

## Purification and Characterization of a Membrane-bound Nonlysosomal Ceramidase from Rat Brain\*

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We have purified a membrane bound ceramidase 22,300-fold to apparent homogeneity. The purification scheme included Triton X-100 extraction of membranes followed by Q-Sepharose, blue Sepharose, phenyl-Sepharose, and MonoS column chromatography. The purified enzyme showed an apparent molecular mass of 90 kDa as estimated by SDS-polyacrylamide gel electrophoresis under reducing conditions and 95 kDa by chromatography on Superose 12. Using C<sub>16</sub>-ceramide as substrate, the enzyme showed a broad pH optimum in the neutral to alkaline range. A mixed micelle assay was developed, and using Triton X-100/ceramide mixed micelles, the enzyme exhibited classical Michaelis-Menten kinetics, with a  $K_m$  of 1.29 mol % and a  $V_{max}$  of 4.4  $\mu$ mol/min/mg. When dihydroceramide was used as substrate, these values were 3.84 mol % and 1.2  $\mu$ mol/min/mg, respectively, indicating that the enzyme hydrolyzes ceramides preferentially. The activity of the purified ceramidase did not require cations, and it was inhibited by reducing agents. Phosphatidylcholine and sphingomyelin were without effect on the enzyme activity, whereas phosphatidic acid and phosphatidylserine stimulated the activity 3-fold. Sphingosine acted as a competitive inhibitor with an IC<sub>50</sub> of 5–10  $\mu$ M. These results indicate that the purified enzyme is a novel ceramidase.

Sphingolipid metabolites are now recognized as important components in signal transduction, not only in mammalian cells, but also in yeast, where they are implicated in heat stress responses. Ceramide (Cer)<sup>1</sup> is one of these sphingolipid metabolites, and it has been shown to play a role in apoptosis, cell cycle arrest, and differentiation (for recent reviews, see Refs. 1–3).

As a consequence of this diverse biology, the study and characterization of enzymes that regulate ceramide levels has become an essential area of study. Ceramidases (CDases) are enzymes that cleave the *N*-acyl linkage of ceramide into sphingosine (SPH) and free fatty acid, and recent studies suggest that CDase may exert important functions in the regulation of its substrate (Cer) or in the regulation of its immediate product (SPH) or the downstream metabolite sphingosine 1-phosphate (SPP). Indeed, current understanding indicates that the major

pathway for the formation of sphingosine is via the degradation of ceramide and not from the *de novo* pathway (4, 5). This suggests that CDases are the key enzymes to regulate levels of SPH. Two reports implicate an alkaline CDase activity in signal transduction. Using cell homogenate of rat glomerular mesangial cells, Coroneos *et al.* (6) have shown that an alkaline CDase activity was stimulated by the platelet-derived growth factor and not by the inflammatory cytokines (tumor necrosis factor  $\alpha$  and interleukin-1) or the vasoconstrictor peptide endothelin-1. In another report, Nikolova-Karakashian *et al.* (7) showed in primary cultures of rat hepatocytes that alkaline CDase activity is stimulated by low concentrations of interleukin-1. The activation of CDase in these cells resulted in the formation of SPH, and these authors suggested that SPH or SPP may mediate some of the effects of low concentrations of interleukin-1. In addition, studies using inhibitors of CDases (*N*-oleoylethanolamine and *D*-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol) have also shown that inhibition of these enzymes causes an elevation in the endogenous level of ceramide that is either sufficient to inhibit growth or augments the effects of other inducers of growth arrest (8, 9). Taken together, these observations underscore the potential importance of CDases and their roles in different process such as apoptosis and proliferation.

Three ceramidases have been described that differ by their pH optima. An acid ceramidase was first described by Gatt (10) in rat brain. The enzyme has been purified and cloned from human urine (11) and recently from mouse tissue (12). This enzyme is located in the lysosomes, and it plays a role in the catabolic pathway of ceramide, and the inherited deficiency of this enzyme causes Farber disease (13). A neutral activity has been described in liver plasma membranes (14) and in rat intestinal brush border membranes (15); little is known about this enzyme. An alkaline activity was described in human cerebellum (16), fibroblasts (17), and in many rat tissues (18). Alkaline CDases were best characterized in Guinea pig skin epidermis (19), where two enzymes were purified, one to apparent homogeneity and the other only partially. These two enzymes are membrane-bound, and their estimated molecular masses on SDS-PAGE were 60 and 148 kDa, respectively.

In this study, we have purified a membrane-bound ceramidase to apparent homogeneity. On SDS-PAGE, the enzyme appeared as a single protein of 90 kDa. The activity of this enzyme is independent of cations, inhibited by reducing agents, and stimulated by PS and phosphatidic acid. Based on these results, this enzyme appears to be a novel CDase.

### EXPERIMENTAL PROCEDURES

**Materials**—Frozen rat brains were purchased from Pel-Freez Biologicals (Rogers, AK). Hitrap Q-Sepharose high performance, Hitrap blue Sepharose high performance, MonoS (HR 5/5), MonoP (HR 5/5), and Superose 12 (HR 10/30) columns and phenyl-Sepharose high performance, polybuffer 96, and polybuffer 74 media were purchased from Amersham Pharmacia Biotech. Centriprep and Centricon sample con-

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<sup>1</sup> The abbreviations used are: Cer, ceramide; CDase, ceramidase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PS, phosphatidylserine; SPH, sphingosine; SPP, sphingosine 1-phosphate.

TABLE I  
Fractionation of rat brain homogenate

Rat brains (2) were homogenized, and the postnuclear supernatant was fractionated as described under "Experimental Procedures." CDase activity was measured at pH 9.5 using glycine buffer at 100 mM.

Step	Proteins	Activity	Specific activity
	mg (%)	units (%)	units/mg
Postnuclear supernatant	148.4 (100)	17.5 (100)	0.12
10,000 × g pellet	64.1 (43.2)	9.9 (56.5)	0.15
100,000 × g pellet	11.3 (7.6)	1.1 (6.3)	0.1
Cytosol	72.3 (48.7)	5.1 (29.1)	0.07

centrators were from Amicon, Inc. (Beverly, MA). Pro-blue staining and silver staining kits were from Owl Separation Systems (Portsmouth, NH). Triton X-100 was from Sigma. Bradford protein assay, isoelectric focusing gels (pH 3–10), isoelectric focusing standard mixture, and gel electrophoresis apparatus were from Bio-Rad. BCA protein assay, CHAPS, and  $\beta$ -octyl glucoside were from Pierce. Polyacrylamide gels were from Novex. [ $^3\text{H}$ ]C<sub>16</sub>-ceramide, ceramides with various chain length, sphingosine, and dihydrosphingosine were synthesized as described (9), and other lipids were from Avanti Polar Lipids.

**CDase Assay**—CDase activity was measured according to the protocol described by Yavin and Gatt (20). Briefly, 10 nmol of [ $^3\text{H}$ ]C<sub>16</sub>-ceramide were mixed with 100  $\mu\text{l}$  of Triton X-100 (0.2%) and 100  $\mu\text{l}$  of sodium cholate (0.4%) in chloroform/methanol (2:1), and the solvent was dried. The dried mixture was resuspended in water by heating at 80 °C for 5 s, and the appropriate buffer and amount of enzyme were added. The reaction was terminated by adding 2 ml of isopropyl alcohol/heptane/1 N NaOH, 4:1:0.1 (Dole solution), followed by 1 ml of water and 1 ml of heptane. After centrifugation, the upper phase was discarded, and the lower phase was washed twice with heptane. Finally, 1 ml of sulfuric acid (1 N) and 2 ml of heptane were added, the mixture was centrifuged, and the upper phase containing the fatty acid was counted in liquid scintillation. In the experiments of enzyme characterization or where the effects of other lipids were tested, these lipids were dried with the substrate and then resuspended in 100  $\mu\text{l}$  of Triton X-100 (1%). The final Triton X-100 concentration in the assay was 0.5%. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 nmol of ceramide/min at 37 °C.

**Protein Assay and SDS-PAGE**—Protein concentration was determined using the Bradford assay or the BCA assay in samples containing Triton X-100. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (21). Proteins were visualized by Pro-blue staining followed by silver staining.

**Fractionation and Triton X-100 Extraction**—Frozen rat brains (53–58 g) were thawed in 150 ml of 20 mM cold phosphate buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride (homogenization buffer). Brains were then homogenized using a Dounce homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min, and the pellet of this centrifugation was homogenized again using 80 ml of homogenization buffer. After centrifugation at 1,000 × g for 10 min, the pellet was washed twice with 50 ml of homogenization buffer. All supernatants were combined and designated as the postnuclear supernatant fraction. The postnuclear supernatant fraction was then centrifuged at 10,000 × g for 30 min. The pellet of this centrifugation was resuspended in 145 ml of Tris 20 mM, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100. After stirring for 1 h, the Triton X-100-solubilized fraction was obtained by centrifuging the mixture at 10,000 × g for 30 min. The supernatant (Triton X-100 extract) was used as a source for ceramidase purification. All steps were carried out at 4 °C.

In initial experiments, aiming to determine the ceramidase-rich fraction, the 10,000 × g supernatant was further centrifuged at 100,000 × g to obtain the plasma membrane rich fraction (pellet) and the cytosol (supernatant).

**Q-Sepharose**—The Triton X-100 extract was applied directly to a Q-sepharose high performance column (25 ml) equilibrated with buffer A (20 mM Tris, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% Triton X-100) at 1 ml/min. Unbound proteins were eluted by washing the column with 75 ml of buffer A. The bound proteins were then eluted with a 250-ml linear gradient of NaCl from 0 to 0.3 M in buffer A. The salt concentration was then increased to 1.5 M for 100 ml, and then for another 75 ml it was combined with 0.5% Triton X-100 to wash out tightly bound proteins. Fractions of 5 ml were collected, ceramidase activity was measured in these fractions, and

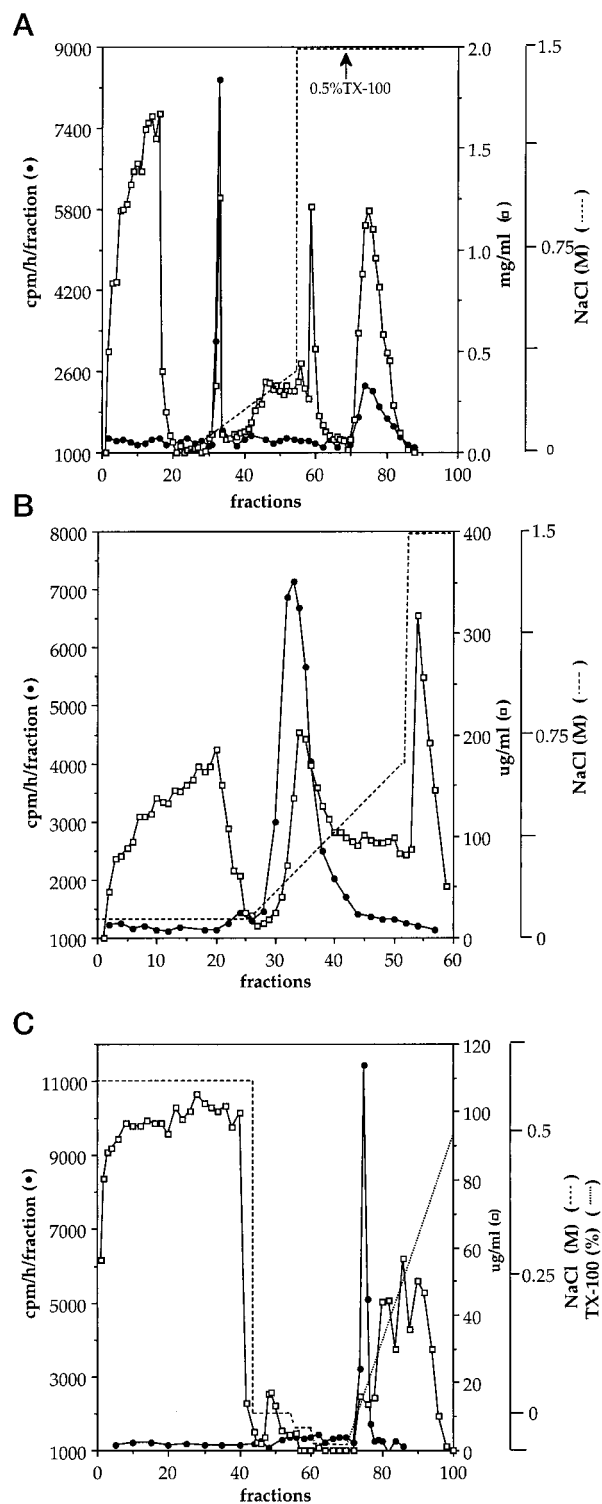


FIG. 1. Purification of CDase. A, the Triton X-100 (TX-100)-solubilized fraction was applied to a Q-Sepharose column equilibrated with buffer A. After washing the column, CDase activity was eluted with a linear gradient from 0 to 0.3 M NaCl in buffer A. Fractions of 5 ml were collected. B, the active fraction obtained from Q-sepharose (NaCl peak) was applied directly to a blue Sepharose Hitrap column equilibrated with buffer B. After washing the column, CDase activity was eluted with a linear gradient of NaCl from 0.075 to 0.4 M. Fractions of 2 ml were collected. C, the active fractions obtained from blue Sepharose were adjusted to 0.6 M NaCl and applied to a phenyl-Sepharose column equilibrated with buffer C. After washing the column with buffer C and eluting more proteins by decreasing the NaCl concentration (see "Experimental procedures"), CDase activity was eluted with a Triton X-100 gradient (0–0.5%) in 10 mM Tris buffer, pH 7.5. Fractions of 1 ml were collected. CDase activity and proteins were measured as described under "Experimental Procedures."

TABLE II  
Purification of CDase from rat brain

Step	Proteins	Activity	Specific activity	Recovery	Purification
	mg	units	units/mg	%	-fold
Postnuclear supernatant	2504	323.7	0.129	100	1
10,000 × g pellet	923	168.6	0.18	52	1.4
Triton X-100 extract	582	155.5	0.27	48	2.1
Q-Sepharose	19.8	69.2	3.5	21	27
Blue Sepharose	5.7	58.8	10.3	18	80
Phenyl-Sepharose	0.086	50.0	580.5	15	4,488
MonoS	0.0126	36.5	2,894.9	11	22,383

CDase was purified from 30 rat brains (55 g) as described under "Experimental Procedures."

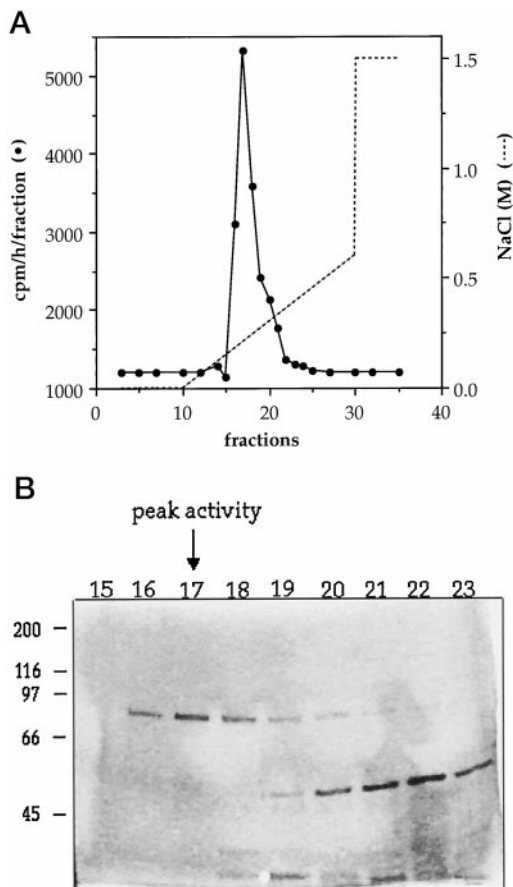


FIG. 2. A, purification of CDase on MonoS cation exchange chromatography. The active fractions from phenyl-Sepharose were adjusted to 50 mM acetate buffer (pH 5.0) and then applied to a MonoS column equilibrated with buffer D. CDase activity was eluted with a linear gradient of NaCl from 0 to 0.6 M. Fractions of 1 ml were collected. B, double stained SDS-PAGE of the MonoS fractions. SDS-PAGE of the MonoS peak of activity fractions was stained with Pro-blue followed by silver staining. The molecular weights of standard proteins are indicated.

fractions with peak activity were pooled.

**Blue Sepharose**—The pooled Q-Sepharose fractions were applied directly to a blue Sepharose column (2 ml) equilibrated with buffer B (buffer A plus 0.075 M NaCl) at a flow rate of 1 ml/min. After the sample was applied, the column was washed with 12 ml of buffer B. Then a 50-ml linear gradient from 0.075 to 0.6 M NaCl in buffer A was applied, and the NaCl concentration was then stepped up to 1.5 M for 10 ml. Fractions of 2 ml were collected, and those containing ceramidase activity were pooled.

**Phenyl-Sepharose**—Fractions of blue Sepharose were adjusted to 0.6 M NaCl and then applied to a phenyl-Sepharose high performance column (0.3 ml) equilibrated with buffer C (buffer A plus 0.6 M NaCl) at a flow rate of 0.2 ml/min. After the sample was applied, the flow rate was increased to 0.5 ml/min, and the column was washed with 5 ml of

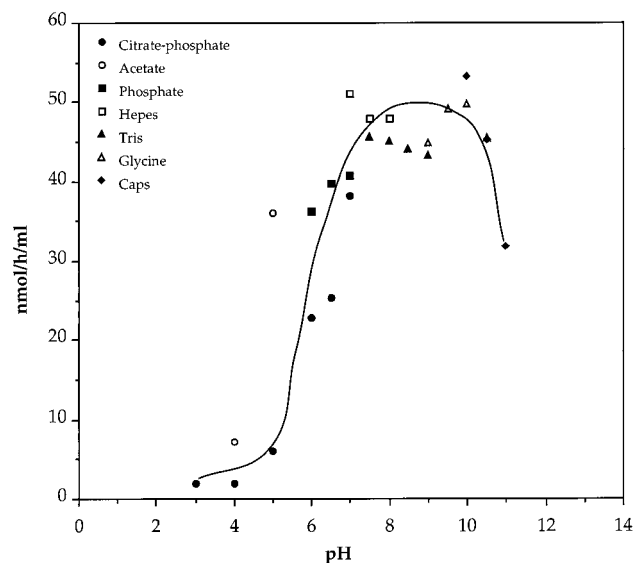


FIG. 3. pH dependence of CDase activity. The activity of CDase was measured on 5–10 ng of protein (in 5  $\mu$ l) from MonoS column (fractions 16 and 17) as described under "Experimental Procedures." The pH was adjusted by the addition of the indicated buffers at a final concentration of 100 mM.

buffer C. A stepwise elution was then applied using 2 ml of buffer A, then 2 ml of 10 mM Tris buffer (pH 7.5), and then 2 ml of 1 mM Tris buffer (pH 7.5). Finally, a 5-ml gradient from 0 to 0.5% of Triton X-100 in 10 mM Tris buffer (pH 7.5) was applied. Fractions of 1 ml were collected, and ceramidase activity was measured. Fractions containing peak activity (recovered in the Triton X-100 gradient) were combined.

**MonoS**—The pooled fractions from phenyl-Sepharose were adjusted to 50 mM (pH 5.0) acetate buffer, diluted 3 times, and then applied to a MonoS column (1 ml) equilibrated with buffer D (50 mM acetate buffer, pH 5.0, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) at a flow rate of 1 ml/min. After washing the column with 5 ml of buffer D to remove unbound proteins, ceramidase activity was eluted with a 20-ml linear gradient of NaCl from 0 to 0.6 M in buffer D. The column was finally washed with 5 ml of 1.5 M NaCl in buffer D. Fractions of 1 ml were collected, and ceramidase activity was measured in these fractions.

**MonoP and Isoelectric Focusing Gels**—To determine the isoelectric point of ceramidase, the enzyme was subjected to chromatofocusing on MonoP. The column was equilibrated with buffer A (pH 8.2), and the sample in the same buffer was then applied. Proteins were then eluted by decreasing the pH using a mixture of polybuffer 96 and polybuffer 74 adjusted to pH 5.0 as elution buffer. Samples of 1 ml were collected, and ceramidase and the pH were measured in these fractions. The isoelectric point was also determined using isoelectric focusing gels and by comparing the focusing distance of the nonlysosomal CDase (obtained from the MonoS single band fractions 16 and 17) to a standard curve of protein standards of known isoelectric points.

**Superose 12**—When Superose 12 column was used, the pooled fractions from the MonoS column were first concentrated around 10–20-fold to 200  $\mu$ l using Centricon and Microcon 10-kDa membrane cut-off and then applied to the column equilibrated with buffer E (buffer A plus 0.1 M NaCl) at a flow rate of 0.5 ml/min. Proteins were then eluted with 30 ml of buffer E. Fractions of 0.5 ml were collected, and ceramidase activity was measured.

**Characterization Experiments**—In these experiments, the substrate was solubilized by adding the detergent in aqueous solution. In the purification experiments, the enzyme was assayed at pH 9.5 according to Yavin and Gatt (20) by drying down the detergent (which is in chloroform/methanol, 2:1) with the substrate and by resuspending the mixture in water. Experiments for optimization of assay were performed on the blue Sepharose-purified enzyme. All other characterization experiments were performed on the enzyme obtained from the single band fractions of the MonoS column. When reducing agents were tested, the enzyme was preincubated with these agents for 2 min prior to the assay. When lipid effects were tested, lipids were dried down with the substrate, and the mixture was resuspended with Triton X-100 at a final concentration of 0.5%.

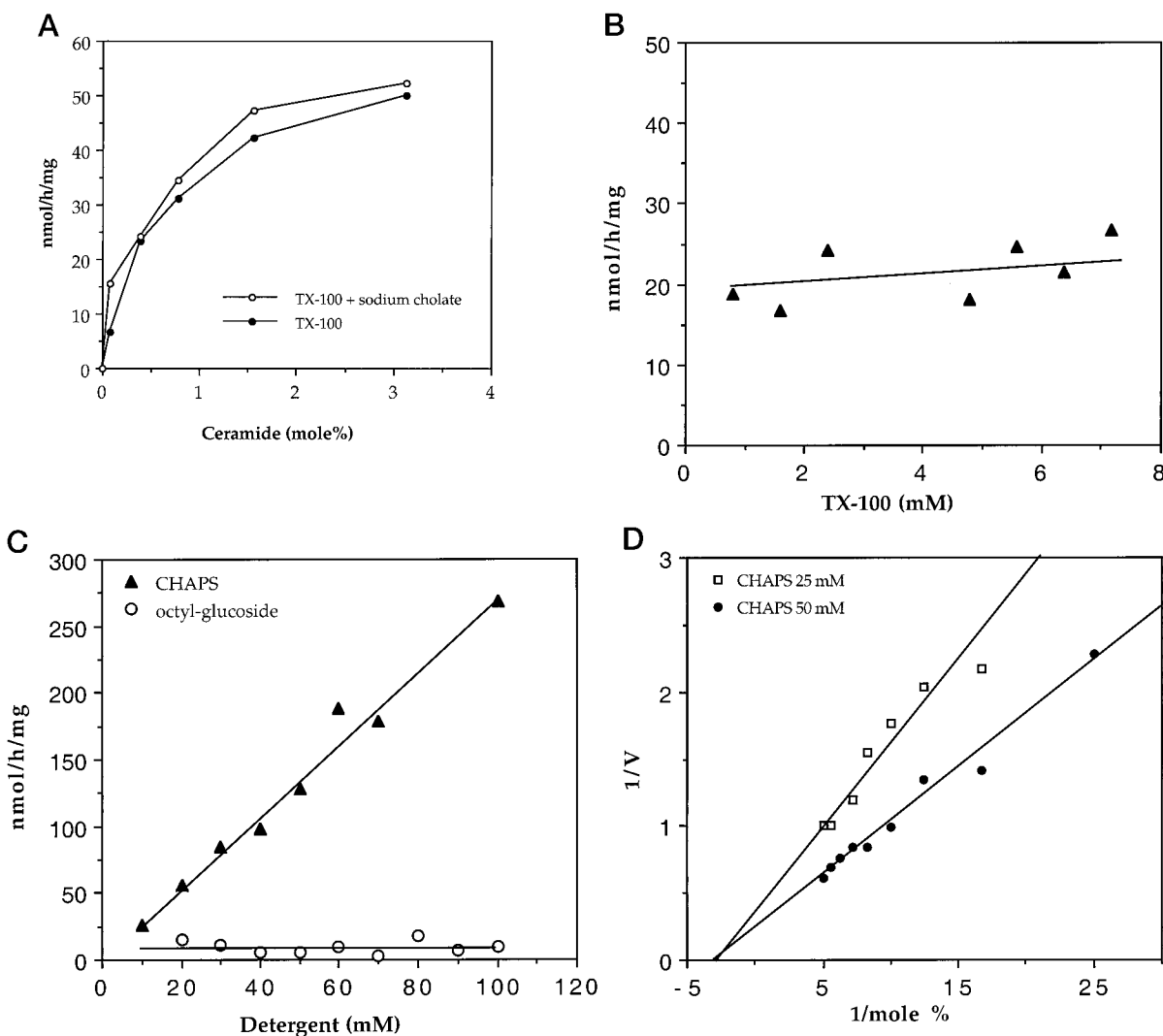


FIG. 4. **Optimization of CDase assay.** A, effect of sodium cholate on CDase activity as measured according to Yavin and Gatt (20). B and C, CDase assay was performed in the presence of increasing concentrations of the indicated detergents. The mol % ratio of ceramide was kept constant at 1 mol % (Cer concentrations were increased from 8 to 72  $\mu$ M) for Triton X-100 experiments and 0.1 mol % (Cer concentrations were increased from 10 to 100  $\mu$ M) for CHAPS and  $\beta$ -octyl glucoside experiments. D, Lineweaver-Burk representation of CHAPS effect on CDase activity.

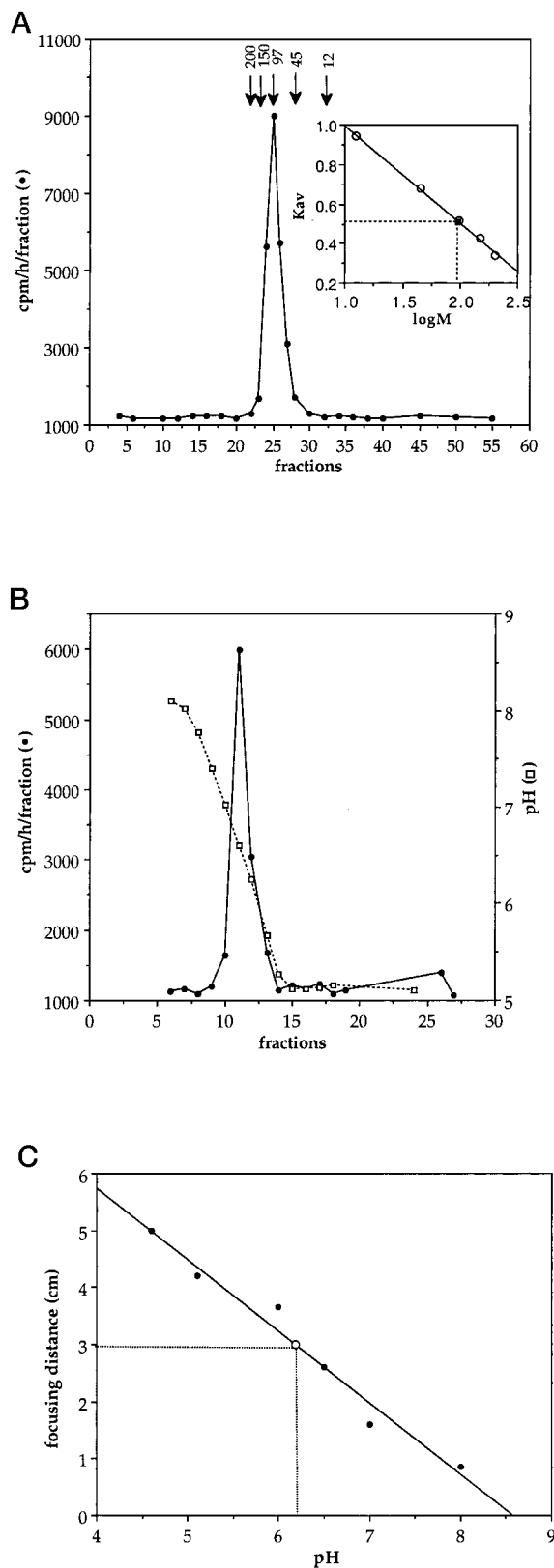
## RESULTS

**Purification of Ceramidase**—Ceramidase activity appears to be distributed in all tissues, and it is highly expressed in kidney and in brain (18). At pH 9.5, we found higher activity in rat brain (7 nmol/h/mg of protein) compared with rat liver (2 nmol/h/mg of protein). Therefore, we chose to purify the enzyme from rat brain. Preliminary experiments showed that more than one CDase exists in rat brain tissue. At least two characteristics, the intracellular localization (subfractionation) and the pH optimum, differ among these enzymes. Fractionation studies showed that most of the membrane-bound CDase activity can be recovered in the 10,000  $\times$  g pellet fraction (Table I). The pH profile of this fraction showed that it contained a ceramidase with a broad pH optimum in the neutral to alkaline range (not shown). Thus, we used this fraction to purify this membrane-bound CDase activity. We solubilized the enzyme with Triton X-100 at a concentration of 0.5%, since this detergent has been shown to be efficient in solubilizing and stabilizing several membrane proteins.

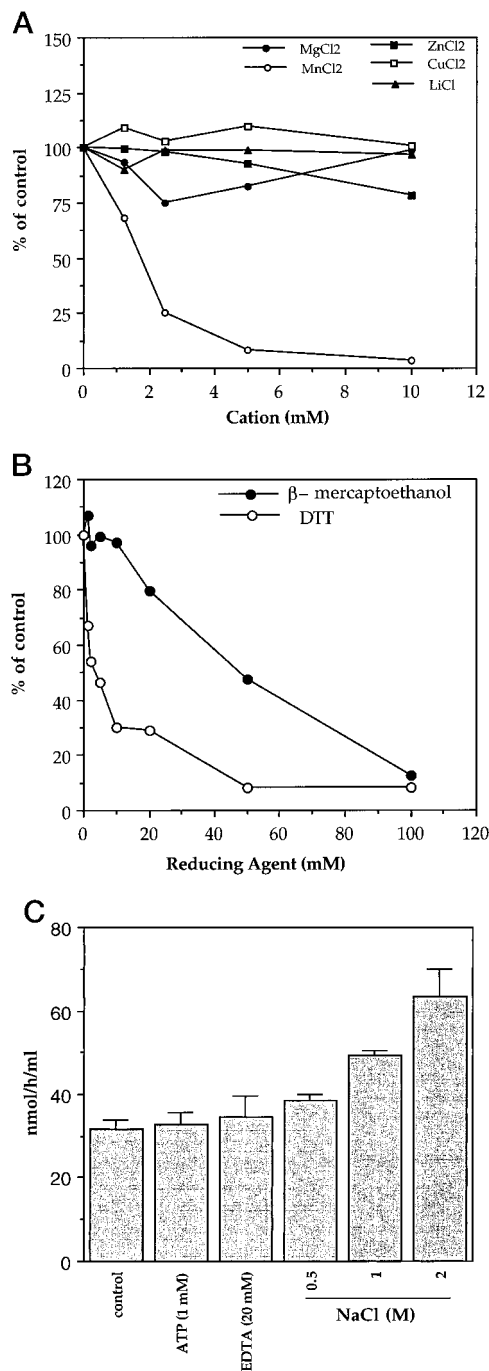
As a first step, the Triton X-100 extract was applied to a Q-sepharose column. Ceramidase bound to this column, and it was then eluted with a very shallow NaCl gradient. As shown in Fig. 1A, CDase activity eluted from the column with low NaCl concentrations (0.07–0.08 M). When the column was

washed with high salt concentrations (up to 1.5 M NaCl), we could not detect any remaining activity bound to the column. However, when Triton X-100 (0.5%) was then applied in combination with the salt, another peak of CDase activity eluted from the column. This peak of activity represented 40–50% of the total activity loaded on the column, and it could represent the same enzyme eluted with salt but in a more hydrophobic conformation, or it could represent another isoenzyme. We did not purify any further this salt/Triton X-100-eluted enzyme, and we focused our purification on the salt-eluted enzyme, since we suspected this form of the enzyme was less hydrophobic. In this step of purification, the enzyme was enriched about 20-fold. This step was also important because it allowed us to keep the sample concentrated by using smaller columns in the following steps. The enzyme was recovered from this column with 80% yield, and 40–50% of the activity eluted when Triton X-100 was added to the salt (Table II).

Next the active fractions were applied to a blue Sepharose column. The enzyme eluted from the column upon application of a NaCl gradient as a single broad peak with NaCl concentrations of 0.2–0.4 M (Fig. 1B). The specific activity after this step was increased 4–5 times. The combined fractions from the blue Sepharose separation were adjusted to 0.6 M NaCl and then applied to a phenyl-Sepharose column. As the amount of



**FIG. 5. Physical properties of CDase.** A, CDase activity, concentrated to 200  $\mu$ l, was applied to a Superose 12 gel filtration column equilibrated with buffer E. Proteins were then eluted with 30 ml of buffer E. Standard molecular mass markers were loaded onto the column, and the elution volumes were used to calibrate the column. B, CDase activity in 20 mM Tris buffer (pH 8.2) was applied to a MonoP chromatofocusing column equilibrated with the same buffer. Proteins were eluted by decreasing the pH using a mixture of polybuffers 97 and 74 adjusted at pH 5.0. Fractions of 1 ml were collected, and CDase activity and the pH were measured on these fractions. C, CDase



**FIG. 6. Effects of cations, reducing agents, and NaCl.** CDase activity was assayed using  $C_{16}$ -ceramide at 0.64 mol % (50  $\mu$ M) and 5–10 ng of protein (in 5  $\mu$ l) from MonoS column (fractions 16 and 17). The final Triton X-100 concentration was 0.5%. A, effect of cations; B, effect of reducing agents; C, effect of EDTA, ATP, and NaCl. DTT, dithiothreitol.

protein was low, we used a small column having a volume of 0.3 ml. When the salt concentration was decreased to zero (elution with buffer A), the CDase activity did not elute from the column, but when a Triton X-100 gradient was applied, CDase eluted from the column (Fig. 1C). In this step, a 50-fold increase in the specific activity was obtained.

obtained from fractions 16 and 17 of the MonoS column was applied on isoelectric focusing polyacrylamide gel (pH 3–10). After running according to the manufacturer's procedure, proteins were visualized by silver stain, and the focusing distances of CDase and standards were measured.

The isoelectric point of CDase was determined to be 6.2–6.5 (see Fig. 5), and based on this we used a cation exchange chromatography on MonoS as the next step. Using acetate buffer, pH 5.0, CDase bound to the MonoS column and eluted with NaCl concentrations around 0.2 M (Fig. 2A). Aliquots from fractions of MonoS separation subjected to SDS-PAGE showed that the peak of activity of CDase (fractions 16–18) corresponded to a single protein band with an apparent molecular mass of 90 kDa (Fig. 2B). This final step increased the specific activity by another 5-fold, yielding an overall increase of the specific activity of 22,300-fold. The overall recovery was 11%, but if we take into consideration that some activity from the postnuclear supernatant fractionated into the cytosol and that on the first column two peaks of activity were obtained, the overall recovery of this specific CDase is estimated to be even higher.

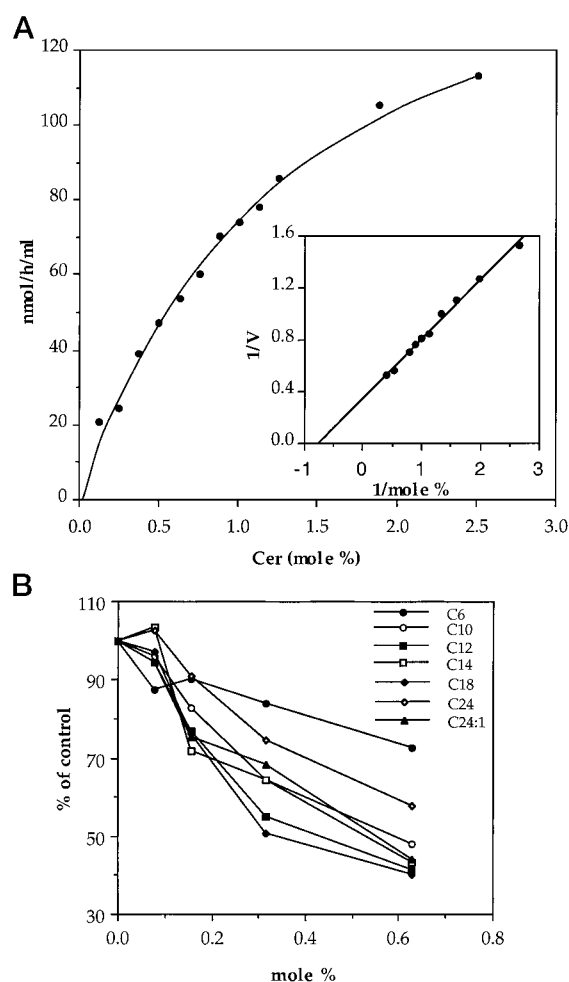
**Characterization of Ceramidase pH Optimum**—The purified CDase was active over a broad pH (Fig. 3). The pH optimum ranged from 7 to 10. At pH 11 significant activity was still observed, whereas at pH lower than 4.5 no activity was noted, indicating clearly that this enzyme is different from the lysosomal acid ceramidase. We therefore chose to name this enzyme as nonlysosomal CDase, since it was neither strictly neutral nor alkaline.

**Optimization of Ceramidase Assay**—In the purification experiments, we assayed the activity according to the protocol described by Yavin and Gatt (20) in the presence of 0.1% Triton X-100 and 0.2% sodium cholate. To optimize the assay, we first examined whether sodium cholate is required for the assay, and therefore we performed kinetic analysis of substrate dependence in the presence or absence of sodium cholate. Fig. 4A shows that there was no significant difference between the curves when the enzyme was assayed with or without sodium cholate, indicating that the addition of sodium cholate was not necessary for the assay of this purified enzyme.

Next, we tested other detergents to solubilize ceramide. To do this, we tested the effects of increasing concentrations (up to 10 times the critical micellar concentration) of Triton X-100, CHAPS, and  $\beta$ -octyl glucoside on CDase activity while keeping the ceramide concentration at a fixed mol % (ceramide and Triton X-100 molar concentrations were increased in parallel to keep the mol % concentration of ceramide fixed). This was approached as such since most lipid-acting or lipid-regulated enzymes respond to the mole fraction of the lipid substrate/regulator in the micelle rather than to the bulk (molar) concentration. For a thorough discussion, refer to Hannun *et al.* (22) and Robson and Dennis (23). Fig. 4B shows that the enzyme activity was independent of Triton X-100 concentration. Octyl glucoside inhibited the enzyme, whereas CHAPS stimulated CDase activity (Fig. 4C). Fig. 4D shows that CHAPS stimulated ceramidase activity by increasing the  $V_{max}$  of the reaction. From these experiments, we concluded that Triton X-100 did not affect ceramidase activity, and we therefore used Triton X-100-based mixed micelles in all subsequent experiments.

**Physical Properties of CDase**—Upon SDS-polyacrylamide electrophoresis, the enzyme appeared as a single protein with an apparent molecular mass of 90 kDa (Fig. 2B). Using gel filtration chromatography on Superose 12, the estimated molecular mass was around 95 kDa (Fig. 5A). In this step, Triton X-100 concentration (0.005%) was lower than the critical micellar concentration ( $\sim 0.02\%$ ), indicating that the estimated molecular weight corresponds the native enzyme and not to the enzyme in Triton X-100 micelles.

The isoelectric point of CDase was also determined. Using chromatofocusing on MonoP column and isoelectric focusing polyacrylamide gels as described under "Experimental Proce-



**Fig. 7. Kinetics of CDase.** CDase activity was measured on 5–10 ng (in 5  $\mu$ l) of protein from MonoS column (fractions 16 and 17). A, Michaelis-Menten and Lineweaver-Burk representations for CDase activity toward increasing concentrations of  $C_{16}$ -ceramide. The Triton X-100 concentration was kept fixed at 0.5%, and ceramide concentration was increased from 10 to 200  $\mu$ M. B, competition of ceramides with different acyl chain with [ $^3$ H] $C_{16}$ -ceramide. [ $^3$ H] $C_{16}$ -ceramide was kept fixed at 0.16 mol %.

dures," the isoelectric point of ceramidase was determined to be 6.5 and 6.2, respectively (Fig. 5, B and C).

**Effects of Cations**—The addition of  $MgCl_2$ ,  $CuCl_2$ ,  $ZnCl_2$ , and  $LiCl$  was without any effect on CDase activity (Fig. 6A).  $MnCl_2$  inhibited the enzyme, and total inhibition was observed around 5–10 mM.  $CaCl_2$  up to 1 mM had no effect on CDase activity (not shown).

**Effect of Other Agents**—As shown in Fig. 6B, the reducing agents dithiothreitol and  $\beta$ -mercaptoethanol inhibited CDase activity; dithiothreitol was more effective. Since the molecular mass of CDase estimated by SDS-PAGE (90 kDa) corresponded to the one obtained by gel filtration on Superose 12 column (95 kDa), these results indicate that the protein is a monomer. Thus, these reducing agents do not appear to disrupt intermolecular interactions, but they probably act by reducing intramolecular disulfide bonds or by reducing a critical residue in the enzyme. The addition of EDTA (20 mM) and ATP (1 mM) did not affect ceramidase activity. NaCl at high concentrations increased ceramidase activity moderately (Fig. 6C).

**Kinetics of CDase**—Fig. 7A shows the substrate dependence of CDase toward  $C_{16}$ -ceramide in mixed micelles. The enzyme showed classical Michaelis-Menten kinetics, and from Lineweaver-Burk plots, a  $K_m$  of 1.29 mol % and a  $V_{max}$  of 4.4  $\mu$ mol/min/mg were calculated. The substrate specificity was

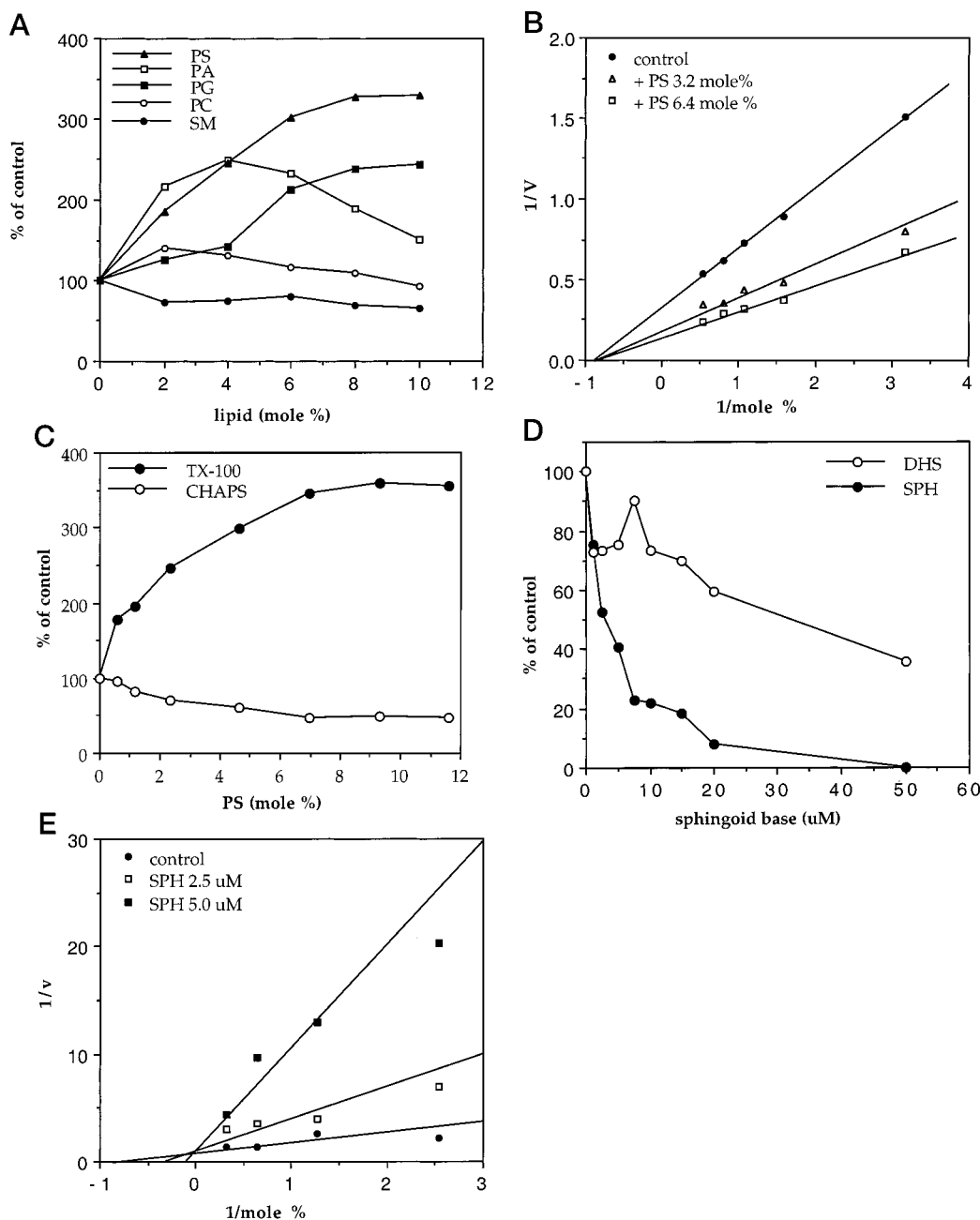


FIG. 8. **Effect of lipids on CDase activity.** Lipids were dried down with the substrate and the mixture resuspended with Triton X-100 at a final concentration of 0.5%. CDase activity was measured on 5–10 ng of protein (in 5  $\mu$ l) from MonoS column (fractions 16 and 17). A, effects of phospholipids and Sphingomyelin; B, double reciprocal representation of PS effects; C, PS effect on CDase activity in the presence of CHAPS (20 mM) or Triton X-100 (0.5%); D, effect of SPH and dihydrospingosine (DHS) on CDase activity; E, competitive inhibition by sphingosine.

studied next. It has been shown for lysosomal acid ceramidase, purified from urine (24) or from fibroblast homogenate (17), that  $C_{12}$ -ceramide is the best substrate. Also, acid ceramidase hydrolyzed  $C_6$ - and  $C_{18}$ -ceramides with about the same efficiency. In our experiments, the nonlysosomal enzyme was assayed by competing [ $^3$ H] $C_{16}$ -ceramide with unlabeled ceramides having various fatty acyl chains. To avoid chain length solubility effects, [ $^3$ H] $C_{16}$ -ceramide was used at a concentration of 0.16 mol %, and cold ceramides were added up to 0.64 mol %. As shown in Fig. 7B, we found that  $C_6$ -ceramide was a poor substrate compared with ceramides having longer fatty acyl chains, for which we did not observe any specific pattern. Comparing  $C_{24:0}$ - to  $C_{24:1}$ -ceramides showed that the introduction of the double bond increased the affinity of the substrate to the enzyme. When dihydro- $[^3$ H] $C_{16}$ -ceramide was used as substrate, the  $K_m$  and  $V_{max}$  values were 3.84 mol % and 1.2

$\mu$ mol/min/mg, respectively. This indicates that the enzyme hydrolyzes preferentially ceramides over dihydroceramides. Sphingomyelin labeled on the choline head group was also tested as substrate. After Bligh and Dyer extraction and TLC separation, we could not detect any lysosphingomyelin formation, indicating that sphingomyelin was not a substrate for the purified ceramidase (data not shown) and that this enzyme is different from a previously described sphingolipid *N*-deacylase (25).

**Effects of Lipids**—The effects of various phospholipids and sphingolipids were tested on the enzyme. These lipids were added at the indicated mol % concentrations with the substrate. Fig. 8A shows that the acidic phospholipids PS and phosphatidic acid stimulated the enzyme, and a 3-fold stimulation was observed with PS at around 8 mol %. At higher concentrations (>15 mol %), the stimulatory effect of PS de-

creased. Phosphatidylglycerol increased the activity to a lesser extent, whereas phosphatidylcholine was without any effect. Kinetic analysis of PS stimulation showed that PS increased the  $V_{\max}$  of the reaction (Fig. 8B).

Since CHAPS showed a similar activation mechanism, we tested the effect of PS on the enzyme activity in the presence of CHAPS. Fig. 8C shows that the stimulation by PS was no longer observed in the presence of 20 mM CHAPS. This indicates that the stimulating effect of CHAPS and PS probably involves the negative charge of the molecules. On the other hand, at 10 mol % PS (in Triton X-100) the activity was lower than that in the presence of 20 mM CHAPS (105.4 versus 284.7 nmol/h/mg), indicating that CHAPS has an additive stimulating effect.

Next we tested the effect of sphingomyelin, sphingosine, and dihydrosphingosine. Sphingomyelin up to 10 mol % did not affect ceramidase activity (Fig. 8A). Sphingosine, a product of the reaction, inhibited the enzyme with an  $IC_{50}$  of 5–10  $\mu$ M (Fig. 8D). Sphingosine acted as a competitive inhibitor with a  $K_i$  of 1  $\mu$ M (Fig. 8E). Dihydrosphingosine was less effective than sphingosine (Fig. 8D).

#### DISCUSSION

In this study, we have purified and characterized a membrane-bound nonlysosomal CDase from rat brain. The activity of this enzyme was maximal at pH in the neutral to alkaline range, distinguishing this enzyme from the lysosomal acid CDase. Because of this broad pH optimum, the enzyme could not be classified as either alkaline or neutral, and we suggest a classification of this enzyme as a nonlysosomal CDase. Measurement of CDase activity of excised gel from SDS-PAGE of the MonoS column (Fig. 2B) was attempted, but no activity could be detected in any molecular weight region, before and after SDS removal. However, our results strongly suggest that the activity of CDase corresponds to the 90-kDa protein on SDS-PAGE and to a 95-kDa protein on gel filtration.

Two alkaline ceramidases have been described in Guinea pig skin by Yada *et al.* (19). The estimated molecular masses of these proteins were 60 and 148 kDa. The activity of these enzymes was inhibited by PS, phosphatidic acid, phosphatidylcholine, and sphingomyelin. Recently, a CDase of 70 kDa was purified from *Pseudomonas aeruginosa* (26), and calcium was found to be a cofactor of this enzyme. The nonlysosomal CDase purified from rat brain in this study has a molecular mass of 90 kDa; its activity is stimulated by PS and phosphatidic acid and does not require cations. Based on these results, the rat brain enzyme appears to be a novel ceramidase. Also, this enzyme did not hydrolyze other sphingolipids, thus distinguishing it from a previously described sphingolipid, *N*-deacylase (25).

In our purification protocol, we noticed that the order of the columns used was important as the behavior of CDase on these columns was different. For example, when the phenyl-Sepharose column was used as a second step (after Q-Sepharose), CDase activity eluted from the column when the salt concentration was decreased to zero (elution with buffer A). When blue Sepharose was used prior to phenyl-Sepharose, Triton X-100 was needed to elute the enzyme from the phenyl-Sepharose column. One possible explanation is that CDase exhibits different conformations, one more hydrophobic than the other, and this affects the elution of the protein from each column. This can also explain the need for Triton X-100 to elute a second peak of CDase from the Q-Sepharose column used in the first step.

The purified enzyme showed specificity for the hydrolysis of ceramide, *i.e.* dihydroceramide was a relatively poor substrate, and sphingomyelin, a structurally close sphingolipid, was not hydrolyzed by this enzyme.

Ceramidases appear to be a class of enzymes composed of multiple isoforms, and they can be distinguished at the biochemical level. Moreover, our results show that the nonlysosomal CDase is stimulated by PS. Many membrane-bound enzymes have been shown to be stimulated by acidic phospholipids, in particular PS, and this may suggest the localization of these proteins in a PS-rich compartment, such as the inner leaflet of the plasma membrane.

It will be important to elucidate the role of these isoforms in the regulation of Cer, SPH, and SPP levels, since these sphingolipid metabolites do not induce the same physiological effects. For example, inhibition of CDase by *D*-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol in HL-60 cells resulted in the accumulation of Cer and the arrest of the cell cycle at the  $G_0/G_1$  phase (9). In contrast, treatment of mesangial cells with platelet-derived growth factor (6) or treatment of primary cultured hepatocytes with low concentrations of interleukin-1 (7) resulted in stimulation of CDase, decreases in Cer levels, and increases in SPH or SPP levels. This was suggested to drive SPH and SPP-dependent activities.

In conclusion, the regulation and localization of CDases are key aspects for future studies, especially in important processes such as signaling, cell proliferation, and apoptosis. The availability of purified and characterized enzymes is essential for these studies.

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