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Original Article

Purification and biochemical characterization of membrane-bound neutral ceramidase from camel brain (*Camelus dromedarius*)

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Abstract: Ceramidases cleave the N-acyl linkages of ceramide to generate sphingosine and its subsequent product sphingosine-1-phosphate (S1P). Ceramide and S1P are important bioactive lipids, and ceramidases are important in regulating the availability of these lipids. In this study, we report the purification and characterization of camel brain neutral ceramidase (CBCDase). The novel CBCDase was purified from camel brain using sequential chromatography of DEAE-Sepharose, Phenyl-Sepharose, Superdex, and Mono Q column. The Mono Q fractions containing ceramidase activity were used for enzyme characterization. The purified CBCDase showed a single band corresponding to a molecular weight of ~100 kDa, displaying classical Michaelis-Menten kinetics, with maximum enzymatic activity at pH 7.0. Deglycosylation of the enzyme yields an apparent molecular weight of ~80 kDa. The purified CBCDase was inhibited by Zn^{2+} and Cu^{2+}, while Ca^{2+} stimulates the activity. Phosphatidic acid, phosphatidylserine and phosphatidylcholine completely inhibited enzyme activity at low concentrations. Thiol-containing compounds inhibited the CBCDase activity. Among the nucleotides, ADP, UMP, and TMP inhibited the enzyme activity at low concentrations, whereas, ATP inhibited the activity at higher concentrations only. The CBCDase catalysed both ceramide hydrolysis and reverse CDase reactions. For the first time, we have purified to apparent homogeneity of a ~100 kDa nCDase from camel brain.

Keywords: Ceramidase, characterization, chromatography, glycosylation, pH optimum, purification

Introduction

Sphingolipids have been shown to modulate various cell functions such as proliferation, differentiation, senescence, and apoptosis. Amongst these sphingolipids, ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) are the three most studied sphingolipids. These lipids have been shown to play important role in cellular regulation under stress conditions such as heat [1], UV or γ-radiations [2, 3], chemical stress [4], phytochemicals [5], and oxidative stress [6]. Therefore, characterization and biochemical analysis of enzymes responsible for modulation of the level of these bioactive lipids are very important.

Ceramidases (CDases, EC 3.5.1.23) cleave the N-acyl linkage of Cer to form Sph and fatty acid. After its generation, Sph is phosphorylated to form S1P by the enzyme sphingosine kinase. Several CDases also carry out the synthesis of Cer by condensing Sph and a fatty acid. Therefore, CDases can be considered as key enzymes that control intracellular levels of Cer, Sph, and S1P, hence, controlling the cellular responses mediated by these very important bioactive lipids [7, 8]. Ceramidases are composed of multiple isoforms which have been further classified as acid, neutral or alkaline, depending on the optimum pH of their activities [7]. Acid ceramidase (aCDase), which hydrolyses Cer in lysosomes, was first identified and purified from rat brain [9]. The human form was isolated and characterized from urine [10] and Koch *et al.* cloned cDNA encoding aCDase from human [11]. Site-directed mutagenesis of the aCDase identified the existence of six N-glycosylation sites [12]. Alkaline CDases (alkCDases) were identified by Yada *et al.* during purification of two membrane bound enzymes having a molecular mass of 60 and 148 kDa from guinea pig skin [13]. Mao *et al.* cloned and partially characterized two types of alkaline CDases from yeast (*Saccharomyces cerevisiae*); YPC1p, having phytoceramidase, as well as, ceramide synthase activities, and YDC1p having dihydroceramidase activity [14, 15].

A CDase having a broad optimum pH from neutral to alkaline range, with a molecular mass of 90 kDa (RBCDase I) and another having a 110 kDa (RBCDase II) were purified from rat brain [16, 17]. Neutral CDases (nCDases) have been also cloned and characterized from mouse liver, rat kidney, fruit fly, zebra fish, and human [18-23]. Moreover, Galadari *et al.* identified a novel amidase motif containing a serine residue that is critical for the catalytic activity of nCDase [19]. In a recent study, the molecular mechanism of hydrolysis and synthesis of Cer by nCDase has been well characterized [24]. Interestingly, the data demonstrated that the breakdown or synthesis of N-acyl linkage of Cer occured through a Zn^{2+} dependent mechanism [24].

The involvement of nCDase in the metabolism of Cer, and regulation of sphingolipid-mediated signaling at the plasma membrane, and the extracellular milieu has been reported in nCDase over-expressing CHOP cells [25]. It has been shown that mouse nCDases were mainly localized in the plasma membrane, whereas, the human homologue of RBCDase was detected in the mitochondria, and the human kidney isoform was transported to the plasma membrane when expressed in HEK 293 cells [26, 27]. Very recently, the knock down of nCDase using siRNA has been shown to increase cellular Cer level, and arrest cell cycle in gemcitabine, a chemotherapeutic agent, treated murine epithelial cells [28]. Similarly, a number of studies have reported that nCDase can be regulated by cytokines and growth factors [29-31]. These findings indicate that nCDases may have a critical role for the cellular function through the regulation of ceramide metabolism. However, the putative regulatory molecule(s) which regulate the activation or inactivation of these enzymes have not yet been identified. Therefore, a detailed enzymological study is required for the characterization of the physiological function of these enzymes. Ceramidase activity appears to be distributed in all tissues of rat and is highly expressed in the brain and the kidneys [16, 32].

In the present study, we have purified and biochemically characterized a ~100 kDa protein having nCDase activity from camel brain. The results of this study imply that this purified enzyme may be a novel CDase.

Materials and methods

Fresh camel brains were obtained from a local slaughter house. DEAE-Sepharose high performance, phenyl-Sepharose HP, Superdex 200 HR 10/30, Mono Q HR 5/5, and PD-10 columns were purchased from Amersham Bioscience (Uppsala, Sweden). Centriprep and Centricon sample concentrators were from Amicon, Inc. (Beverly, MA 01915 USA). Pre-coated Silica Gel 60 TLC plates were obtained from Whatmann (Germany). D-*erythro*- C_{12}-NBD-Cer was kindly provided by the Lipidomics Core Facility at the Medical University of South Carolina (Charleston, SC., USA). All other lipids were from Avanti Polar Lipid (USA). Rabbit polyclonal anti-nCDase antibodies were generated as described previously [17]. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was from sigma (St. Louis, MO, USA). All SDS-PAGE reagents were purchased from Bio-Rad, (USA). Silver staining kits, Triton X-100, detergent removing gel, BCA proteins assay kits, and enhanced chemiluminescence reagent were from Pierce (Rockford. IL, USA). Glycosidase F was purchased from Calbiochem (USA). All other chemicals used were purchased from Sigma (St. Louis, M.I., USA).

Neutral CDase enzyme assay and biochemical characterization

CDase activity was measured using C_{12}-NBD-Cer as a substrate as described previously [17]. Briefly, 25 µl of 100 µmol of D-*erythro*-C_{12}-NBD-Cer was incubated at 37°C for 1 h with an appropriate amount of the enzyme (10 µl). The reaction was stopped by adding 100 µl chloroform/methanol (1:1). After drying it in a speed vacuum concentrator (Savant Instruments, Inc.), the sample was dissolved in 25 µl of chloroform/methanol (2:1) and applied to a TLC plate which was developed with chloroform/methanol/ammonia (75:15:0.9). The spot corresponding to NBD-dodecanoic acid and C_{12}-NBD-Cer were scraped and then incubated with ethanol at 37°C for 5 min to extract the compounds. Their fluorescence was measured at (485/535 nm) excitation/emission wavelength in a Perkin-Elmer spectrofluorophotometer. The compounds were quantified using a standard curve of known amounts of C_{12}-NBD-Cer and NBD-dodecanoic acid. One enzyme unit is defined as the amount capable of catalyzing the release of 1 μmole of NBD-dodecanoic acid/min from C_{12}-NBD-Cer. For the optimum pH determination, the substrate was dissolved in the following buffers: pH 3-5, 100 mM acetate buffer; pH 6-7, 100 mM Phosphate buffer; pH 7-8, 100 mM Tris or Hepes buffer and pH 8-10, 100 mM glycine buffer.

Fractionations and Triton X-100 extraction

Tissue fractionation and Triton X-100 extraction was carried out as previously described [17]. Briefly, fresh camel brain was homogenized in homogenization buffer (500 ml of 20 mM cold phosphate buffer of pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, and 0.2 mM phenylmethylsulphonyl fluoride) using Dounce homogenizer. The homogenate was centrifuged at 1000 g for 10 min, and the pellet was further homogenized using 100 ml of homogenization buffer. After centrifugation at 1000 g for 10 min, the pellet was washed twice with a 100 ml homogenization buffer. All supernatants were combined and designated as the post nuclear supernatant fraction. The post nuclear supernatant fraction was then centrifuged at 10000 g for 30 min and the pellet of this centrifugation was resuspended in solubilisation buffer (150 ml of 20 mM Tris buffer of pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100). After mixing for 1 h, the Triton X-100 solubilized fraction was obtained by centrifugation of the mixture at 10000 g for 30 min. The supernatant (Triton X-100 extract) was used as a source for ceramidase purification. All the steps were carried out at 4°C.

DEAE-Sepharose

The Triton X-100 extract (150 ml) was applied to DEAE-Sepharose column (25 ml) equilibrated with buffer A1 (20 mM Tris, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethyl sulfonyl fluoride, and 0.005% Triton X-100) at 1 ml/min. The unbound proteins were eluted by washing the column with 200 ml linear gradient of NaCl from 0 to 0.3 M in buffer B1 (20 mM Tris, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.005% Triton X-100 and 1.5 M NaCl). The salt concentration was then increased to 1.5 M for 100 ml. After the B1 buffer the column was washed again with A1 buffer for 50 ml, the tightly bound proteins were eluted by a linear gradient of buffer B2 (0-0.5% Triton X-100 in B1). The CDase activity was measured in the 5 ml-fractions that were collected and fractions containing activity were pooled.

Phenyl-Sepharose HP

The active fractions obtained from the salt gradient of DEAE-Sepharose were collected and loaded on to a phenyl-Sepharose column which was equilibrated with buffer B1 (buffer A1 plus 0.3 M NaCl) at a flow rate of 0.5 ml/min. After the sample was applied, the flow rate increased to 1 ml/min. The column, on the other hand, was washed with decreasing concentrations of the buffer B1. A stepwise elution was applied using 100 ml of a 70% buffer B2 (1 mM Tris, pH 7.4, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride) then 100 ml of 100% buffer B2. Finally, 100 ml gradient from 0-1% Triton X-100 in buffer B2 was applied. Fractions of 1 ml were collected, and CDase activity was measured. Fractions containing CDase activity (recovered in 100% buffer B2) were combined.

Superdex 200 HR 10/30

The pooled phenyl-Sepharose active fractions were concentrated with Centriprep and then load on to a Superdex 200 column (25 ml) equilibrated with a buffer A1 at flow rate of 0.1 ml/min and 1 ml-fractions were collected. Fractions containing activity were pooled.

Mono Q

The pooled fractions from the Superdex column were 5 times diluted with buffer A1 and applied to a Mono Q column (1 ml) equilibrated with buffer A1 at a flow rate of 1 ml/min. After washing the column with 10 ml of buffer A1 to remove the unbound protein, CDase activity was eluted with a 20 ml-linear gradient of NaCl (0-0.6 M). The column was finally washed with 15 ml of 1 M NaCl in buffer A1. One ml-fractions were collected, and CDase activity was measured.

Protein assay, SDS-PAGE and western blotting analysis

The protein concentration was determined using the Bradford assay and the BCA assay also used for samples containing Triton X-100. The SDS-PAGE of the reducing condition was carried out according to previously reported [33]. The purified enzyme preparation was subjected to SDS-PAGE (10%). The separated proteins were electrophoretically transferred on to a nitrocellulose membrane. After blocking with 5% non-fat milk in Tris buffer saline containing 0.1% Tween 20, the membrane was incubated with anti-nCDase antibody followed by secondary antibody conjugated horseradish peroxidase. Proteins were visualized by using enhanced chemiluminescence system.

Glycosidase F treatment

The deglycosylation treatment was performed according to the manufacturer's protocol (Calbiochem). Briefly, CDase was denatured in a SDS-PAGE 6X sample buffer (375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-Mercaptoethanol, and 0.03% bromophenol blue) for 3 min. The denatured enzyme was then incubated at 37°C for 18 h with 0.5 milliunits of glycosidase F in the presence of 0.5% Triton X-100. After incubation, the samples were subjected to SDS-PAGE and Western blot analysis using anti-nCDase antibody.

Substrate specificity assay using HPLC

HPLC assay was used to quantitate the amount of released sphingoid base as described previously with a little modification [17]. Ceramide species were dissolved in 50 mM Tris (pH-7.4) containing 0.4% IGEPAL CA 630. The final concentration of IGEPAL CA 630 in the assay was 0.2%. The reaction was started by adding 20 ng enzyme (10 μl) into the tube containing 10 μl of different Cer species, and incubated for 1 h at 37°C. The reaction was stopped by adding 55 μl of stopping buffer (1:9, 0.07 M potassium hydrogen phosphate buffer:methanol). The released Sph was derivatized with *o*-phthaladehyde (OPA) reagent. After stopping the reaction add 25 μl of freshly prepared OPA reagent (12.5 mg OPA dissolved in 250 μl ethanol and 12.5 μl mercaptoethanol and made up to 12.5 ml with 3% (w/v) boric acid, pH 10.3). The mixture was allowed to stand for 30 min. A 25 μl of aliquot is injected into the HPLC. HPLC analysis was done using *Waters 1525* binary pump system. Waters XTerra C18 column was equilibrated with a mobile phase (20% methanol, 80% 1:9, 0.07 M potassium hydrogen phosphate buffer:methanol) at a flow rate of 1 ml/min. The fluorescence detector (*Waters 2475*) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Neutral CDase reverse reaction (ceramide synthesis) assay

The reverse activity of purified CBCDase was measured by using C_{12}-NBD-dodecanoic acid and D-*erythro*-sphingosine as substrates [34]. The standard reaction mixture contained 5 ng of enzyme (10 μl) and 25 μl of 50 mM Tris buffer (pH 7.5) containing 100 μm NBD-dodecanoic acid, 100 μM sphingosine and 0.3% Triton X-100. The reactions were incubated at 37°C for 3h and the reactions were terminated by adding 100 μl of chloroform/ methanol (1:1). After drying in a speed vacuum concentrator (Savant Instruments, Inc.), the sample was redissolved in 25 μl of chloroform/methanol (2:1) and applied to a TLC plate, which was developed with chloroform, methanol, and ammonia (75:15:0.9). The spots corresponding to NBD-dodecanoic acid and C_{12}-NBD-Cer were scraped, incubated with ethanol at 37°C for 15 min to extract the compounds from Silica and their fluorescence was measured at (485/535 nm) excitation/emission wavelength in a Perkin-Elmer spectrofluorometer.

Results

Purification of camel brain CDase (CBCDase)

In order to determine the camel organ containing the highest nCDase activity, several camel organs, such as brain, kidney, liver, heart and lung, were homogenized and measured the nCDase activity. The highest nCDase activity was detected in brain followed by lung. Activities detected in other organs were very weak (data not shown). Therefore, it was decided to use camel brain as enzyme source for purification. Camel brain homogenates was solubilized with 0.5% Triton X-100, and then subjected to a series of column chromatographies in order to purify CBCDase, as described under "Materials and methods". Following purification using chromatography on DEAE-Sepharose, phenyl-

Table 1. Purification of nCDase from camel brain[a]

Purification step	Proteins	Activity	Specific activity	Recovery	Purification
	mg	units	units/mg	%	fold
Post-nuclear supernatant	45137	4846.6	0.107	100	1.0
Triton X-100 Extract	4486	823.2	0.184	17	1.7
DEAE Sepharose	266	538.6	2.02	11	18.9
Phenyl Sepharose	17.8	157.3	8.84	3.2	82.6
Superdex 200 HR	3.31	55.6	16.8	1.1	157.0
Mono Q	0.124	28.6	230.6	0.6	2147.1

[a]nCDase was purified from camel brain (250 g) as described under "Material and methods".

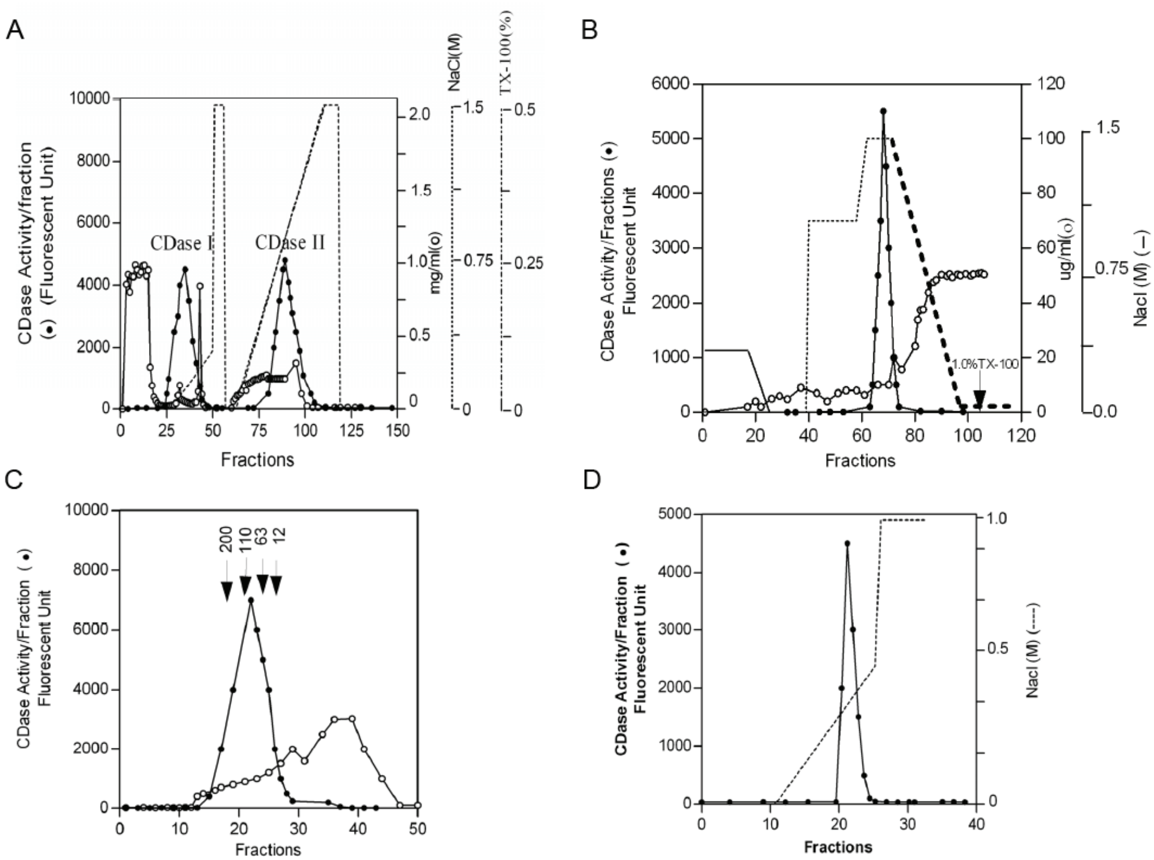

Figure 1. Purification of CBCDase. A: The Triton X-100 solubilized fraction was applied to DEAE sepharose column equilibrated with buffer A1. After washing the column CDase activity was eluted with a linear gradient from 0 to 0.3 M NaCl in buffer A1. Fractions of 5 ml were collected. B: The active fractions obtained from DEAE sepharose was adjusted to 0.3 M NaCl, then applied to a phenyl sepharose column equilibrated with 0.3 M NaCl. The active fractions eluted with 1 mM of Tris buffer were collected. C: The active fractions obtained from phenyl sepharose were applied to a Superdex gel filtration column equilibrated with buffer A1. D: The active fractions from Superdex column were diluted with buffer A1 and applied to Mono Q ion exchange column. Then a 20 ml of linear gradient from 0.0 to 0.4 M NaCl in buffer A1 was applied, and the NaCl concentration was then stepped upto 1 M for 10 ml. 1mL fractions were collected and those containing CDase activity were pooled.

Sepharose, Superdex 200, and Mono Q columns, pure fractions of homogenous protein with CDase activity were obtained. The final fractions showed 2147-fold purification of CDase and yielded 0.6%, as summarized in **Table 1**. After applying the Triton X-100 extract to a DEAE-Sepharose column, the active fractions were detected using fluorescent CDase assay. The first active fractions (CDase I) were eluted using a very shallow gradient of NaCl as shown in **Figure 1A**. When we applied a combination of 0.5% Triton X-100 with NaCl, another

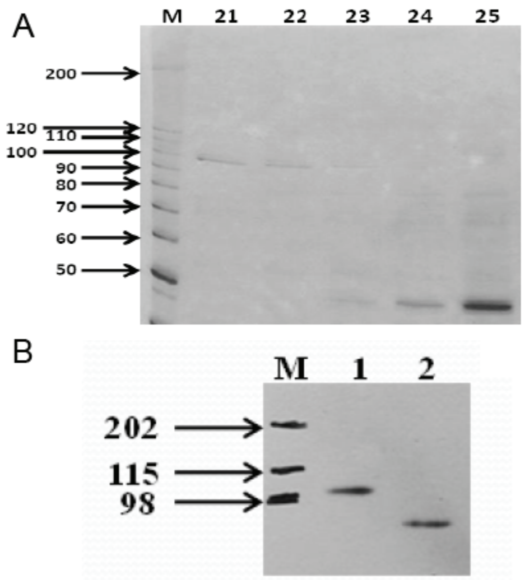

Figure 2. Identification of purified CBCDase. A: The active fractions obtained after Mono Q were subjected to SDS-PAGE and then silver staining. The molecular weights of the standard proteins are indicated. B: Purified CBCDase was treated without or with glycosidase F (*Lane 1* and *2* respectively) as described under "Materials and methods" and was subjected to SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-nCDase antibody.

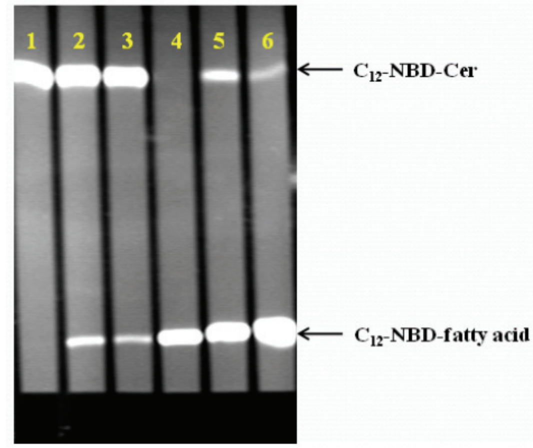

Figure 3. Mode of action of CBCDase. The CDase activity and ceramide synthase activity reactions were carried out and developed on a TLC plate as described under "Materials and methods". *Lane 1*, C_{12}-NBD-Cer only; *lane 2 and 3,* C_{12}-NBD-Cer *with 10 ng and 5 ng of protein respectively, lane 4,* C_{12}-NBD-fatty acid and *D-erythro*-Sph only (substrate for Cer synthase activity); *lane 5 and 6,* C_{12}-NBD-fatty acid, *D-erythro*-Sph *with 10ng and 5ng of protein respectively*.

peak of enzyme activity (CDase II) was obtained which could represent a more hydrophobic form of the CBCDase. In the present study we only focused on purification and characterization of the salt-eluted first peak of enzyme activity (CDase I).

The first active fractions obtained from the DEAE-Sepharose column were adjusted to 0.225 M NaCl, and then loaded on to phenyl-Sepharose column. After a step gradient in order to remove unbound proteins, the active fractions were eluted with 1 mM Tris buffer (**Figure 1B**). Here we obtained an increase of specific activity from 18.9 to 82.6 fold. The active fractions were then loaded onto a Superdex 200 column to remove other impurities, followed by Mono Q column to reach an almost homogenous protein (**Figure 1C** and **1D**). The purified fractions from Mono Q were subjected to SDS-PAGE followed by silver staining. On SDS-PAGE, the purified CBCDase appeared pure with a single band at ~100 kDa (**Figure 2A**). It has been reported that other nCDases, such as RBCDase I, II and rat kidney CDase are highly glycosylated [16, 17, 20]. In order to verify whether CBCDase is glycosylated or not, we treated the purified enzyme with N-Glycosidase F which catalyzes the hydrolysis of asparagine-linked high mannose as well as hybrid and complex oligosaccharides from glycoproteins. After the treatment, the sample was subjected to SDS-PAGE, and then immuno blotted using an anti-neutral CDase antibody. As shown in **Figure 2B**, the ~100 kDa protein band shifted to ~80 kDa by the treatment of glycosidase F, which confirmed that the purified CDase is highly glycosylated.

Mode of action of purified camel brain ceramidase

Previously, it has been proposed that a single protein can catalyze the hydrolysis of Cer (CDase activity) and the reverse reaction (Ceramide synthase) through a CoA-independent mechanism [8]. Recent studies using several cloned neutral and alkaline CDases have confirmed these early observations, and revealed that these enzymes can, indeed, catalyze both Cer hydrolysis and Cer synthesis reaction *in vitro* [7, 35, 36]. The purified fractions obtained from Mono Q were pooled and used to characterize the enzyme. Investigation of the purified CBCDase revealed

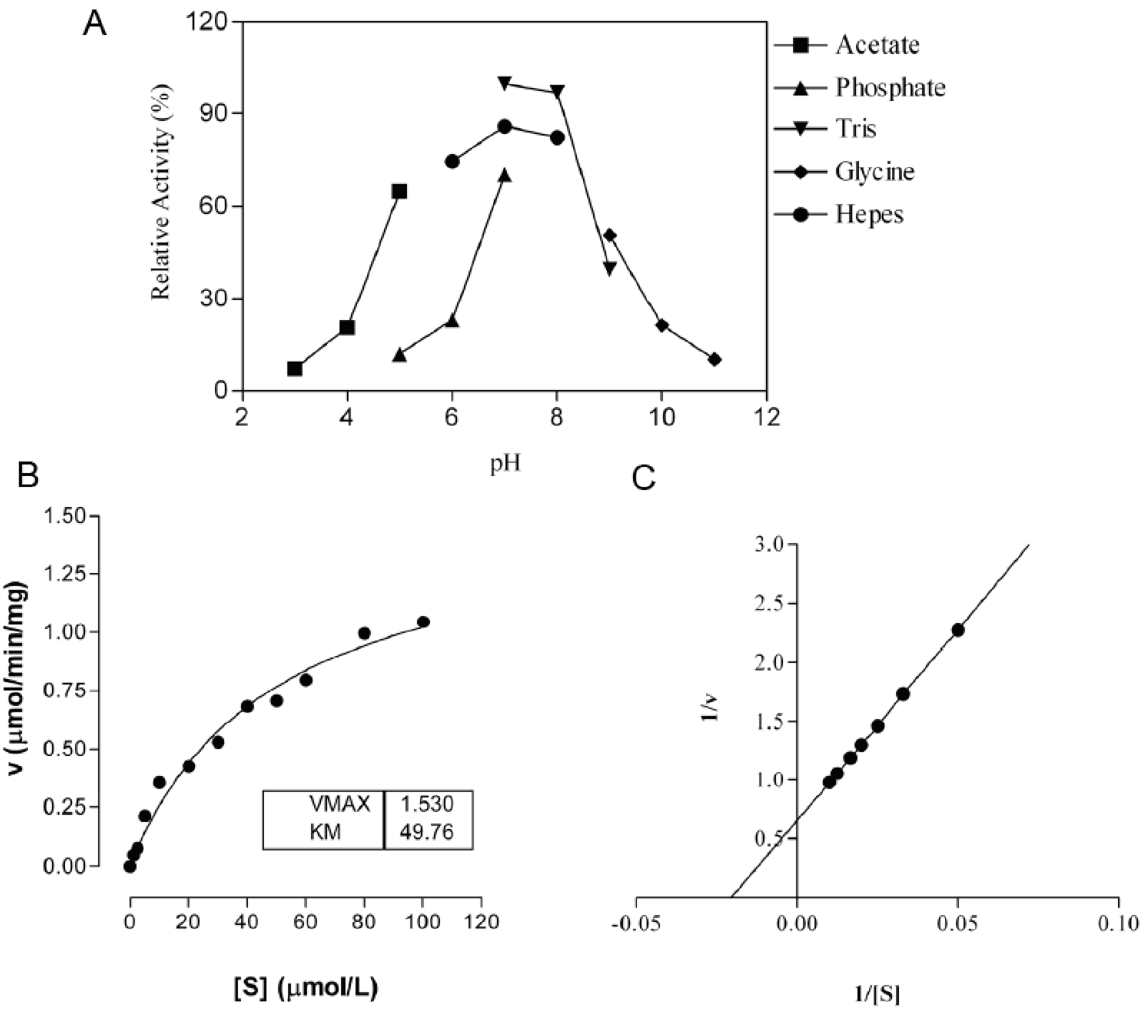

Figure 4. pH dependence and kinetics of purified CBCDase enzyme. A: The activity of CDase pooled from Mono Q column was measured as described under "Materials and methods". The pH was adjusted by the addition of the indicated buffers at a final concentration of 100 mM. B: Michaelis-Menten representation for CDase activity. Ceramidase activity was measured as described under "Materials and methods" in the presence of increasing concentration of C_{12}-NBD-Cer from 0 to 100 μM. C: Lineweaver-Burk representation for CDase activity toward increasing concentration of C_{12}-NBD-Cer. Data are the average of three independent experiments.

that this enzyme also catalyzes the forward and the reverse reaction. As demonstrated in **Figure 3**, CBCDase catalyzed both the hydrolysis of C_{12}-NBD-Cer to Sph and NBD-fatty acid (lane 2 and 3), and the condensation of D-*erythro*-Sph and C_{12}-NBD-fatty acid into C_{12}-NBD-Cer (*lane 5 and 6*).

Optimum pH and kinetics of CBCDase activity

The purified CBCDase showed a broad optimum pH activity ranging from pH 6–8 when assayed using C_{12}-NBD-Cer as substrate (**Figure 4A**). The optimal activity was observed at pH 7.0. The hydrolytic capacity of purified CBCDase in 50 mM Tris buffer was examined with C_{12}-NBD-Cer as substrate. The enzyme showed a classical Michaelis-Menten kinetics. Lineweaver-Burk plots, with C_{12}-NBD-Cer as substrate, derived a K_m of 49.76 μmol and a V_{max} of 1.53 μmol/min/mg for the CBCDase (**Figure 4B** and **4C**).

Substrate specificity of camel brain ceramidase

The substrate specificity of CBCDase was examined using various Cers as substrates. For the determination of substrate specificity the amount of released Sph measured by HPLC as

Table 2. Substrate specificity of CBCDase: Various substrates were dissolved in 50 mM Tris-HCl buffer, pH 7.5, containing 0.2% IGEPAL CA 630 and then incubated with 20 ng CBCDase (in 10 μl) at 37 °C for 1 h. The extent of hydrolysis of substrate was determined as described under "Materials and methods"

Substrate	Hydrolysis (%)
$C_{6:0}$-Cer	58.76
$C_{14:0}$-Cer	100.00
$C_{16:0}$-Cer	64.70
$C_{18:0}$-Cer	23.47
$C_{24:0}$-Cer	7.44
Dh-C_6-Cer	0.00
Dh-C_{14}-Cer	17.87
Dh-C_{16}-Cer	0.00

described under "Materials and methods". As shown in **Table 2**, CBCDase hydrolyzed various species of Cers. Amongst the Cers tested, $C_{14:0}$-Cer (N-myristoyl-D-*erythro*-sphingosine) was most efficiently hydrolysed, followed by $C_{16:0}$-Cer (N-palmitoyl-D-*erythro*-sphingosine), $C_{6:0}$-Cer (N-Hexanoyl-D-*erythro*-sphingosine), and $C_{18:0}$-Cer (N-stearoyl-D-*erythro*-sphingosine). Camel brain CDase does not show any specificities for dihydroCers, and only to a lesser extent the enzyme hydrolyzed Dh-$C_{14:0}$-Cer (**Table 2**).

Effect of cations on camel brain ceramidase activity

The effect of metal ions on the hydrolytic activity of the purified CBCDase were tested with 1 to 10 mM concentration of Mg^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Cu^{2+} (**Figure 5A**). The addition of $MgCl_2$ and $MnCl_2$ had no effect on the CBCDase activity, while $ZnCl_2$ and $CuCl_2$, even at 1 mM concentration, inhibited the enzyme activity by 50%. Intriguingly, the addition of $CaCl_2$ appeared to be slightly stimulatory with respect to CBCDase activity, which increased with increasing cation concentration.

Effect of phospholipids on camel brain ceramidase activity

To investigate the effect of various phospholipids on purified CBCDase activity, an equal volume of different concentrations of phospholipids, ranging from 0.25 to 2.0 mM, were added and the lipids then dried. The dried lipids were re-dissolved with an equal amount of substrate before the purified enzyme was added in order to initiate the assay. After the assay, the products were subjected to TLC chromatography, and the separated NBD-fatty acid was quantified as described under "Materials and methods". As observed in **Figure 5B** and **5C**, all phospholipids had an inhibitory effect on the hydrolytic activity of the purified CBCDase enzyme. Amongst these lipids, PA, PC, and PS were all potent inhibitors. However, PA was the most potent inhibitor of the purified CBCDase enzyme (**Figure 5B**).

Effect of reducing agents on camel brain ceramidase activity

The effect of reducing agents on the hydrolytic activity of purified CBCDase was tested by adding thiol-containing compounds such as GSH, GSSG, NAC, and cysteine, at different concentrations starting from 5 to 20 mM. All tested reducing agents inhibited the hydrolytic activity of the purified CBCDase. Amongst these, GSSG had the most potent inhibitory effect. At 10 mM concentration all reducing agents fully inhibited CBCDase (**Figure 5D**).

Effect of nucleotides on camel brain ceramidase activity

The effect of purine and pyrimidine nucleotides on the purified CBCDase activity was tested. As can be seen in **Figure 6A**, ATP inhibited CBCDase activity of the purified enzyme in a concentration dependent manner, while ADP completely repressed the enzyme activity at a low concentration (6 mM). AMP showed the least effect on CBCDase activity. **Figure 6B** represents the effect of the three guanosine nucleotides on purified CBCDase activity. These three nucleotides did not have a recognizable effect on the enzyme activity. Amongst the uridine nucleotides, only UMP showed inhibition towards the enzyme activity. However, UDP and UTP did not have a recognizable effect on the hydrolytic activity of the purified enzyme (**Figure 6C**). Similarly, amongst the thymidine nucleotides, only TMP inhibited the enzyme activity, while TDP and TTP did not have any significant effects on the CBCDase activity (**Figure 6D**).

Discussion

This study is the first report of the purification and biochemical characterization of camel brain neutral CDase (CBCDase). Through a

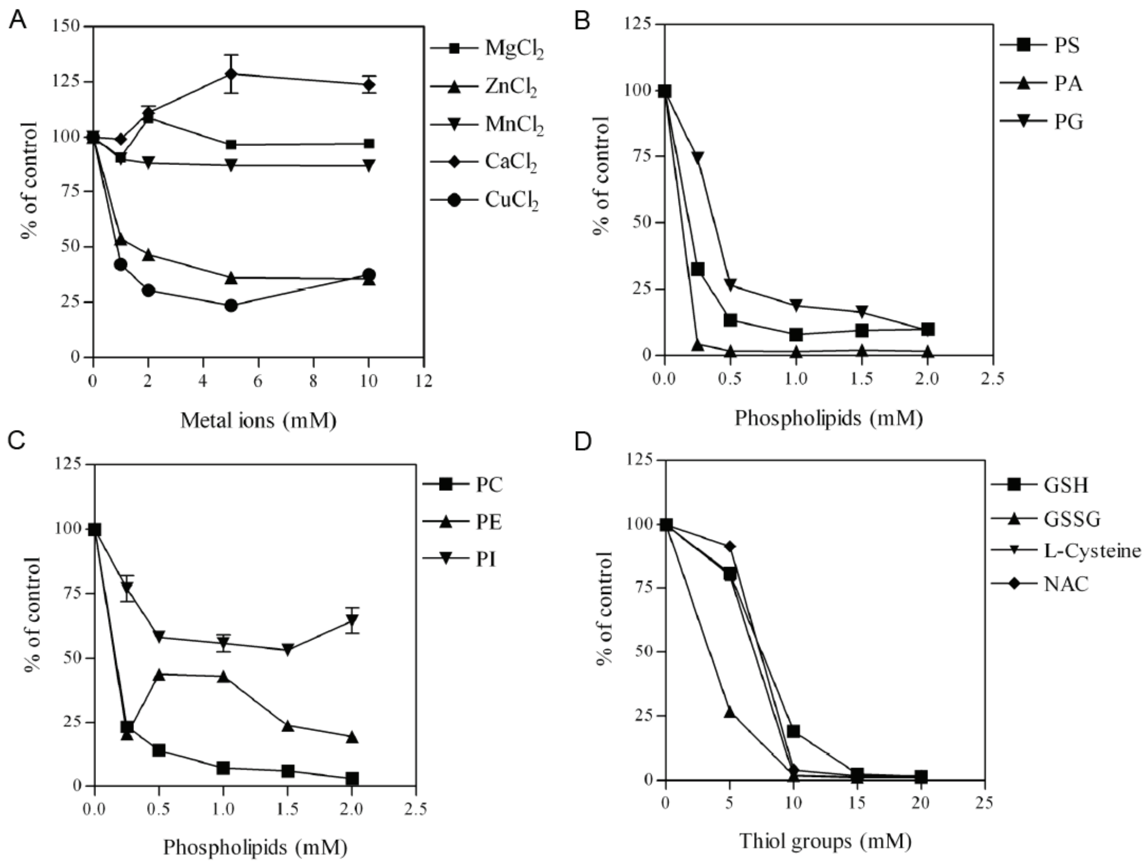

Figure 5. Effect of other metal ions, phospholipids and antioxidants on CDase activity. A: Effect of metal ions on neutral CDase activity was measured as described in "Materials and methods" by incubating the purified enzyme with C_{12}-NBD-Cer and indicated concentrations of metal ions as chloride salts. B: The indicated concentrations of phospholipids were dried down in the assay tubes and then resuspended with the substrate C_{12}-NBD-Cer and incubated with purified enzyme. The neutral CDase activity was measured as described under "Materials and methods". C: The neutral CDase activity of the purified enzyme was determined in the presence of indicated concentrations of reducing agent containing compounds such as GSH, GSSG, cysteine and NAC. Data are average of three independent experiments.

series of chromatographic steps, a protein appearing as a single band on SDS-PAGE, and with an apparent molecular mass of ~100 kDa was obtained. With respect to molecular mass, deglycosylation, and optimum pH, CBCDase showed similarity only to that of the zebrafish CDase [22]. During the purification of CBCDase, it was noticed that the order of the columns used was important, since the behaviour of the CBCDase on these columns was different to that of other recently reported rat brain CDases from our laboratory [17]. It was found that similar to RBCDases, at least two types of CDase are present in camel brain [16, 17]. The two active peaks are different in that the second peak is more hydrophobic than the first. The need for Triton X-100 to elute the second peak from the DEAE Sepharose column used in the first step, explains possible difference in the conformation and/or hydrophobicity of the camel brain CDases. In this study we have purified and characterized the first CBCDase peak to an apparent homogeneity.

Based on previous reports, C_{12}-NBD-Cer was used as a substrate for all of the characterization experiments [17, 22, 34]. As CDase enzymes are classified based on their optima pH, we looked at the optimal pH for the activity of purified CBCDase enzyme. Our study revealed that the purified CDase enzyme present in the camel brain has a neutral optimal pH of 7.0. However, the enzyme shows a recognizable conversion of substrate at the acidic pH starting from pH 5.0. In the alkaline pH range the enzyme shows a low or almost no hydrolytic

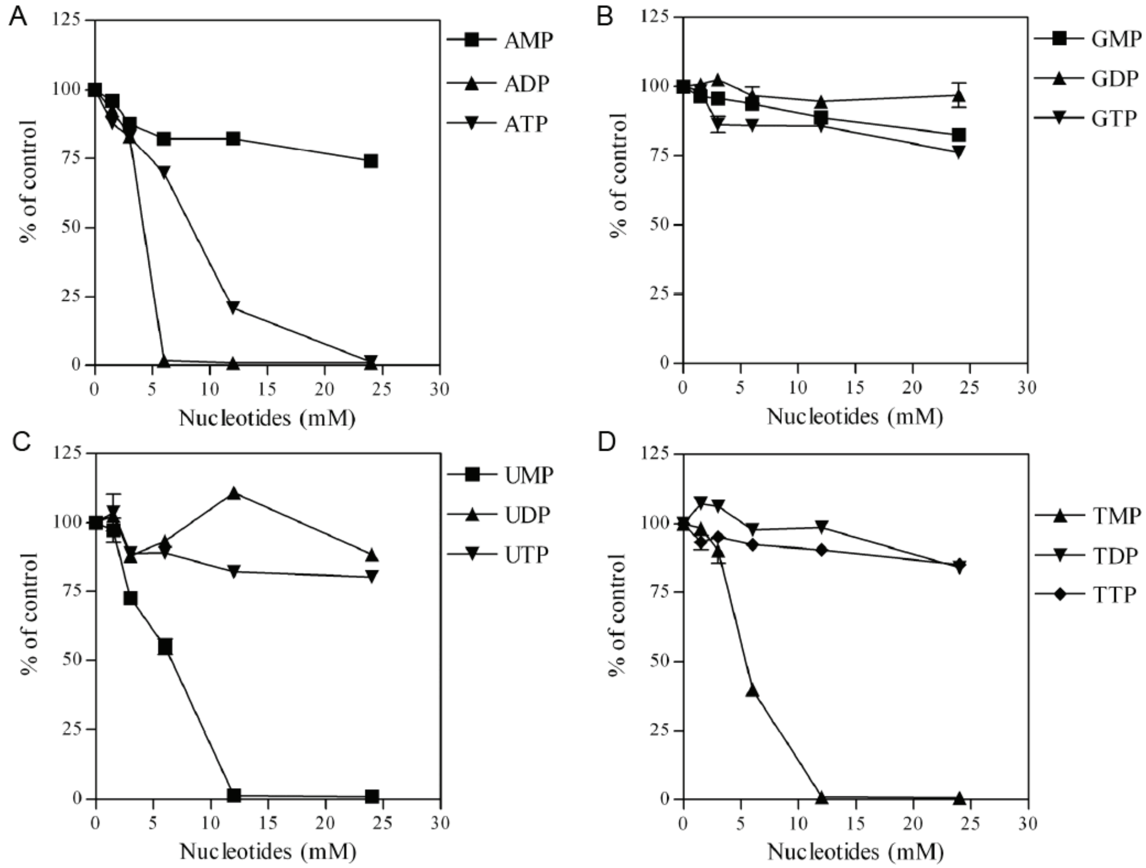

Figure 6. Effect of nucleotides on neutral CBCDase activity. A: Effect of adenosine nucleotides such as AMP, ADP and ATP on CDase activity was measured by incubating the purified enzyme with C_{12}-NBD-Cer and indicated concentrations of nucleotides. B: CDase activity was determined in the presence of the indicated concentrations of guanosine nucleotides such as GMP, GDP and GTP. C: Effect of uridine nucleotides such as UMP, UDP and UTP on CDase activity was measured in the presence of indicated concentrations. D: Indicated concentrations of thymidine nucleotides TMP, TDP and TTP were added with purified enzyme and the substrate C_{12}-NBD-Cer. The CDase activity was measured as described under "Materials and methods". Data are average of three independent experiments.

activity. These observations indicate that the purified CBCDase enzyme is a member of the neutral CDases with a broad pH profile, particularly in the acidic range. Gel filtration, silver staining, and western blot analysis using anti-neutral CDase antibody confirmed that the purified CBCDase possesses an apparent molecular mass of ~100 kDa, which shifts to ~80 kDa following deglycosylation as can be seen in **Figure 2B**. These results demonstrate that similar to RBCDase I and II, the purified CBCDase is glycosylated and is clearly different from the acid/neutral/alkaline CDases isolated from other origins [17].

The effects of metal ions on the purified CBCDase were found to be similar to other nCDases, as CBCDase activity was inhibited by Cu^{2+} and Zn^{2+} ions. The effect of Cu^{2+} ions on CBCDase activity was most pronounced, however, the activity was not influenced by Mg^{2+} and Mn^{2+}. This is contrary to rat brain CDase, where Mn^{2+} was shown to inhibit enzyme activity [16]. Interestingly, CBCDase activity was stimulated by Ca^{2+} ions.

Unlike RBCDase I where PS and PI have been shown to stimulate the enzyme activity, CBCDase activity is inhibited by all of the phospholipids tested (**Figure 4B** and **4C**). Interestingly, amongst these phospholipids, PA behaved as a potent inhibitor of CBCDase since it fully inhibited the enzyme activity at a very low concentration. Similar results are reported in the case of the RBCDase II [17].

All of the reducing agents that were tested on CBCDase, such as GSH, GSSG, NAC, and cysteine, inhibited the enzyme activity. This may be a mode of regulation of CBCDase enzyme activity

Table 3. Biochemical characterizations of CBCDase reverse activity. The table shows the effect of biochemical agents such as metal ions, phospholipids and reducing agents on both forward and reverse activity of CBCDase

Agents used	Forward activity			Reverse Activity		
	Optimum pH 7.0			Optimum pH 7.0		
	Activation	Inhibition	No effect	Activation	Inhibition	No effect
Metal ions	Ca^{2+}	Cu^{2+}, Zn^{2+}	Mg^{2+}, Mn^{2+}		Zn^{2+}, Cu^{2+}	Mn^{2+}, Mg^{2+}, Ca^{2+}
Phospholipids		PA, PG, PC, PS, PE, PI			PA, PG, PC, PS, PE, PI	
Reducing agents		GSH, GSSG, NAC, L-Cysteine			GSH, GSSG, NAC, L-Cysteine	

by these reducing agents in order to regulate the level of bioactive sphingolipids. Inhibition of another important sphingolipid metabolizing enzyme neutral sphingomyelinase (nSMase) by GSH has been previously reported [37]. The purified CBCDase activity was more inhibited by the oxidised form of GSH (GSSG), suggesting that the sulfhydryl group of GSH may not be required for the inhibition of the CBCDase as with the nSMase finding [37]. However, in our study, other reducing agents such as NAC and cysteine also inhibited CBCDase activity. To identify the mechanism and significance of the inhibitory action of these reducing agents on the CBCDase activity further mechanistic studies are required.

Furthermore, we verified the effect of nucleotides on the activity of the purified CBCDase. Unlike RBCDase II, ADP completely inhibited the activity of the CBCDase at low concentration, while ATP, UMP and TMP inhibited the activity only at higher concentrations [17]. Other nucleotides such as, AMP, UDP, UTP, GMP, GDP, GTP, TDP and TTP had no significant effect on CBCDase activity. Further studies are required to identify the exact role of these nucleotides in the regulation of CBCDase enzyme.

Recent studies have been shown that different CDases have distinct roles in regulating cellular responses, likely due to the difference in their cellular localization and their substrate specificities [38, 39]. The purified CBCDase showed high specificity towards medium chain ceramide $C_{14:0}$-Cer (N-myristoyl-D-*erythro*-sphingosine) and then $C_{16:0}$-Cer and $C_{6:0}$-Cer. The CBCDase enzyme showed least specificity towards long chain Cer species and dihydroceramide (dhCer) species. Further studies are required to identify the role of CBCDase in regulating cellular level of $C_{14:0}$-Cer and their cellular responses.

Several studies demonstrated that CDases could catalyze both Cer hydrolysis and Cer synthesis reactions. We found that purified CBCDase can synthesize Cer *in vitro* by using C_{12}-NBD-fatty acid and D-*erythro*-Sph as substrates. Recent studies, using several cloned neutral and alkaline CDases, have confirmed these early observations and have revealed that these enzymes can catalyze both Cer hydrolysis and Cer synthesis *in vitro* [6, 14, 15, 18]. This study also demonstrates that the purified CBCDase-associated reverse reaction is different from the major *de novo* Cer synthesis reaction, because the reaction proceeds *in vitro* without a requirement for acyl-CoA, ATP or Mg^{2+}. Unlike RBCDase, which showed a broad pH range (5.5-10) for the forward reaction [16], the optima pH for the CBCDase was 7.0 for both forward and reverse reactions. This study also supports the possibility of regulation of the level of bioactive lipids such as Cer, Sph and S1P through the reverse CBCDase activity. The CBCDase associated reverse reaction may be a pathway for Cer synthesis that is utilized only following cell stress and/or stimulation of signal transduction pathways requiring Cer. **Table 3** shows the effect of biochemical agents such as metal ions, phospholipids and reducing agents on both forward and reverse activity of CBCDase.

In conclusion, we have purified and characterized a novel CDase enzyme from the brain of *Camelus dromedarius* which can catalyse both forward and reverse reaction. There are biochemical differences between the purified CBCDase enzyme and the recently identified CDase enzymes. The biochemical responses of the CBCDase with different biomolecules are important to our understanding of the regulation of CBCDase enzyme since it controls the balance between important bioactive lipids, and hence, regulating their biological effects.

Further mechanistic studies are required to identify how molecules such as phospholipids, reducing agents, and nucleotides are regulating the CBCDase activity.

Abbreviations

aCDase, acid ceramidase; alkCDase, alkaline ceramidase; C_{12}-NBD-Cer, 4-nitrobenzo-2- oxa-l,3-diazole ceramide; CBCDase, camel brain ceramidase; CDase, ceramidase; Cer, ceramide; Cers, ceramides; nCDase, neutral ceramidase; RBCDase I, rat brain ceramidase I; RBCDase II, rat brain ceramidase II; PA, Phosphatidic acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; S1P, sphingosine-1-phosphate; SDS-PAGE, sodium dodecyl suphate-polyacrylamide gel electrophoresis; Sph, sphingosine.

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References

[1] Yabu T, Imamura S, Yamashita M, Okazaki T. Identification of Mg2+ -dependent neutral sphingomyelinase 1 as a mediator of heat stress-induced ceramide generation and apoptosis. J Biol Chem 2008; 283: 29971-82.

[2] Grether-Beck S, Salahshour-Fard M, Timmer A, Brenden H, Felsner I, Walli R, Füllekrug J. Ceramide and raft signaling are linked with each other in UVA radiation-induced gene expression. Oncogene 2008; 27: 4768-4778.

[3] Ardail D, Maalouf M, Boivin A, Chapet O, Bodennec J, Rousson R, Rodriguez-Lafrasse C. Diversity and complexity of ceramide generation after exposure of jurkat leukemia cells to irradiation. Int J Radiat Oncol Biol Phys 2009; 73: 1211-1218.

[4] Rath G, Schneider C, Langlois B, Sartelet H, Morjani H, Btaouri HE, Dedieu S, Martiny L. De novo ceramide synthesis is responsible for the anti-tumor properties of camptothecin and doxorubicin in follicular thyroid carcinoma. Int J Biochem Cell Biol 2009; 41: 1165-1172.

[5] Sánchez AM, Malagarie-Cazenave S, Olea N, Vara D, Chiloeches A, Díaz-Laviada I. Apoptosis induced by capsaicin in prostate PC-3 cells involves ceramide accumulation, neutral sphingomyelinase, and JNK activation. Apoptosis 2007; 11: 2013-2024.

[6] Franzen R, Fabbro D, Aschrafi A, Pfeilschifter J, Huwiler A. Nitric oxide induces degradation of the neutral ceramidase in rat renal mesangial cells and is counterregulated by protein kinase C. J Biol Chem 2002; 277: 46184-46190.

[7] El Bawab S, Mao C, Obeid LM, Hannun YA. Ceramidases in the regulation of ceramide levels and function. Subcell Biochem 2002; 36: 187-205.

[8] Mao C, Obeid LM. Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate. Biochim Biophys Acta 2008; 1781: 424-434.

[9] Gatt S. Enzymic hydrolysis and synthesis of ceramides. J Biol Chem 1963; 238: 3131-3133.

[10] Bernardo K, Hurwitz R, Zenk T, Desnick RJ, Ferlinz K, Schuchman EH, Sandhoff K. Purification, characterization, and biosynthesis of human acid ceramidase. J Biol Chem 1995; 270: 11098-11102.

[11] Koch J, Gärtner S, Li CM, Quintern LE, Bernardo K, Levran O, Schnabel D, Desnick RJ, Schuchman EH, Sandhoff K. Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification of the first molecular lesion causing Farber disease. J Biol Chem 1996; 271: 33110-33115.

[12] Ferlinz K, Kopal G, Bernardo K, Linke T, Bar J, Breiden B, Neumann U, Lang F, Schuchman EH, Sandhoff K. Human acid ceramidase: processing, glycosylation, and lysosomal targeting. J Biol Chem 2001; 276: 35352-35360.

[13] Yada Y, Higuchi K, Imokawa G. Purification and biochemical characterization of membrane-bound epidermal ceramidases from guinea pig skin. J Biol Chem 1995; 270: 12677-12684.

[14] Mao C, Xu R, Bielawska A, Obeid LM. Cloning of an alkaline ceramidase from Saccharomyces cerevisiae. An enzyme with reverse (CoA-independent) ceramide synthase activity. J Biol Chem 2000; 275: 6876-6884.

[15] Mao C, Xu R, Bielawska A, Szulc ZM, Obeid LM. Cloning and characterization of a Saccharomyces cerevisiae alkaline ceramidase with specificity for dihydroceramide. J Biol Chem 2000; 275: 31369-31378.

[16] El Bawab S, Bielawska A, Hannun YA. Purification and characterization of a membrane-

bound nonlysosomalceramidase from rat brain. J Biol Chem 1999; 274: 27948-27955.
[17] Thayyullathil F, Chathoth S, Hago A, Patel M, Szulc ZM, Hannun YA, Galadari S. Purification and characterization of a second type of neutral ceramidase from rat brain: A second more hydrophobic form of rat brain ceramidase. Biochim Biophys Acta 2011; 1811: 242-2452.
[18] Tani M, Okino N, Mitsutake S, Tanigawa T, Izu H, Ito M. Purification and characterization of a neutral ceramidase from mouse liver. A single protein catalyzes the reversible reaction in which ceramide is both hydrolyzed and synthesized. J Biol Chem 2000; 275: 3462-348.
[19] Galadari S, Wu BX, Mao C, Roddy P, El Bawab S, Hannun YA. Identification of a novel amidase motif in neutral ceramidase. Biochem J 2006; 393: 687-695.
[20] Mitsutake S, Tani M, Okino N, Mori K, Ichinose S, Omori A, Iida H, Nakamura T, Ito M. Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney. J Biol Chem 2001; 276: 26249-26259.
[21] Yoshimura Y, Okino N, Tani M, Ito M. Molecular cloning and characterization of a secretory neutral ceramidase of Drosophila melanogaster. J Biol Chem 2002; 132: 229-236.
[22] Yoshimura Y, Tani M, Okino N, Iida H, Ito M. Molecular cloning and functional analysis of zebrafish neutral ceramidase. J Biol Chem 2004; 279: 44012-44022.
[23] El Bawab S, Roddy P, Qian T, Bielawska A, Lemasters JJ, Hannun YA. Molecular cloning and characterization of a human mitochondrial ceramidase. J Biol Chem 2000; 275: 21508-13.
[24] Inoue T, Okino N, Kakuta Y, Hijikata A, Okano H, Goda HM, Tani M, Sueyoshi N, Kambayashi K, Matsumura H, Kai Y, Ito M. Mechanistic insights into the hydrolysis and synthesis of ceramide by neutral ceramidase. J Biol Chem 2008; 284: 9566-9577.
[25] Tani M, Igarashi Y, Ito M. Involvement of neutral ceramidase in ceramide metabolism at the plasma membrane and in extracellular milieu. J Biol chem 2005; 280: 36592-36600.
[26] Tani M, Iida H, Ito M. O-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein. J Biol Chem 2003; 278: 10523-10530.
[27] Hwang YH, Tani M, Nakagawa T, Okino N, Ito M. Subcellular localization of human neutral ceramidase expressed in HEK293 cells. Biochem Biophys Res Commun 2005; 331: 37-342.
[28] Wu BX, Zeidan YH, Hannun YA. Downregulation of neutral ceramidase by gemcitabine: Implications for cell cycle regulation. Biochim Biophys Acta 2009; 1791: 730-739.
[29] Coroneos E, Martinez M, McKenna S, Kester M. Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. Implications for cellular proliferation and differentiation. J Biol Chem 2005; 270: 23305-23309.
[30] Nikolova-Karakashian M, Morgan ET, Alexander C, Liotta DC, Merrill AH Jr. Bimodal regulation of ceramidase by interleukin-1beta. Implications for the regulation of cytochrome p450 2C11. J Biol Chem 1997; 272: 18718-18724.
[31] Franzen R, Pautz A, Bräutigam L, Geisslinger G, Pfeilschifter J, Huwiler A. Interleukin-1beta induces chronic activation and de novo synthesis of neutral ceramidase in renal mesangial cells. J Biol Chem 2001; 276: 35382-35389.
[32] Spence MW, Beed S, Cook HW. Acid and alkaline ceramidases of rat tissues. Biochem Cell Biol 1986; 64: 400-404.
[33] Thayyullathil F, Chathoth S, Hago A, Patel M, Galadari S. Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. Free Radic Biol Med 2008; 45: 1403-1412.
[34] Galadari S, Thayyullathil F, Chathoth S, Patel M, Hago AK. Biochemical characterization of reverse activity of human recombinant neutral ceramidase. J Med Sci 2009; 2: 128-135.
[35] Okino N, Tani M, Imayama S, Ito M. Purification and characterization of a novel ceramidase from Pseudomonas aeruginosa. J Biol Chem 1998; 273: 14368-14373.
[36] El Bawab S, Birbes H, Roddy P, Szulc ZM, Bielawska A, Hannun YA. Biochemical characterization of the reverse activity of rat brain ceramidase. A CoA-independent and fumonisin B1-insensitive ceramide synthase. J Biol Chem 2001; 276: 16758-16766.
[37] Liu B, Hannun YA. Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. J Biol Chem 1997; 272: 16281-16287.
[38] Sun W, Jin J, Xu R, Hu W, Szulc ZM, Bielawski J, Obeid LM, Mao C. Substrate specificity, membrane topology, and activity regulation of human alkaline ceramidase 2 (ACER2). J Biol Chem 2010; 285: 8995-9007.
[39] Hu W, Xu R, Sun W, Szulc ZM, Bielawski J, Obeid LM, Mao C. Alkaline ceramidase 3 (ACER3) hydrolyzes unsaturated long-chain ceramides, and its down-regulation inhibits both cell proliferation and apoptosis. J Biol Chem 2010; 285: 7964-7976.