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QUARTERLY

Hydrophobic nature of mammalian ceramide glycanases: Purified from rabbit and rat mammary tissues*°

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The ceramide glycanase (CGase) activities, which cleave the intact oligosaccharide chain and the ceramide moiety of a glycosphingolipid, have been characterized from two mammalian sources. The enzymatic activities are almost comparable in rabbit and rat mammary tissues. The majority of the activities has been concentrated in the soluble fraction which could be partially purified using hydrophobic columns. The rabbit mammary ceramide glycanase activity has been purified up to 1438-fold using ion exchange and hydrophobic columns in tandem. The purified protein exhibited a molecular mass of 54 kDa which could be immunostained on the Western blot with clam anti-CGase polyclonal antibody. In addition, a 98 kDa protein also exhibited positive immunostain in a successive purified fraction with that antibody and is under investigation. The requirement for the optimal enzymatic activities are similar for both rabbit and rat CGase activities. The CGase activity requires the presence of detergent for optimal activity but is not dependent on the presence of any divalent cations. However, Hg^{2+}, Zn^{2+}, and Cu^{2+} are inhibitory to the enzymatic activities. It has been observed that rat as well as rabbit CGases are inhibited by both D- and L-PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl) and its higher analogue PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol·HCl). Alkyl amines containing C_{12} and higher chains are also found to inhibit both rat and rabbit CGase ac-

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Abbreviations: CARS, carbohydrate-site recognizing enzyme; CGase, ceramide glycanase; EGCase, endoglyceroceramidase; FACE, fluorophore assisted carbohydrate electrophoresis; GSL(s), glycosphingolipid(s); PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl; PPMP, 1-phenyl-2-palmitoyl-amino-3-morpholino-1-propanol·HCl; RMH, rabbit mammary homogenate; RMS, rabbit mammary supernatant; RMSH, heat-treated RMS; RMSHAc, acid RMSH; TNF, tumor necrosis factor.

tivities. Substantial levels of CGase activities have also been observed in various human tumor cells as well as in developing avian brains. These observations are significant in view of the recent findings that ceramide, which is one of the enzymatic reaction products of CGase activity, is mediating different cellular events like signal transduction and apoptosis. The role of this enzyme in development, metastasis and cellular regulation are anticipated.

Recent interest in glycosphingolipid research has taken a turn toward the investigation of their functional aspects. A number of biological and pathological functions are attributed to different glycosphingolipids. The role of oligosaccharide moieties of glycosphingolipids in antigenicity and tumorigenicity is well accepted [1, 2]. The expression of various gangliosides on the cell surfaces has been correlated with cell transformation and tumor progression [3]. Gangliosides also serve as differentiation markers and they are known to participate in various cell regulatory functions [4]. Previously, very commonly occurring glycoconjugate epitope sialyl Le^x has been implicated in receptor function during lymphocyte homing [5, 6]. More recently, other related glycolipids, sialyl Le^a and a higher analogue of sialyl Le^x present in various tumor cells have been shown to bind to the vascular endothelium with equal affinity [7, 8].

Ceramide and its breakdown products sphingosine, dimethyl sphingosine, and sphingosine-1-phosphate, have been emerging as the key molecules in various signaling pathways [9–12]. The ceramide has recently been shown to be involved in the process of programmed cell death or apoptosis also [13, 14], and the major pathway for the production of ceramide is believed to be the hydrolysis of sphingomyeline by the action of neutral sphingomyelinase [15]. Various tissue factors like TNFα and others are also involved in these signaling processes by stimulating the sphingomyelinase [16, 17]. The recent discovery of mammalian ceramide glycanase which cleaves glycosphingolipids (GSLs) in a one-step process liberating ceramide [18] could also play a significant role in cellular signaling processes. This enzyme was reported almost simultaneously from bacteria [19, 20] and leech [21, 22]. Later the same activity was reported from earthworm [23, 24] and clam [25, 26]. The endoglycoceramidase (EGCase) from *Rhodococcus* species has been extensively purified and found to contain three molecular species with unique specificities [19, 20, 27].

The first evidence for the mammalian CGase came from our laboratory with the characterization of the enzyme in rabbit mammary tissues [28]. The rationale for the presence of this enzyme in mammary tissue came from the fact that the milk is enriched in lactose and oligosaccharides containing glucose as reducing sugar. So it was anticipated that there might be a high turnover for both synthesis and degradation of GSLs in mammary tissues. Recently, CGase activity has been characterized and partially purified from another mammalian source, rat mammary tissue [18]. The enzyme is ubiquitous in nature in different organs of rat though the highest activity was observed in the mammary tissues. We have also observed that the activity of CGase gradually increases during gestation period and stays high during lactation whereas virgin rat mammary tissue has a minimum level of activity. It is important to investigate whether the CGase is hormonally regulated as is reported for bovine mammary glucosidase 1 [29].

The reaction catalyzed by ceramide glycanase is given in Scheme 1:

MATERIALS AND METHODS

Materials

Lactating rabbit mammary tissue were purchased from Pel-Freez (Rogers, AR, U.S.A.). Rat mammary tissues of different days of gestation and lactation have been obtained from the Frieman Life Science Centre at the University of Notre Dame (Notre Dame, IN, U.S.A.). Galactose oxidase, Triton X-100, sodium taurocholate and taurodeoxycholate were purchased from Sigma Chemical Company (St. Louis, MO). Tritiated sodium borohydride ($NaBT_4$) was purchased from Du Pont Company (Boston, MA). All other chemicals used were of reagent grade. Pentaglycosylceramide (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; nLcOse5Cer) was isolated from bovine blood and purified by previously published methods [30, 31]. Lacto-neo-tetraosylceramide (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; nLcOse4Cer) was obtained from partial hydrolysis of nLcOse5Cer using α-galactosidase isolated from fig following published methods [32].

Gangliotetraosylceramide (Galβ1-3GlcNAcβ-1-4Galβ1-4Glc-Cer; GgOse4Cer) was obtained by formic acid-hydrolysis of ganglioside, G_{M1} isolated from bovine brain and purified as previously described [33]. All other glycolipids used in this study were prepared according to published methods [30, 34].

Radiolabeling of the substrate

The glycosphingolipid substrates were tritiated at the double bond of ceramide according to the method of Schwarzman & Sandhoff [35]. Briefly, 1 mg of nLcOse5Cer (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), GgOse4Cer (Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer), or any other glycolipid was suspended in tetrahydrofuran and radiolabeled with $NaBT_4$ in the presence of palladium chloride as catalyst at room temperature for 6 to 8 h with shaking followed by treatment with excess $NaBH_4$ for another 4 to 6 h. The unreacted $NaBT_4$ was removed by passing the reaction mixture through a SepPak C-18 column. The labeled GSL was further purified by Biosil column, and specific activity was determined with a densitometric scanner before using as a substrate [18, 25].

CGase purification from mammary tissue

The CGase from rat mammary tissue has been prepared as described recently using hydrophobic column [18]. Rat mammary tissues are homogenized in 20 mM Hepes buffer containing 0.32 M sucrose using Polytron 10 ST. The supernatant, buffy coat, and pellets are separated after the homogenate is spun at 100000 × ***g*** for 1 h. The soluble supernatant has been purified through hydrophobic columns for part of the study. The supernatant is passed through an octyl-Sepharose column which has been equilibrated with high salt concentration (1 M sodium chloride) in 50 mM Hepes buffer, pH 7.0, containing 0.1% β-mercaptoethanol.

The enzyme, bound to the column, has been eluted with 1% octylglucoside in the same buffer in absence of salt. Recently we have used phenyl-Sepharose column and the bound CGase activity eluted in the same fashion. All procedures were performed at 4°C, unless mentioned otherwise.

Lactating rabbit mammary tissues (30 g) were homogenized in 4 volumes of a buffer containing 0.32 M sucrose, 1.0% dextran, 0.1% β-mercaptoethanol, 10 mM $MgCl_2$ and 20 mM Hepes, pH 7.0 (Buffer A). Homogenization was performed with a Polytron 20ST (Kinematica, Lucerne, Switzerland) followed by a Potter-Elvehjem tissue grinder. The rabbit mammary homogenate (RMH) was centrifuged for 60 min at 100000 × ***g***. The resulting supernatant (RMS) was collected, incubated at 60°C in a water bath for 1 min, and recentrifuged for 30 min at 100000 × ***g***. The heat treated supernatant (RMSH) was adjusted to pH 5.0 by the dropwise addition of acetic acid.

After stirring for 30 min at 4°C, this mixture was centrifuged for 30 min at 100000 × *g*. The resulting heat-treated, acid-supernatant (RMSHAc) was decanted and could be stored at −70° for more than 2 years.

S-Sepharose column chromatography. RMSHAc fraction was applied to a 1.5 cm ×15 cm S-Sepharose Fast Flow column, pre-equilibrated in 20 mM Hepes, pH 6.0, containing 0.1%, dextran and 1 mM β-mercaptoethanol (Buffer B). After collecting the effluent, the column was washed extensively with Buffer B until no protein could be detected by the UV monitor at 280 nm. Proteins were eluted off the column with a linear gradient of NaCl (0–1.0 M) in Buffer B. Absorbance was monitored at 280 nm and fractions containing protein were collected (S-Seph. elu.) and assayed for CGase activity as described below.

Octyl-Sepharose column chromatography. CGase active fractions from the S-Sepharose eluent were pooled together, and ammonium sulfate was added to the pooled sample to a final concentration of 1.0 M. The sample was then loaded on an octyl-Sepharose column (1.0 cm × 10 cm) which was pre-equilibrated with Buffer B containing 1.0 M ammonium sulfate. After extensively washing the column with the eqiulibration buffer, proteins were eluted off the column with a reverse gradient of ammonium sulfate (1.0–0 M). Absorbance was monitored at 280 nm and fractions were assayed for CGase activity.

HPLC size-exclusion column chromatography. CGase active fractions from octyl-Sepharose eluent were pooled and concentrated over a Diaflo PM30 membrane (Amicon, MA) and applied onto an Ultraspherogel SEC 3000 column at a flow rate of 1 ml/min. The column was eluted with Buffer B, and the elution of proteins was monitored at 280 nm. Peaks were collected, concentrated by Centricon 30 and assayed for CGase activity.

SDS-PAGE analysis. Samples of CGase active eluents from S-Sepharose, octyl-Sepharose, and Ultraspherogel SEC 3000 columns, as well as the heat and acid-treated rabbit mammary supernatant, were electrophoresed on a 10% slab gel of 0.75 mm thickness under reducing conditions according to Laemmli [36]. The gel was stained with 0.2% Coomassie Brillant Blue G-250 for visualization of the protein bands [37].

Western-immunoblot

The rat or rabbit ceramide glycanase was resolved on 10% polyacrylamide gel/sodium dodecylsulfate according to the published method as described above and the proteins were identified on one half of the gel using Coomassie Blue stain [36, 37]. The protein bands from the other half of the gel were transblotted onto a nitrocellulose paper at 80 volts for 1 h in Tris/glycine/methanol (pH 8.0) buffer. The blot was immunostained with anti-clam CGase antibody. In brief, the nitrocellulose paper was first treated with 1% milk in phosphate-buffered saline (PBS) for 1 h at 37°C, followed by anti-CGase antibody at a specific dilution for the same period of time. The blot was washed three times with PBS containing 0.1% Tween 20, after which it was treated with horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 h at 37°C. After washing off the second antibody with PBS, the antibody-conjugated protein band was visualized using 4-chloro-1-naphthol reagent.

Enzyme assay

Assay conditions with radiolabeled substrates. The incubation mixture contained the following components in micromoles, unless otherwise stated, in a final volume of 50 μl: [^{3}H]GSL substrate (nLcOse5Cer), 1 nmol (20 × 10^6 c.p.m./μmol); detergent, taurodeoxycholate, 20–30 μg; sodium acetate buffer, pH 5.5, 10 μmol; enzyme protein up to 50 μg. After incubation of 2–4 h at 37°C, 50 μl each 2-propanol and hexane was added to the reaction mixture, vortexed, and spun at 5000

r.p.m. for 5 min. The upper layer was then quantitatively spotted on SG81 paper, and descending chromatography was performed using chloroform/methanol (9:1, v/v) solvent. The cleaved ceramide moved one inch behind the solvent front which was then quantitatively determined using toluene scintillation counting technique [18].

Previously FACE (Fluorophore Assisted Carbohydrate Electrophoresis) N-linked oligosaccharide profiling kit (Glyko Inc.) was used in our laboratory for the analysis of the oligosaccharides generated by the action of purified clam ceramide glycanase when unlabeled glycolipids were used. Fluorophore labeling of the cleaved oligosaccharides were performed using ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid), without any modification, as described in the instruction manuals accompanying the kits and electrophoresed [25]. The oligosaccharide profiling gels were analyzed using FACE Imager and FACE Analytical Software (Glyko, Inc.).

RESULTS

Purification and characterization of mammary CGase

The majority (65%) of ceramide glycanase activity of both rabbit and rat mammary tissues was found to be concentrated in the soluble supernatant fraction after the mammary homogenate was centrifuged at 100000 × *g*.

Further purification of the enzyme was carried out using this 100000 × *g* supernatant fraction in both cases. The CGase protein from both rat and rabbit tissues was found to be hydrophobic in nature and the enzyme was partially purified using hydrophobic column. The partial purification of the rat mammary CGase has been reported recently [18]. Hence, the detail purification of the rabbit mammary CGase is being reported in the present article. The CGase activity, in general, was found to be partially heat stable [18, 28] and that particular characteristic of the protein was utilized for purification of rabbit mammary enzyme. As seen in Table 1 about 2-fold purification of the rabbit mammary CGase was obtained without any loss of enzyme activity when the supernatant was heated at 60°C for 1 min. The heat-treated protein was then pH-adjusted with acetic acid before it was purified on an ion-exchange column. The S-Sepharose eluent fraction was further purified on a hydrophobic octyl-Sepharose column. The rat mammary CGase was also purified using the same hydrophobic column. However, 1% detergent was needed to elute the rat mammary CGase from this hydrophobic column whereas the rabbit mammary CGase eluted out from the octyl-Sepharose column just by elimination of the salt from the wash buffer. That indicates the stronger hydrophobic nature of the rat CGase. The final purification of the rabbit CGase was obtained using spherogel SEC 3000, and approximately 1438-fold purification was achieved for rabbit mammary ceramide glycanase as seen in Table 1.

The purified CGase was found to be a 54 kDa protein (fraction E, Fig. 1a) which could be immunostained with clam anti-CGase antibody. However, in the successive Spherogel SEC 3000 fraction (fraction F, Fig. 1a) a 98 kDa protein also co-immunostained in addition to the 54 kDa protein (Fig. 1b). The molecular mass of clam CGase was found to be around 64 kDa [26] and that for rat CGase was found to be 63 kDa [18].

Different fractions from rabbit mammary tissues were tested for exoglycosidase activities during purification using *para*-nitrophenyl-sugar substrates as indicated in Table 2. As seen in the Table 2, majority of the exoglycosidase activities were completely separated from the CGase activity. The most purified fraction obtained after spherogel SEC 3000, was almost devoid of exoglycosidase activities.

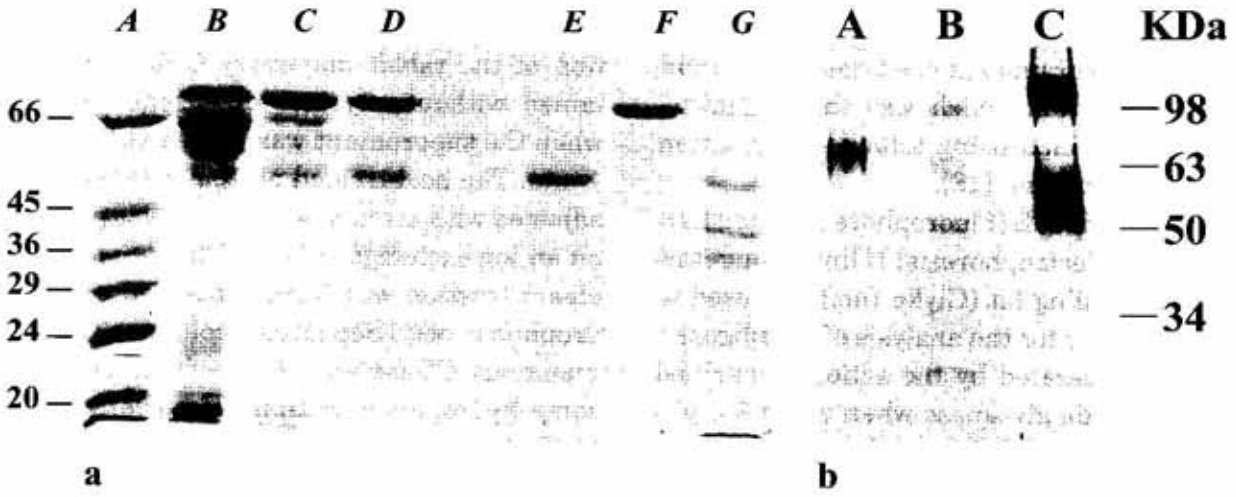

Figure 1 (a and b). SDS/PAGE-Western blot analysis of rabbit ceramide glycanase.

a. Different fractions of rabbit mammary (RM) CGase has been used for SDS/PAGE analysis. After electrophoresis the gel is stained with Coomassie Blue. The samples shown on the gel are as follows: Lane A, molecular mass standard; Lane B, heat-treated RM CGase; Lane C, S-Seph. efflu.; Lane D, S-Seph.elu.; Lanes E and F, Spherogel SEC elus. P1 and P2; Lane G, mixed fractions. b. Western-immunoblot of clam and rabbit mammary CGases. The SDS/PAGE electrophoresed samples have been electroblotted on nitrocellulose membrane as described in the text and immunostained with anti-CGase antibody against clam CGase. Lane A, purified clam CGase fraction; Lane B, molecular mass standard; Lane C, purified rabbit mammary CGase fraction (fraction F of Fig. 1a).

Table 1. Purification of rabbit ceramide glycanase.

The purification method of rabbit CGase is described in the text. The substrate used for this purification procedure is GgOse4[^{3}H]Cer. Different fractions are identified as follows: RMH, rabbit mammary homogenate; RMS, rabbit mammary supernatant; RMSH, rabbit mammary heat treated supernatant; RMSHAc, rabbit mammary heat-, and acid-treated supernatant; S-Seph., S-Sepharose eluent; Octyl-Seph., octyl-Sepharose eluent; SEC 3000, SEC 3000 eluent.

Fraction	Tot. vol. (ml)	Tot. prot. (mg)	Tot. act. (pmol)	Sp. act. (pmol/mg)	Recovery %	Purity fold
RMH	100	2007.0	28880	14.4	100	1
RMS	72	702.7	84326	120.0	292	8
RMSH	68	321.0	80730	251.5	277	18
RMSHAc	66	221.1	85180	385.3	295	27
S-Seph.	36	23.8	25891	1087.9	90	76
Octyl-Seph.	78	3.32	10469	3153.2	36	219
Secretary 3000	12	0.32	6634	20731.3	23	1438

Table 2. Exoglycosidase activities in purified rabbit CGase fractions.

The exoglycosidase activites of different fractions from rabbit mammary tissue were measured during purification using synthetic substrates *para*-nitro-phenylglycosides. After incubation for 2 h at 37°C (pH 4.0) the cleaved *para*-nitrophenol was measured by addition of sodium carbonate at 400 nm.

Substrate (0.5 mM)	RMSHAc	S-Seph. Eluent	SEC
	(nmol/h)	(nmol/h)	(nmol/h)
pNP-β-D-Gal	2.93	0.63	0.17
pNP-α-D-Gal	0.00	0.00	0.00
pNP-β-D-GlcNAc	1.91	0.00	0.00
pNP-α-D-GlcNAc	0.00	0.00	0.00
pNP-β-D-GalNAc	6.34	0.53	0.12
pNP-α-D-GalNAc	4.59	0.00	0.00
pNP-α-L-Fuc	1.01	0.29	0.00
pNP-β-D-Glc	0.20	0.00	0.00

Requirement for CGase

The requirement for the optimal CGase activity from rabbit mammary tissues is shown in Table 3. Taurodeoxycholate was found to be the best detergent for optimal rabbit CGase activity compared to either taurocholate or Triton X-100 as indicated in the Table.

Similar detergent requirement was also found for rat CGase [18]. The optimum pH for both rat and rabbit CGase activities was found to be between 5.0 and 5.8 in sodium acetate buffer.

Either heat-killed (5 min at 100°C) or trypsin-treated (1 h at 37°C with 2 mg/ml trypsin) rabbit CGase was completely inactive for the hydrolysis of the labeled glycolipid substrate (Table 3). Like all other CGase activities, rat as well as rabbit CGase activities were not

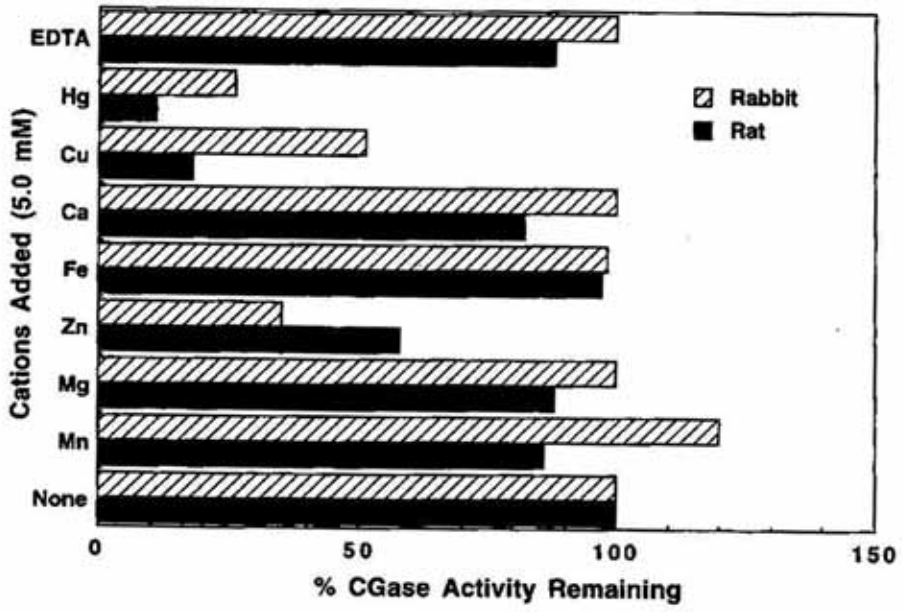

Figure 2. Effect of divalent cations on mammalian CGase activities.

Assay conditions are the same as described in the text using nLcOse5[^{3}H]Cer as substrate. Different divalent cations are used as indicated.

Table 3. Requirement of rabbit CGase.

The complete incubation mixture contained the following in a final volume of 50 μl [^{3}H]GSL substrate (nLcOse5Cer), 1 nmol (20×10^6 c.p.m./μmol); detergent, taurodeoxycholate, 20–30 μg; sodium acetate, pH 5.0, 20 μmol; enzyme protein up to 50 μg (RMS fraction). After incubation for 4 h at 37°C, 50 μl each 2-propanol and hexane were added to the incubation mixture, vortexed, and spun for 5 min at 5000 r.p.m. The upper layer was quantitatively spotted on SG81 paper and chromatographed in descending fashion using chloroform/methanol (9:1, by vol.) solvent. The cleaved ceramide moved near the solvent front and quantitatively estimated using scintillation technique.

Incubation conditions	[^{3}H]Ceramide released (nmol/mg protein per 2 h)
Complete mixture	3.05
minus TDC	1.0
minus TDC plus TC	0.1
minus TDC plus Triton X-100	0.08
plus Trypsin treated enzyme (2 mg/ml)	0.4
plus heat inactivated enzyme	0.2

TDC, taurodexycholate.

stimulated by any divalent cations neither were they inhibited in the presence of EDTA. However, some divalent cations were inhibitory to both rat and rabbit CGase activities as seen in Fig. 2.

Mercuric ion was found to be the most inhibitory to both rat and rabbit CGase activities as found for all other CGase proteins (Fig. 2). Whether this common mercury ion inhibition of CGase activities from all different sources is indicative of some structural similarities has yet to be investigated.

Substrate specificity of CGase activities

Various glycosphingolipid substrates with labeled ceramide (at sphingosine double bond) were tested with rabbit ceramide glycanase. The V_{max} values for different substrates used with rabbit CGase were found to be comparable as seen in Table 4. However, the K_m values for different substrates were different. The apparent K_m value for GgOse4Cer was found to be 0.27 mM with rabbit ceramide glycanase (Fig. 3).

We have used both galactose[6-^{3}H]-nLcOse5Cer and -GgOse4Cer as substrates with rabbit enzyme and corresponding cleaved oligosaccharides comigrated with standard oligosaccharides on thin-layer plate after developing with butanol/acetic acid/water (2:1:1, by vol.).

Table 4. Substrate specificities of rabbit ceramide glycanase activity.

The assay conditions are the same as described in the text except that different labeled glycolipid substrates were used.

Substrates (0.3 mM)	[^{3}H]Ceramide produced (nmol/ml per h)
Endogenous	0.2
Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-[^{3}H]Cer (nLcOse5Cer)	6.1
Galβ1-3GalNAcβ1-4Galβ1-4Glc-[^{3}H]Cer (GgOse4Cer)	6.3
Galβ1-3GalNAcβ1-4Galβ1-4Glc-[^{3}H]Cer I α2-3 NeuAc (G_{M1})	4.2
Galβ1-4Glc-[^{3}H]Cer I α2-3 NeuAc (G_{M3})	5.4

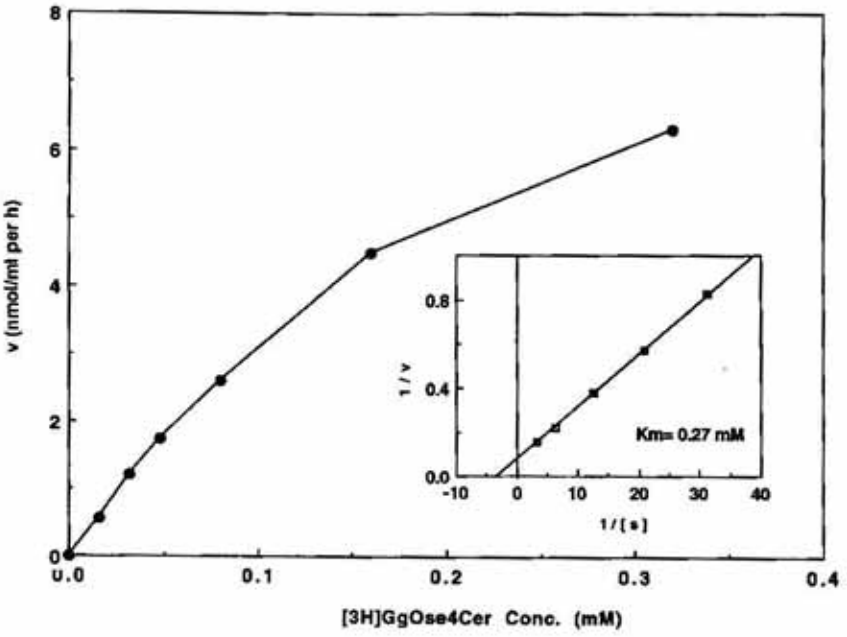

Figure 3. Substrate saturation of rabbit CGase with [^{3}H]GgOse4Cer

Assay conditions are the same as described for Fig. 2 except that different concentrations of [^{3}H] GgOse4Cer has been added in the incubation mixture as indicated.

Thin-layer chromatographic (TLC) assay was used when unlabeled substrates were tested. The cleaved ceramides were identified on TLC using Coomassie stain [38] when globo-, lacto, and ganglio-series glycolipids were tested as substrates with CGases from both rat and rabbit mammary tissues (not shown). The rabbit mammary CGase appears to cleave the acidic glycolipids with almost equal specificity also when radiolabeled (at sphingosine doublebond) substrates were used (Table 4). The characterization of oligosaccharides using FACE technology has been under investigation in the purified enzyme preparation of both rat and rabbit mammary CGase fraction as used previously for clam CGase [25]. Exo β-galactosidase inhibitor p-NH_2-β-galactose did not inhibit either rat or rabbit CGase activities and was added to the incubation mixture routinely to inhibit any β-galactosidase activity.

Inhibitors of CGase activities

The glycolipid:glycosyl transferases have been classified as CARS (carbohydrate-site recognizing enzyme) and HY-CARS (hydrophobic as well as carbohydrate-site recognizing enzyme) according to their substrate recognition site specificities [39]. The CGase, an enzyme which cleaves between hydrophobic ceramide moiety and the carbohydrate chain of a glycosphingolipid might also exhibit such a specificity. PDMP (1-phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl), the well-known inhibitor for GSL biosynthetic enzyme glucosyltransferase (GlcT-1), [40] and its analogue PPMP (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol·HCl) [41, 42] has been used to study the inhibition of CGase activity. Previously, both D- and L-PPMP had been shown inhibitory to the rat mammary CGase activity [18] and this was confirmed in the present work (Fig. 4a). It is apparent from the Figure that inhibition by PPMP is stronger than inhibition by both D- and L-PDMP. The same observation is also true for the rabbit mammary CGase activity (Fig. 4b). However, quite high concentrations of both PDMP and PPMP are needed in either case for inhibition of CGase activity which is in contrast to the D-PDMP concentration needed for inhibition of glycosyltransferases [43]. L-PDMP rather stimulated GlcT-1 activity at the concentrations tested.

The inhibition by PPMP is also found with purified clam CGase and the inhibition is of mixed nature [26]. Recently, myristoyl-amino-

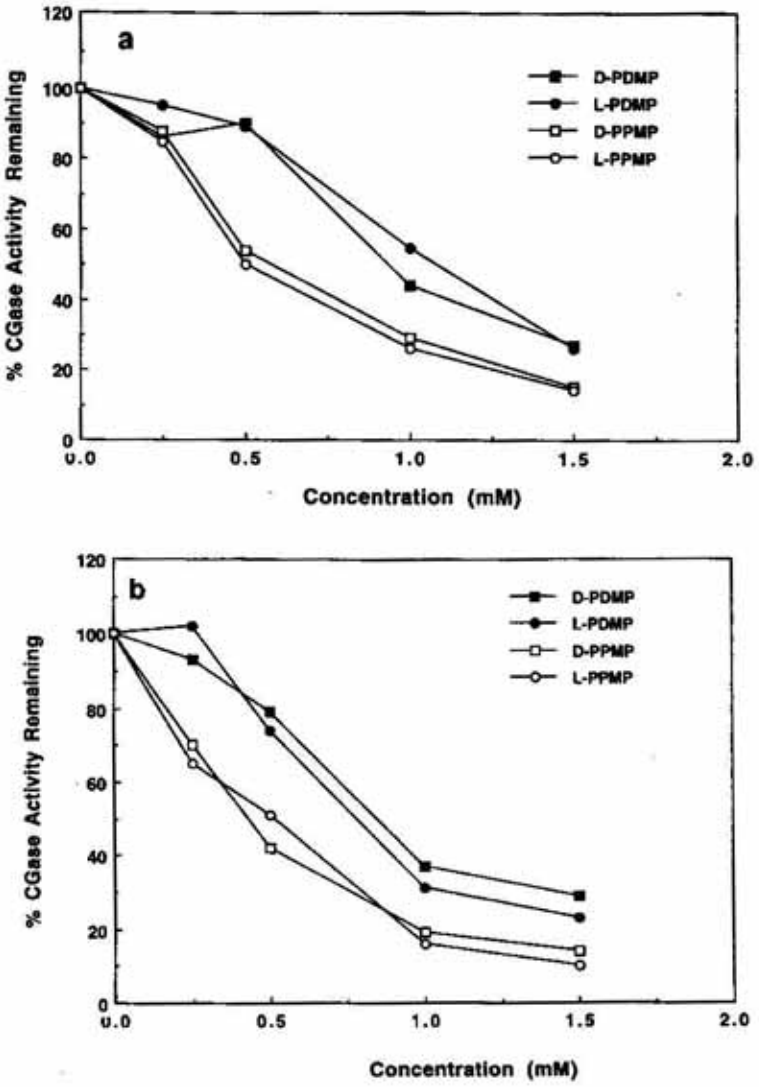

Figure 4 (a and b). Inhibition of mammalian CGase activities by PDMP and PPMP.

a. Rat CGase; b. Rabbit CGase. Different concentrations of D- and L-*threo*-PDMP and PPMP were added in the incubation mixture under the assay conditions as described in the text using nLcOse5[^{3}H]Cer as substrate.

propanol has been shown to inhibit the ceramidase activity [44]. A similar kind of inhibition has also been observed with cerebroside, sphingosine, and ceramide when either rat or rabbit CGase fractions are used as enzyme (not shown).

These results in combination with stronger inhibition of CGase activity by PPMP sug-

Table 5. Ceramide glycanase activities in carcino-embryonic cells.

The assay conditions are the same as described in the Method section.

Source	Ceramide glycanase activity* (nmol/mg per 2 h)
Rat mammary tissue	5.6
Rabbit mammary tissue	4.1
Human colon carcinoma cells (Colo-205)	3.6
Human neuroblastoma cells (IMR-32)	2.8
Embryonic chicken brains (15-day-old)	3.5

*Substrate: nLcOse5[^{3}H]Cer (0.1 mM); the values are mean of three or more experiments.

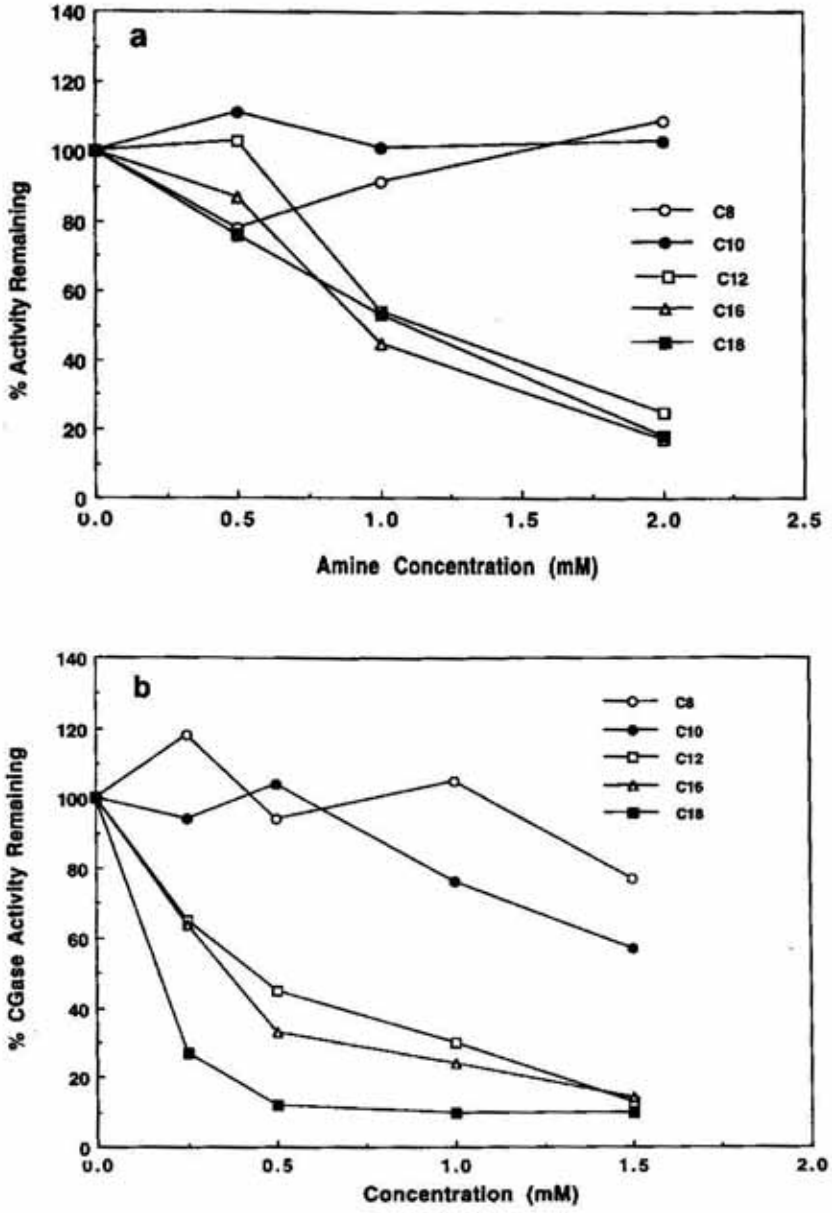

Figure 5 (a and b). Alkyl amine inhibition of mammalian CGase activities.

a. Rat CGase; b. Rabbit CGase. The assay conditions are the same as described in the text except that different concentrations of alkyl amines containing various fatty acyl chains have been added.

gests the possibility of involvement of the hydrocarbon side chain of the ceramide at the active site. Figures 5a and 5b show that alkylamines containing longer chains (C_{12} and higher) inhibited the CGase activities more than 90%. Similar results are also obtained when fatty alcohols of different chain lengths have been used (not shown). From these observations it is anticipated that either one of the hydrocarbon chains of ceramide might be effective in enzyme substrate recognition. Inhibition studies are in progress using substituted sphingosine with C_2 and higher hydrocarbon chains.

CGase activity in higher animals

It has been suggested recently that apoptosis which is a normal process in development and morphogenesis could contribute to pathological conditions when improperly regulated [45]. Our recent studies show that high levels of CGase activities are present in human colon carcinoma Colo-205 and also in human neuroblastoma IMR-32 cells when radiolabeled neolactopentaosylceramide (at the sphingosine double bond) is used as substrate (Table 5). The significance of such a high level of CGase activity in tumor cells is not under-

stood. These tumor cell CGase activities are also found to be inhibited by both PDMP and PPMP (not shown). Whether ceramide plays any role during development is also not known yet. However, it has also been shown in Table 5 there is significant CGase activity in embryonic chicken brains. Studies on the CGase activities from developing chicken brains indicate that there might be a gradual increase of CGase activity in embryonic chicken brain as previously found for GalT-3 (UDP-Gal:G_{M2} β1-3galactosyltransferase) [46–48].

DISCUSSION

The present report is mainly concerned with characterization, purification, and inhibition studies of the two mammalian ceramide glycanases. Purification of CGase was based on the intrinsic nature of the enzyme. Since the enzyme activity was found to be quite resistant to acidic pH (4–5) and heat treatment, precipitation with acetic acid and heat-treatment at 60°C were performed to remove other proteins from the crude enzyme fraction. This step provided considerable purification of rabbit mammary ceramide glycanase protein with 100% recovery of enzyme activity (Table 1). Further purification of CGase was achieved by S-Sepharose column chromatography followed by hydrophobic chromatography on an octyl-Sepharose column and finally gel-filtration chromatography on a HPLC size-exclusion column from which the CGase activity was recovered as a single peak. The purified protein showed one major band on SDS/PAGE with a molecular mass of 54 kDa (Fraction E, Fig. 1) which was immunoprecipitated with anti-clam CGase antibody prepared previously in our laboratory [26]. The associated exoglycosidase activities in the original enzyme preparation are removed by successive column chromatography (Table 2). Ceramide glycanase activities have been detected and characterized from various non-mammalian sources [19–27]. A comparison of the physical and biochemical properties of these enzymes with the CGase characterized and purified from rat and rabbit mammary tissues reveals important and interesting differences. The bacterial enzyme appears to exist as three distinct isoforms of which two (endoglycoceramidase I and II) have been further purified and characterized [19, 20, 28]. Except for the CGase from leech (330 kDa), the molecular mass of the other enzymes are similar (55.9 kDa for endoglycoceramidase I, 58.9 for endoglycoceramidase II, 43.7 kDa for earthworm CGase) [19–26] compared to that obtained from rabbit (54 kDa) (Fig. 1). Rat CGase exhibited a molecular mass about 63 kDa [18] and for clam CGase it has been found to be 64 kDa [26].

Most of the CGases are active at pH 5.0 except for earthworm and clam where the optimum pH was found to be 4.0 [23, 25, 26]. However, the mammalian ceramide glycanase seems to be optimum between pH 5.0 and 6.0 [18]. Although EDTA or most of the divalent cations have no effect on any of the CGases, all of them appear to be sensitive to the inhibition by Cu^{2+}, Hg^{2+} and Zn^{2+} ions, with varying extents (Fig. 2). It is possible that the heavy metal ions are binding to catalytically important residues on CGase or are disrupting an ordered structure in the protein, the maintenance of which is essential for catalysis. It appears that none of the CGases are active in the absence of detergents; however, the detergent requirements vary. Triton X-100 and sodium cholate (up to 0.4%) are the detergents of choice for the bacterial enzymes. Sodium cholate is also the most stimulatory detergent for both leech and earthworm CGase. Sodium taurodeoxycholate was the next best detergent for leech but had no effect on earthworm CGase which was stimulated by Triton X-100. For mammalian CGases, taurodeoxycholate has been found to be the best detergent and no effects are observed with either sodium taurocholate or Triton X-100 (Table 3). Such wide differences in detergent effects

in the presence of the same GSL substrate indicates that the detergent-enzyme interaction is defined by subtle but critical interaction between the functional groups of the detergent and amino-acid residues on the protein. The detergent probably mediates the role of an amphipathic, physiological activator(s) for this class of enzymes.

Substrate specificity studies indicate that, excepting for endoglycoceramidase II (which does not hydrolyze globo-series GSLs and cleaves sialyl-neolactotetrosylceramide most efficiently), all the other CGases work best with the ganglio-series glycosphingolipids. The leech CGase is reported to work better on globo-series GSLs than on the neolacto-core GLSs while the reverse is true for earthworm and rabbit CGases. However, the enzymes appear to differ widely in their kinetic properties. Thus the rabbit CGase has an apparent K_m of 0.27 mM for GgOse4Cer (asialo-G_{M1}; Fig. 3) which is comparable to the K_m's for G_{M1} for endoglycoceramidase I (0.1–0.25 mM) and endoglycoceramidase (0.2–0.5 mM) but is very different from the K_m for G_{M1} for leech (15.4 μM) and earthworm (16.7 μM) CGase. None of the CGases is active on galα-series GSLs or cerebrosides but cleave lactotriaosylceramide, thereby indicating a minimal requirement of a trisaccharide moiety in the glycan portion of the molecule. Both PDMP and its higher analogue PPMP inhibit the enzymatic reaction at high concentrations (Fig. 4) which is in contrast to the concentration needed for the inhibiton of the synthetic enzymes. The binding of the CGase protein to the hydrophobic column and inhibition by PDMP and PPMP indicates the presence of a hydrophobic center at the active site of the enzyme [39, 48]. To investigate this hypothesis, inhibition studies were conducted with substrate analogues. Both sphingosine and ceramide (non-hydroxy fatty acid or hydroxy fatty acid) inhibited both rat and rabbit mammary CGase activities (not shown). Alkyl amines and alkyl alcohols of different chain lengths have been used as inhibitors to investigate the importance of the hydrocarbon chains in these inhibitions. The alkyl amines of C_{12} and higher chain lengths inhibited the CGase activities (Fig. 5) and the same observation was true for alkyl alcohols. These inhibiton studies suggest that the two hydrocarbon tails of ceramide moiety in the substrate contribute significantly in the binding to the active center by hydrophobic interaction [39, 48]. Further experiments are needed to understand these interactions.

The presence of CGase activity in human tumor cells as well as its presence in developing avian brains indicate probable involvement of CGase and ceramide in developmental process. Previously we have reported that the CGase activity increases in rat mammary tissues with the progression of gestation, and during lactation it stays in high level which declines after lactation period ends. This indicates probable involvement of CGase during hormonal regulation also.

Identification of a ceramide glycanase from mammary tissues provides the first evidence that this activity is ubiquitously distributed across species, from bacteria to mammals and perhaps, possess considerable significance. The necessity to cleave intact oligosaccharides from glycolipids might arise to fulfill a diverse array of little-known physiological functions (such as metabolizing the glycosphingolipids of blood in leech, or enriching the milk with GSL-type oligosaccharide in rabbit mammary tissue). Ceramide, being a key modulator of apoptosis, might be involved in the initiation of a lytic cycle of the cancer cells. Further studies on the role of animal ceramide glycanases in cancer cell lytic cycle, apoptosis and embryonic development are under investigation.

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