v) trifluoroacetic acid and 90% (v/v) acetonitrile. Sample volume injected was 250 μl. Panel A illustrates a typical chromatogram obtained from buffer containing LDAO. B shows the resolution of unique SAT-1 proteins from the same LDAO containing buffer.

peaks obtained by HPLC. The endogenous proteolytic activity is suspected to be a cathepsin D-like activity (23) and is believed to be important in the release of the soluble catalytic domain of the glycosyltransferase from the membrane anchor (22).

One other consideration for the use of LDAO is important. Since it is an amphipathic compound, some is recoverable from SAT-1 product assays on Sep Pak C_{18} cartridges. This does not present a problem for optimal recovery of the GM_3 product under the conditions described by Melkerson-Watson *et al.* (9). We recommend, however, that an alternative method of analysis be employed for studies of enzyme specificity as the LDAO and PC, used in the Sep Pak C_{18} recovery, have relative mobilities on HPTLC in the range of some glycolipid substrates. Modifications of the method of Yu *et al.* (24), reported elsewhere (4), were well-suited for analyzing sialyltransferase activity with glycolipids chromatographed on HPTLC plates.

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