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 Zn²⁺ and Cu²⁺, while Ca²⁺ 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...
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(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 occurred through a Zn2+ 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- C12-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 C12-NBD-Cer as a substrate as described previously [17]. Briefly, 25 µl of 100 µmol of D-erythro-C12-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 cor-
responding to NBD-dodecanoic acid and C\textsubscript{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\textsubscript{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\textsubscript{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 Heps 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 phenylmethylsulfonyl 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 sulfonil 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 phenylmethylsulfonil 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 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 milli-units 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-phthalaldehyde (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 X Terra 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 following 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-
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**Table 1. Purification of nCDase from camel brain**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Proteins</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
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<td></td>
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<td>units/mg</td>
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<td>fold</td>
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<td>Triton X-100 Extract</td>
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<td>16.8</td>
<td>1.1</td>
<td>157.0</td>
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<tr>
<td>Mono Q</td>
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<td>28.6</td>
<td>230.6</td>
<td>0.6</td>
<td>2147.1</td>
</tr>
</tbody>
</table>

*aCDase was purified from camel brain (250 g) as described under “Material and methods”.

![Figure 1.](image)

**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 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 up to 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
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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 immunoblotted 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...
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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...
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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”.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6:0-Cer</td>
<td>58.76</td>
</tr>
<tr>
<td>C14:0-Cer</td>
<td>100.00</td>
</tr>
<tr>
<td>C16:0-Cer</td>
<td>64.70</td>
</tr>
<tr>
<td>C18:0-Cer</td>
<td>23.47</td>
</tr>
<tr>
<td>C24:0-Cer</td>
<td>7.44</td>
</tr>
<tr>
<td>Dh-C6-Cer</td>
<td>0.00</td>
</tr>
<tr>
<td>Dh-C14-Cer</td>
<td>17.87</td>
</tr>
<tr>
<td>Dh-C16-Cer</td>
<td>0.00</td>
</tr>
</tbody>
</table>

described under “Materials and methods”. As shown in Table 2, CBCDase hydrolyzed various species of Cers. Amongst the Cers tested, C14:0-Cer (N-myristoyl-D-erythro-sphingosine) was most efficiently hydrolysed, followed by C16:0-Cer (N-palmitoyl-D-erythro-sphingosine), C6:0-Cer (N-Hexanoyl-D-erythro-sphingosine), and C18:0-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-C14-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 Mg2+, Zn2+, Mn2+, Ca2+, and Cu2+ (Figure 5A). The addition of MgCl2 and MnCl2 had no effect on the CBCDase activity, while ZnCl2 and CuCl2, even at 1 mM concentration, inhibited the enzyme activity by 50%. Intriguingly, the addition of CaCl2 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
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A 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, C12-NBD-Cer was used as a substrate for all of the characterization experiments [17, 22, 34]. As CDase enzymes are classified based on their optimal 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 activity.

**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 C12-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 C12-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.

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Purification and biochemical characterization of neutral ceramidase 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 Cu²⁺ and Zn²⁺ ions. The effect of Cu²⁺ ions on CBCDase activity was most pronounced, however, the activity was not influenced by Mg²⁺ and Mn²⁺. This is contrary to rat brain CDase, where Mn²⁺ was shown to inhibit enzyme activity [16]. Interestingly, CBCDase activity was stimulated by Ca²⁺ 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.
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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

<table>
<thead>
<tr>
<th>Agents used</th>
<th>Forward activity</th>
<th>Reverse Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimum pH 7.0</td>
<td>Optimum pH 7.0</td>
</tr>
<tr>
<td></td>
<td>Activation</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Metal ions</td>
<td>Ca²⁺</td>
<td>Cu²⁺, Zn²⁺</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>PA, PG, PC, PS, PE, PI</td>
<td>PA, PG, PC, PS, PE, PI</td>
</tr>
<tr>
<td>Reducing agents</td>
<td>GSH, GSSG, NAC, L-Cysteine</td>
<td>GSH, GSSG, NAC, L-Cysteine</td>
</tr>
</tbody>
</table>

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 C₁₂-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 Mg²⁺. 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; C₂-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, Phosphatidylycholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; S1P, sphingosine-1-phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sph, sphingosine.

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