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Molecular cloning and characterization of the alkaline ceramidase from *Pseudomonas aeruginosa* PA01

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Abstract

Ceramidase (CDase) hydrolyzes the amide bond in ceramides to yield free fatty acid and sphingosine. From a 3-L *Pseudomonas aeruginosa* PA01 culture, 70 μg of extracellular alkaline, Ca^{2+}-dependent CDase, was purified to homogeneity, the N-terminal sequence was determined, and the CDase gene was cloned. The CDase gene encodes a 670 amino acid protein with a 26 amino acid signal peptide. CDase was expressed in five prokaryotic and eukaryotic expression systems. Small amounts of recombinant active extracellular CDase were expressed by *Pseudomonas putida* KT2440. In *Pichia pastoris* GS115 low amounts of recombinant extracellular glycosylated CDase were expressed. High levels of intracellular CDase were expressed by *Escherichia coli* DH5α and *E. coli* BL21 cells under control of the *lac*-promoter and T7-promoter, respectively. From a 3-L *E. coli* DH5α culture, 280 μg of pure CDase was obtained after a three-step purification protocol. Under control of the T7-promotor CDase, without its signal peptide, was produced in inclusion bodies in *E. coli* BL21 cells. After refolding, 1.8 mg of pure active CDase was obtained from a 2.4-L culture after ammonium sulfate precipitation and gel filtration. Both the recombinant and wild-type CDases have a pH optimum of 8.5. The recombinant enzyme was partially characterized. This is the first report of a high yield CDase production system allowing detailed characterization of the enzyme at the molecular level.

Keywords: Ceramidase; *Pseudomonas*; Gene; Inclusion body; Virulence factor

Sphingolipids are significant constituents of eukaryotic cells and occur mainly in the plasma membrane, the Golgi apparatus, and lysosomes. Furthermore, sphingolipids are highly bioactive compounds, involved in the regulation of cell growth, induction of cell differentiation, and apoptosis (for reviews see [1–4]). In sphingomyelin, the major phosphosphingolipid in mammals, a phosphocholine group, is attached to the terminal hydroxyl group of a ceramide moiety. In cerebrosides, glucose or galactose, and in gangliosides oligosaccharides are linked to the primary hydroxyl group of ceramide. The latter lipids interact with growth factor receptors, the extracellular matrix, and neighboring cells.

Sphingomyelin can be hydrolyzed to ceramide by sphingomyelinase. Subsequently, this ceramide can be hydrolyzed by ceramidase (CDase[2]) to yield a free fatty acid and sphingosine (see Fig. 1).

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[2] *Abbreviations used*: CDase, ceramidase; SMase, sphingomyelinase; IPTG, isopropylthiogalactoside; PMSF, *p*-phenylmethylsulfonyl fluoride; DTT, dithiothreitol; $CHCl_3$, chloroform; MeOH, methanol; NH_4OH, ammonia; NaCl, sodium chloride; X-Gal, 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside; HIC, hydrophobic interaction chromatography; D-*erythro*-MAPP, D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol; HIC, hydrophobic interaction chromatography.

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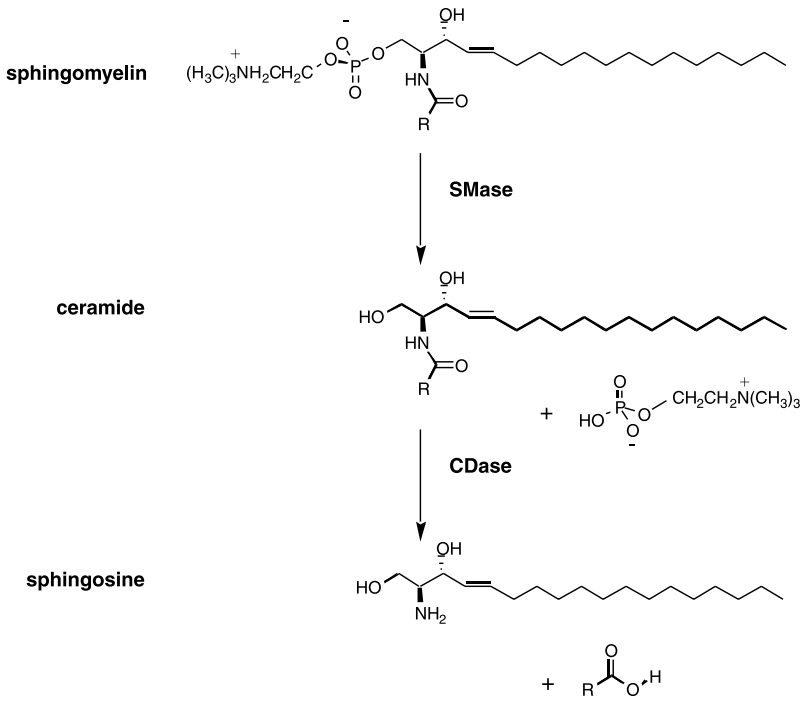

Fig. 1. The subsequent hydrolysis of sphingomyelin by sphingomyelinase (SMase) and ceramidase (CDase). R, alkyl.

Intracellular CDase is present in eukaryotes and prokaryotes and these enzymes have acid [5–9], neutral [10], or alkaline [11–17] pH optima. In mammals, CDase plays an important role in the control of cellular ceramide content and in the regulation of intracellular signal transduction. Acid CDase has been detected in rat brain, kidney, liver and spleen, human kidney and cerebellum, leukocytes, spleen, and plasma. Neutral and alkaline CDase has been found in pig intestinal mucosa, rat organs, human cerebellum and fibroblasts, pig lens epithelium, human leukocytes, and porcine epidermis [18].

The lysosomal acid CDase is the best characterized human CDase. The enzyme acts on ceramide at the lipid–water interface and is activated by anionic phospholipids and sphingolipid activator proteins [19]. In lysosomes, acid CDase plays a critical role in the catabolism of ceramides, and the hereditary deficiency of this CDase leads to the accumulation of ceramide in the lysosomes as it occurs in the lethal Farber disease.

The first extracellular bacterial ceramidases were reported in *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* [11,15]. Bacterial CDase has been implicated to be involved in the ceramide deficiency in the horny layer of the epidermis of patients with atopic dermatitis suffering from bacterial infections [20]. Bacterial CDase generates sphingosine and the latter lipid downregulates macrophages activated by lipopolysaccharides from Gram-negative bacteria like *Pseudomonas* [21], leading to an under-reaction of the body and, hence, CDase may be a virulence factor [22].

CDases are a remarkable class of enzymes because they are lipolytic enzymes that act, like lipases and phospholipases, at the lipid–water interface of membranes and micelles. CDases, however, hydrolyze amide bonds instead of ester bonds. In that respect, CDases resemble proteases. For biochemical studies and for structure elucidation, substantial amounts of CDase are required. First, we tested the occurrence of extra cellular CDase in several *Pseudomonas* strains. We purified the CDase from *P. aeruginosa* PA01, determined its N-terminal amino acid sequence, and cloned its gene. We tested five different prokaryotic and eukaryotic as possible expression systems for an efficient CDase production. Here, we describe an expression system that for the first time allows the production of multi-milligram amounts of active CDase that allows a more detailed characterization of this bacterial CDase.

Materials and methods

Gel electrophoresis and determination of protein concentration

SDS–PAGE (10%) analyses were performed, as described previously [23]. Isoelectric focusing was carried out using a Ready Gel Precast IEF gel (pH 3–10, Bio-Rad) according to manufacturers' instructions.

Protein concentrations were determined with a Bio-Rad DC (Detergent-Compatible) Protein Assay (Bio-Rad) with bovine serum albumin (Sigma) as a standard.

Buffers

Buffer A: 50 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA, Aldrich), and 0.1% (w/v) Thesit (Boehringer–Mannheim), pH 8.0.
Buffer B: 50 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, and 0.1% (w/v) Thesit, pH 8.0.
Buffer C: 25 mM Tris–HCl, 0.25% (w/v) Triton X-100 (Fluka), and 2.5 mM $CaCl_2$, pH 8.5.
Buffer D: 50 mM Tris–HCl, 5 mM EDTA, 1 mM *p*-phenylmethylsulfonyl fluoride (PMSF, Sigma), and 0.25% (w/v) Triton X-100, pH 8.5.
Buffer E: 50 mM Tris–HCl and 40 mM EDTA, pH 8.0.
Buffer F: 10 mM Tris–HCl, pH 8.3.
Buffer G: 50 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol (DTT, Sigma), and 0.1% (w/v) Thesit, pH 8.5.
Buffer H: 50 mM Tris–HCl, 4 mM $CaCl_2$, and 0.1% (w/v) Triton X-100, pH 8.5.

Culture media

All media were prepared with demineralized water and were autoclaved for 30 min at 120 °C. Culture plates contained 1.5% (w/v) agar (Gibco-BRL). LB medium consisted of 1% (w/v) peptone (Difco Laboratories), 0.5% (w/v) yeast extract (Gibco-BRL), and 0.5% (w/v) NaCl.

B medium consisted of 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) $K_2HPO_4 \cdot 3H_2O$, and 0.1% (w/v) glucose.

Screening for SMase and CDase activities

Pseudomonas strains were grown at 30 °C and all other bacteria were grown at 37 °C in 10 mL B medium for 48 h. Cells were removed by centrifugation and the supernatants were assessed for the presence of SMase and CDase, using qualitative assays [19,24] based on the hydrolysis of the fluorescent C6-NBD sphingomyelin and C6-NBD ceramide analogues (Molecular Probes). In short, the fluorescent substrates (1 μg/mL) were dissolved in buffer H containing extra 2.5 mM $MgCl_2$ to determine CDase and SMase activities. To detect the presence of possible acid SMases or CDases, the substrates were also dissolved in 50 mM acetate buffer (pH 6.0) containing 0.5% (w/v) Triton X-100, 2.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$. The reactions were allowed to proceed for 4 h at 37 °C. After termination of the reactions by heating to 95 °C for 10 min., the samples were taken to dryness (Jouan RC 10.22 Speedvac). The residues were dissolved in $CHCl_3$/MeOH (2/1, v/v, 30 μL) and separated by TLC on Merck Kieselgel F254 DC-Fertig plates (20 × 20 cm; 0.25 mm thickness), using $CHCl_3$/MeOH/33% NH_4OH, 90/25/0.5, v/v/v as eluent.

Quantitative radioactive CDase assay

The CDase activities were determined using a solution of 25 μM *N*-palmitoyl sphingosine (Sigma) and 2 μM N-1 [^{14}C]palmitoyl sphingosine (ARC) in buffer C. After 15 min, the incubations were terminated by the addition of MeOH (twice the incubation volume). The samples were taken to dryness and lipids were separated as described above. The relative radioactivities of free palmitic acid and non-hydrolyzed ceramide were determined with a Tracemaster 20 Automatic TLC-linear Analyser (Berthold).

CDase purification from wild-type P. aeruginosa PA01

Pseudomonas aeruginosa PA01 was grown at 30 °C for 3 days in 3-L LB medium containing 0.1% (w/v) taurodeoxycholate and 0.05% (w/v) sphingomyelin [11]. PMSF (1 mM) and EDTA (5 mM) were added to the supernatant and protein was precipitated with ammonium sulfate at 70% saturation (18 h) and centrifugation (GSA rotor, 4 °C, 12,000 rpm, 15 min). The pellet was resuspended in buffer A and dialyzed against the same buffer (3 × 2 L). The dialyzed protein (180 mL) was loaded onto a Resource Q column (6 mL, Pharmacia) and eluted with a linear NaCl gradient (0–1 M in buffer A, 6 mL/min, 30 column volumes) using an Äkta Explorer (Pharmacia Biotech) equipped with a 250-mL superloop. The fractions were tested for CDase with the qualitative fluorescent CDase assay, active fractions were pooled and dialyzed against buffer A (3 × 2 L). CDase was concentrated by loading onto a Resource Q column (6 mL, Pharmacia) and eluted in one step with buffer A containing 1 M NaCl. The concentrated CDase solution (6 mL) was brought to 4 M NaCl, loaded onto a Resource Phe column (1 mL, Pharmacia), and eluted with a linear inverse NaCl gradient (4–0 M NaCl in buffer A, 2 mL/min, 20 column volumes). The CDase containing fractions were pooled and dialyzed against buffer A (3 × 2 L) and concentrated using the Resource Q column as described above. The CDase preparation was filtered (0.22 μm, Millipore) and concentrated to 350 μL by centrifugation (Eppendorf centrifuge, 4 °C, 3000 rpm) through a Millipore UFC 3TTK25 Ultrafree MC Polysulfone 30,000 NMWL filter.

CDase was purified to homogeneity using a SMART system HPLC (Pharmacia) equipped with a Superdex 200 PC3.2/30 column (Pharmacia). CDase was loaded onto the column in 35 μL portions and eluted with buffer B at 50 μL/min. SDS–PAGE analysis showed the presence of a single protein band at 70 kDa.

N-terminal amino acid sequence determination

The N-terminal amino acid sequence was determined via Edman degradation with a gas-phase Sequenator

(Applied Biosystems model 490A protein sequencer, on line equipped with a model 120A phenylthiohydantoin analyser) at the Protein Sequencing Facility, Leiden University, The Netherlands.

Molecular cloning and DNA sequencing

Pseudomonas aeruginosa PA01 was grown in B medium (5 mL) at 30 °C for 24 h.

General cloning techniques and isolation of genomic DNA of *P. aeruginosa* PA01 were carried out essentially as described by Sambrook et al. [25].

Nucleotide sequences were determined on both DNA strands by the dideoxynucleotide chain termination method with a Thermo-Sequenase-Fluorescent-Labelled-Primer-Cycle Sequencing-Kit (Amersham) using a LI-COR DNA sequencer (MWG-Biotech).

Plasmids, DNA fragments, and PCR products were isolated with Qiagen Plasmid Midi Kit, Qiaquik Gel Extraction Kit, and Qiaquik PCR Purification Kit according to the manufacturers' instructions. All nucleases and nuclease buffers were from New England Biolabs. T4 ligase and ligase buffer were from Life Technologies.

PCR was performed using a Primus PCR System (MWG-Biotech). Typically, 45 cycles (each consisting of denaturation at 94 °C for 5 min, annealing for 1 min, and extension at 72 °C for 2.5 min) were performed, using the Expand High Fidelity PCR Kit (Boehringer–Mannheim) according to manufacturer's instructions.

Primers were purchased from MWG-Biotech.

Transformation of *Escherichia coli* and *Pseudomonas putida* KT2440 was performed as described [25,26], using a Gene Pulser equipped with a Gene Controller (Bio-Rad) in a 0.2-cm electroporation cuvette.

PCR amplification and cloning of the CDase gene into pUC18

The CDase gene was identified using the determined N-terminal amino acid sequence in a BLAST search [27–29] in the *P. aeruginosa* PA01 genome (Pseudomonas Genome Project, Pathogenesis, www.pseudomonas.com). The CDase gene was amplified using *P. aeruginosa* PA01 genomic DNA as a template. Primers I (5′-GGAAAACAAGAAGAGGGTCGCCATGTCACGTTCCGC-3′) and II (5′-GATAATTTATCGGTTGATCCCGAAAGCGACTTCG-3′) were designed to amplify the complete CDase gene from 21 bp upstream of the start codon to 246 bp downstream of the stop codon, thus including the Shine–Dalgarno sequence and the terminator region. The amplified PCR product was purified and ligated into a pUC18 vector using the SureClone Ligation Kit (Amersham Pharmacia Biotech) according to manufacturers' instructions and the new plasmid was called pUC18/CDase. The plasmid pUC18/CDase was used to transform *E. coli* (DH5α) and plasmid-containing clones were identified with blue–white screening on B medium plates containing 100 μg/mL ampicillin and 40 μg/mL X-Gal. The transformants were grown in LB medium (50 mL) with ampicillin (100 μg/mL) and CDase production was induced with isopropylthiogalactoside (IPTG, Sigma, 0.4 mM). Cells were recovered by centrifugation (SS34, 4 °C, 5000 rpm, 10 min), resuspended in ice-cold Buffer C, and lysed by sonication (Branson sonifier 250, small tip, level 7, 50% output, 20 times for 30 s with 1-min cooling between sonication bursts). Aliquots (1 mL) were taken and centrifuged (Eppendorf centrifuge, 13,000*g*) and the clear supernatants were used in the fluorescent CDase assay as described above. The sequence of the pUC18/CDase constructs of the active CDase producing transformants was then determined, as described above.

Construction of expression plasmid pT7-5/CDase

The plasmid pUC18/CDase and vector pT7-5 were treated with *Eco*RI and *Hin*dIII, and purified and the ligation product was used to transform *E. coli* BL21 (DE3, Stratagene). Transformants were screened for CDase production as described above.

Construction of expression plasmid pT7-7/CDase

Plasmid pUC18/CDase was used as a template in a PCR to amplify the CDase gene from 72 bp upstream of the start codon (Asp26) to 226 bp downstream of the stop codon using primers III (5′-CCTGCCAGGGCCGACCATATGCCCTACCGC-3′) and IV (5′-GATAATTTATCGGTTGATCCCGAAAGCTTCTTCGATATGACC-3′). The newly introduced *Nde*I and *Hin*dIII cleavage sites are underlined. Vector pT7-7 and the PCR product were treated with *Nde*I and *Hin*dIII, and purified, and the ligation product was used to transform *E. coli* BL21 (DE3, Stratagene). Clones were induced with IPTG (0.4 mM) and their total protein content was analyzed with SDS–PAGE. The DNA sequences of the pT-7-7/CDase constructs of transformants that showed IPTG-inducible protein production were determined.

Construction of expression plasmid pVLT33/CDase

Plasmid pUC18/CDase and vector pVLT33 [26] were treated with *Eco*RI and *Hin*dIII. The ligation product pVLT33/CDase was used to transform *E. coli* DH5α. Transformants were grown at 37 °C on B medium plates containing 75 μg/mL kanamycin. *P. putida* KT2440 was transformed with purified pVLT33/CDase and CDase production by the latter transformants was achieved at 30 °C for 18 h in liquid B medium containing 75 μg/mL

kanamycin and IPTG (1 mM). Cells were removed by centrifugation and with the fluorescent CDase assay the supernatants were tested for CDase activity.

Cloning of CDase in Pichia pastoris

Pichia pastoris GS115 (Invitrogen) was manipulated and genetically modified according to manufacturers' instructions (Pichia Expression Kit and pPIC9K manuals). The CDase gene was amplified using pUC18/CDase as a template and using primers V (5′-GACGACCTG CCCTACGTATTCGGCCTGGGC-3′) and VI (5′-GGC TGGCGAGGATTCCAACCTTGG-3′) to amplify the CDase gene starting from 93 bp (Phe31) to 25 bp downstream of the stop codon. The newly introduced *Sna*BI and *Eco*RI cleavage sites are underlined. After *Sna*BI and *Eco*RI treatment, the PCR product was cloned into the vector pPIC9K.

Recombinant CDase production and isolation from E. coli DH5α

Escherichia coli DH5α (pUC18/CDase) was grown in LB medium, containing ampicillin (100 μg/mL), at 37 °C. Bacteria from an overnight culture were used to inoculate three separate 2-L conical flasks, each containing 1 L LB medium, to $OD_{600} = 0.08$–0.1. The bacteria were grown to $OD_{600} = 0.4$–0.5. CDase production was induced with IPTG (0.4 mM). After 3 h, the cells were collected by centrifugation (GS3 rotor, 4 °C, 5000 rpm, 10 min) and resuspended in ice-cold buffer D. The cells were then lysed by sonication (Branson sonifier 250, big tip, level 7, 50% output, 30 times 1 min with 1-min rest in-between). After centrifugation (SS34 rotor, 4 °C, 14,000 rpm, 10 min), the supernatant was dialyzed against buffer A (3 × 2 L) and recombinant CDase was purified, as described for the wild-type CDase (vide supra).

Recombinant CDase production and isolation from inclusion bodies

Escherichia coli (BL21 containing pT7-7/CDase) were grown in LB medium, containing ampicillin (100 μg/mL), at 37 °C. Bacteria of an overnight culture were used to inoculate six separate 2-L conical flasks, each containing 400 mL LB medium with ampicillin (100 μg/mL), to $OD_{600} = 0.08$–0.1 The bacteria were grown to $OD_{600} = 0.4$–0.5. CDase production was induced with IPTG (0.4 mM). After 3 h, the cells were collected by centrifugation (GS3 rotor, 4 °C, 5000 rpm, 10 min) and stored at −20 °C. Cells (14 g) were resuspended in cold buffer E (60 mL). Sucrose (15.6 g) and lysozyme (15 mg) were added and the cells were incubated on ice for 40 min. A further 60 mL buffer E was added and the cells were incubated on ice for an additional 60 min. The cell suspension was sonicated (Branson sonifier 250, big tip, level 9, 50% output, 3 times 2 min with 2-min rest in-between) in 60 mL portions. Then, 2.5 mL BRIJ-35 (10%, v/v, in water) was added to each portion and the suspensions were sonicated for another 2 min. The inclusion bodies (IB) were isolated by centrifugation (GSA rotor, 4 °C, 400 rpm, 30 min), resuspended in buffer F, and then recovered by centrifugation (SS-34 rotor, 4 °C, 8000 rpm, 15 min).

IB (3 g wet weight) were dissolved in 300 mL of 8 M urea containing 10 mM DTT by stirring for 18 h at 4 °C. The turbid enzymatically inactive solution was centrifuged (GSA rotor, 4 °C, 8000 rpm, 30 min) and the clear supernatant was dialyzed against buffer G (3 × 5 L, 8 h each, 4 °C). Finally, the refolded, enzymatically active, material was dialyzed against buffer A (5 L, 16 h, 4 °C). Protein was recovered by ammonium sulfate precipitation at 75% saturation and centrifugation (SS-34 rotor, 4 °C, 15,000 rpm, 30 min). The pellet was resuspended in buffer A (2 mL) and dialyzed against the same buffer (2× 500 mL, 4 h each, 4 °C). CDase was purified batch-wise (0.5 mL) by gel filtration using a Pharmacia FPLC P-500 pump and Liquid Chromatography Controller LCC-500, on a Superdex 200 prep. grade column (30 cm × 1 cm i.d.; 25 mL, Pharmacia) at 4 °C. Protein was eluted with buffer B at a flow rate of 0.4 mL/min.

Results

CDases are an intriguing class of enzymes because on the one hand they are lipolytic enzymes that, like lipases and phospholipases, act on the lipid–water interface of membranes and micelles. On the other hand CDases, however, are not esterases but hydrolyze amide bonds like proteases. To identify a suitable CDase source to purify and clone an extracellular bacterial CDase, we screened several *Pseudomonas* species for extracellular CDase activity. Because the CDase substrate ceramide is formed after the hydrolysis of sphingomyelin by sphingomyelinase (SMase) and both enzymatic activities may be expressed by the same organism, the SMase activities of the bacteria were also tested. Only the pathogenic *P. aeruginosa* strains PA01, ATCC27853, and ATCC15692 produce extracellular CDase and SMase activities, whereas the non-pathogenic *Pseudomonas fluorescens* (ATCC17400 and ATCC13525), *P. putida* (ATCC12633 and KT2440), *Pseudomonas chlororaphis* ATCC9446, and *Pseudomonas syringae* ATCC19310 strains show no SMase or CDase activities. Here, we describe the purification of the CDase from the pathogenic *P. aeruginosa* PA01 strain and the cloning of its gene. Five different prokaryotic and eukaryotic expression systems for an efficient CDase production are described. The purification of recombinant CDase from *E. coli* DH5α and BL21 is described.

CDase purification from P. aeruginosa PA01

Pseudomonas aeruginosa PA01 was grown at 30 °C in modified LB medium as described [14]. CDase was purified to homogeneity in 5.8% yield in three chromatographic steps (see Table 1). CDase was purified 217-fold and 70 μg of pure CDase was obtained from a 3-L culture. The use of the Resource Q anion exchange column resulted in a 87% loss of activity, whereas the specific activity increased 4.1-fold. With Hydrophobic Interaction Chromatography (HIC) purification, the specific activity was increased 2.9-fold and an almost 2-fold increase of the total activity was achieved. This may be due to the removal of a hydrophobic inhibiting substance which is tightly bound to the CDase. This inhibiting compound may be sphingosine from the culture medium, since the latter compound is a strong CDase inhibitor (vide infra) and may have been formed from sphingomyelin by subsequent hydrolysis by SMase and CDase. In the final gel filtration step, more than 76% of the activity was lost, but the specific activity increased more than 18-fold. CDase was visible as a single protein band at 70 kDa in SDS–PAGE analysis.

N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified CDase was determined via Edman degradation and the found amino acid sequence -LPYRFGLGKA-DITGEAAEVGMMGYS-LEQKTA was used to identify the CDase gene with a BLAST search (see Materials and methods). This indicated that the gene codes for a 670 amino acid protein, with a typical 26 amino acid signal peptide. *Pseudomonas* CDase is not homologous with known eukaryotic CDases. The sequence of the gene (see Fig. 2) has been submitted to the EMBL nucleotide sequence database (Accession No. AJ315932). CDase from *P. aeruginosa* AN17 ([18], Accession No. AB028646) differs from that of *P. aeruginosa* PA01 at four positions, i.e., I90T, N181S, V196A, and E598V (see Fig. 2).

Recombinant CDase with signal peptide: cloning, production, and isolation

The CDase gene, including its Shine–Dalgarno sequence and the putative terminator region, was amplified using *P. aeruginosa* PA01 genomic DNA as a template and was cloned into a pUC18 vector. The CDase was expressed in its active form in *E. coli* DH5α and the active recombinant CDase was found only intracellularly. CDase is most likely present in the periplasm, but is not secreted. We did not investigate the location of the enzyme in further detail. The recombinant CDase was purified using the same protocol as that used for the wild-type enzyme (Table 1). From a 3-L *E. coli* culture, 280 μg of pure CDase was obtained in 2.5% yield. As with the purification of the wild-type enzyme, a significant amount (91%) of the activity was lost in the ResourceQ chromatographic purification step. This step removed most of the protein, but surprisingly also lowered the specific activity. The subsequent HIC purification step resulted in a 70% activity loss and the removal of 88% of total protein. The specific activity increased 2.4-fold. The loss of activity may be due to the irreversible binding of the recombinant CDase to the hydrophobic HIC resin. In the final gel filtration step, the CDase was purified to homogeneity and the specific activity was increased 48-fold. SDS–PAGE analysis showed CDase as a single protein band at 70 kDa.

Attempts to improve the overall yield by reduction of the number of purification steps were unsuccessful.

Expression of CDase in P. pastoris and P. putida

Low amounts of extracellular active recombinant CDase were produced with *P. pastoris* GS115 and *P. putida* KT2440. Because of the low yields, these expression systems were not investigated further. The CDase produced in *P. pastoris* is most likely glycosylated (data not shown).

Table 1
Purification of extracellular wild-type ceramidase (WT-CDase) from *P. aeruginosa* PA01 and intracellular active recombinant ceramidase (rec-CDase) expressed in *E. coli* DH5α

Step	WT-CDase (extracellular)			rec-CDase (intracellular)		
	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
Cell lysate	NA	NA	NA	1008.0	2419.2	2.4
$(NH_4)_2SO_4$	261.0	290	1.1	ND	ND	ND
ResQ	8.3	37.3	4.5	112.6	213.9	1.9
ResPhe	5.4	70.2	13.0	13.7	63.0	4.6
Superdex 200	0.07	16.8	239.0	0.28	61.0	218.0
Overall yield (%)		5.8			2.5	

NA, not applicable; ND, not done; $(NH_4)_2SO_4$, 75% ammonium sulfate precipitation; ResQ, resource Q anion exchange chromatography; ResPhe, resource Phe hydrophobic interaction chromatography; Superdex 200, Superdex 200 gel filtration chromatography. CDase activities were determined by measuring the free [^{14}C]palmitic acid generated by the hydrolysis of N-1 [^{14}C]palmitoyl sphingosine (see Materials and methods).

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-22   GGA AAA CAA GAA GAG GGT CGC C

1     ATG TCA CGT TCC GCA TTC ACC GCG CTC TTG CTG TCC TGC GTC CTG   45
1      M   S   R   S   A   F   T   A   L   L   L   S   C   V   L   15

46    CTG GCG CTC TCC ATG CCT GCC AGG GCC GAC GAC CTG CCC TAC CGC   90
16     L   A   L   S   M   P   A   R   A   D   D   L   P   Y   R   30

91    TTC GGC CTG GGC AAG GCG GAC ATC ACC GGC GAA GCC GCC GAA GTC   135
31     F   G   L   G   K   A   D   I   T   G   E   A   A   E   V   45

136   GGC ATG ATG GGT TAC TCC TCC CTC GAA CAG AAG ACC GCC GGC ATC   180
46     G   M   M   G   Y   S   S   L   E   Q   K   T   A   G   I   60

181   CAC ATG CGC CAG TGG GCG CGT GCC TTC GTG ATC GAG GAA GCG GCC   225
61     H   M   R   Q   W   A   R   A   F   V   I   E   E   A   A   75

226   AGC GGA CGT CGC CTG GTC TAC GTC AAC ACC GAC CTG GGG ATG ATC   270
76     S   G   R   R   L   V   Y   V   N   T   D   L   G   M   I   90

271   TTC CAG GCC GTG CAC CTG AAG GTC CTG GCC CGG CTC AAG GCG AAG   315
91     F   Q   A   V   H   L   K   V   L   A   R   L   K   A   K   105

316   TAC CCC GGT GTC TAC GAC GAG AAC AAC GTG ATG CTC GCC GCC ACC   360
106    Y   P   G   V   Y   D   E   N   N   V   M   L   A   A   T   120

361   CAC ACC CAC TCC GGT CCG GGC GGC TTC TCC CAC TAC GCG ATG TAC   405
121    H   T   H   S   G   P   G   G   F   S   H   Y   A   M   Y   135

406   AAC CTG TCG GTG CTC GGC TTC CAG GAA AAG ACC TTC AAC GCC ATC   450
136    N   L   S   V   L   G   F   Q   E   K   T   F   N   A   I   150

451   GTC GAC GGC ATC GTC CGC TCC ATC GAG CGG GCC CAG GCC AGG TTG   495
151    V   D   G   I   V   R   S   I   E   R   A   Q   A   R   L   165

496   CAG CCC GGC CGC CTG TTC TAC GGC AGC GGC GAG CTG CGC AAC GCC   540
166    Q   P   G   R   L   F   Y   G   S   G   E   L   R   N   A   180

541   AAC CGC AAC CGT TCG CTG CTG TCG CAC CTG AAG AAT CCG GAC ATC   585
181    N   R   N   R   S   L   L   S   H   L   K   N   P   D   I   195

586   GTC GGC TAC GAG GAT GGC ATC GAC CCG CAG ATG AGC GTG CTC AGC   630
196    V   G   Y   E   D   G   I   D   P   Q   M   S   V   L   S   210

631   TTC GTC GAC GCC AAC GGC GAG CTG GCC GGC GCG ATC AGT TGG TTC   675
211    F   V   D   A   N   G   E   L   A   G   A   I   S   W   F   225

676   CCG GTG CAC AGC ACC TCG ATG ACC AAC GCC AAT CAC CTG ATC TCC   720
226    P   V   H   S   T   S   M   T   N   A   N   H   L   I   S   240

721   CCG GAC AAC AAG GGC TAC GCC TCC TAT CAC TGG GAG CAC GAC GTC   765
241    P   D   N   K   G   Y   A   S   Y   H   W   E   H   D   V   255

766   AGC CGC AAG AGC GGT TTC GTC GCC GCC TTC GCC CAG ACC AAT GCC   810
256    S   R   K   S   G   F   V   A   A   F   A   Q   T   N   A   270

811   GGC AAC CTG TCG CCC AAC CTG AAC CTG AAG CCC GGC TCC GGT CCC   855
271    G   N   L   S   P   N   L   N   L   K   P   G   S   G   P   285

856   TTC GAC AAC GAG TTC GAC AAC ACC CGC GAG ATC GGT CTG CGC CAA   900
286    F   D   N   E   F   D   N   T   R   E   I   G   L   R   Q   300

901   TTC GCC AAG GCC TAC GAG ATC GCC GGC CAG GCC CAG GAG GAA GTG   945
301    F   A   K   A   Y   E   I   A   G   Q   A   Q   E   E   V   315

946   CTC GGC GAA CTG GAT TCG CGC TTC CGT TTC GTC GAC TTC ACC CGC   990
316    L   G   E   L   D   S   R   F   R   F   V   D   F   T   R   330

991   CTG CCG ATC CGC CCG GAG TTC ACC GAC GGC CAG CCG CGC CAG TTG   1035
331    L   P   I   R   P   E   F   T   D   G   Q   P   R   Q   L   345

1036  TGC ACC GCG GCC ATC GGC ACC AGC CTG GCC GCC GGT AGC ACC GAA   1080
346    C   T   A   A   I   G   T   S   L   A   A   G   S   T   E   360

1081  GAC GGT CCA GGC CCG CTG GGG CTG GAG GAA GGC AAC AAT CCG TTC   1125
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Fig. 2. The ceramidase gene and protein sequence from *P. aeruginosa* PA01. The signal peptide is underlined and possible glycosylation sites are indicated in bold.

CDase production and isolation from inclusion bodies

The pUC18/CDase plasmid was used as a template to amplify the CDase gene without the sequence coding for the signal peptide. The CDase gene was amplified from position 72 (D26) to +226 and cloned in-frame in the multiple cloning site of the pT7-7 vector via the *Nde*I and *Hin*dIII sites. In this way, only the Asp26 and one

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361    D   G   P   G   P   L   G   L   E   E   G   N   N   P   F    375

1126  CTC TCG GCC CTT GGC GGG TTG CTC ACC GGC GTG CCG CCG CAG GAA   1170
376    L   S   A   L   G   G   L   L   T   G   V   P   P   Q   E    390

1171  CTG GTG CAA TGC CAG GCG GAA AAG ACC ATC CTC GCC GAC ACC GGC   1215
391    L   V   Q   C   Q   A   E   K   T   I   L   A   D   T   G    405

1216  AAC AAG AAA CCC TAC CCC TGG ACG CCG ACG GTG CTG CCG ATC CAG   1260
406    N   K   K   P   Y   P   W   T   P   T   V   L   P   I   Q    420

1261  ATG TTC CGC ATC GGC CAG TTG GAA CTG CTC GGC GCC CCC GCC GAG   1305
421    M   F   R   I   G   Q   L   E   L   L   G   A   P   A   E    435

1306  TTC ACC GTG ATG GCC GGG GTG CGG ATC CGC CGC GCG GTG CAG GCG   1350
436    F   T   V   M   A   G   V   R   I   R   R   A   V   Q   A    450

1351  GCC AGC GAA GCG GCC GGT ATC CGC CAT GTG GTC TTC AAT GGC TAC   1395
451    A   S   E   A   A   G   I   R   H   V   V   F   N   G   Y    465

1396  GCG AAT GCC TAT GCC AGC TAC GTC ACC ACC CGC GAG GAA TAC GCC   1440
466    A   N   A   Y   A   S   Y   V   T   T   R   E   E   Y   A    480

1441  GCC CAG GAA TAC GAA GGC GGC TCG ACC CTC TAC GGC CCC TGG ACC   1485
481    A   Q   E   Y   E   G   G   S   T   L   Y   G   P   W   T    495

1486  CAG GCC GCC TAC CAG CAG TTG TTC GTC GAC ATG GCG GTG GCG CTG   1530
496    Q   A   A   Y   Q   Q   L   F   V   D   M   A   V   A   L    510

1531  CGC GAA CGC CTG CCG GTG GAA ACC TCG GCG ATA GCG CCG GAC CTG   1575
511    R   E   R   L   P   V   E   T   S   A   I   A   P   D   L    525

1576  TCC TGC TGC CAG ATG AAC TTC CAG ACC GGA GTA GTG GCC GAC GAT   1620
526    S   C   C   Q   M   N   F   Q   T   G   V   V   A   D   D    540

1621  CCC TAT ATC GGC AAG TCC TTC GGC GAC GTG TTG CAA CAA CCC AGG   1665
541    P   Y   I   G   K   S   F   G   D   V   L   Q   Q   P   R    555

1666  GAA AGT TAT CGC ATC GGC GAC AAG GTG ACC GTC GCT TTC GTG ACC   1710
556    E   S   Y   R   I   G   D   K   V   T   V   A   F   V   T    570

1711  GGA CAT CCG AAG AAT GAC TTG CGC ACC GAG AAG ACT TTC CTG GAA   1755
571    G   H   P   K   N   D   L   R   T   E   K   T   F   L   E    585

1756  GTG GTG AAT ATC GGC AAG GAT GGC AAA CAG ACG CCC GAG ACC GTT   1800
586    V   V   N   I   G   K   D   G   K   Q   T   P   E   T   V    600

1801  GCC ACC GAT AAT GAC TGG GAT ACT CAA TAC CGC TGG GAG AGA GTG   1845
601    A   T   D   N   D   W   D   T   Q   Y   R   W   E   R   V    615

1846  GGT ATA TCT GCC TCG AAA GCG ACT ATC AGC TGG TCC ATT CCA CCA   1890
616    G   I   S   A   S   K   A   T   I   S   W   S   I   P   P    630

1891  GGG ACC GAG CCC GGC CAT TAC TAC ATC AGG CAC TAC GGC AAC GCG   1935
631    G   T   E   P   G   H   Y   Y   I   R   H   Y   G   N   A    645

1936  AAG AAC TTC TGG ACC CAG AAG ATC AGC GAA ATC GGC GGC TCG ACC   1980
646    K   N   F   W   T   Q   K   I   S   E   I   G   G   S   T    660

1981  CGC TCC TTC GAG GTG CTC GGC ACC ACT CCC TAG CGG GCT CCA GCC   2025
661    R   S   F   E   V   L   G   T   T   P                       670
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Fig. 2. (*continued*)

extra N-terminal Met were introduced as compared with the mature wild-type CDase (see Fig. 2). The recombinant inactive CDase was present in inclusion bodies. After isolation and solubilization of the inclusion bodies, the CDase was refolded to its active state by dialysis in the presence of DTT and the non-ionic detergent Thesit. The refolded material from the inclusion bodies showed only a single 70 kDa band on SDS–PAGE. However, gel filtration analysis showed that the inclusion bodies contained two proteins with similar molecular masses which could be separated on the Superdex 200 column. The separation of the two proteins is possibly due to aspecific interactions of one of the proteins with the gel matrix.

After ammonium sulfate precipitation and gel filtration, 1.8 mg of recombinant CDase was obtained in 24.5% yield (see Table 2), which is an order of magnitude more than that for the recombinant CDase that is produced with its signal peptide (see Table 1). This higher yield is mainly due to the more efficient purification procedure, which does not require the high loss anion exchange chromatographic step. The CDase from inclusion bodies has a p*I* of 5.4, which is in good agreement with the calculated p*I* of 5.8. The somewhat lower specific activity of the refolded CDase compared with that of the wild-type CDase may be explained by the presence of the two additional N-terminal amino acids Met and Asp in the recombinant protein. The

Table 2
Refolding and purification of recombinant ceramidase expressed in *E. coli* BL21

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
Inclusion bodies	3000[a]	0	0
Refolding	150	1140.0	7.6
$(NH_4)_2SO_4$ and Superdex 200	1.8	279.5	155.3
Overall yield (%)		24.5	

$(NH_4)_2SO_4$, 75% ammonium sulfate precipitation; Superdex 200, Superdex 200 gel filtration chromatography. CDase activities were determined by measuring the free [^{14}C]palmitic acid generated by the hydrolysis of N-1 [^{14}C]palmitoyl sphingosine (see Materials and methods).
[a] Wet weight of inclusion bodies.

presence of some incorrectly folded CDases in the sample cannot be excluded.

The second protein fraction with inactive protein was denatured and refolded by following the same procedure as that for the original inclusion bodies, but no CDase activity was found. This indicates that the inactive protein fraction was not incorrectly folded CDase, but a different protein. This protein may be the molecular chaperone Hsp70 [30], also known as DnaK, that is coprecipitated with CDase in the inclusion bodies.

Characterization of CDase

In the presence of 4 mM $CaCl_2$, the enzymatic activities of wild-type CDase, recombinant CDase, and the recombinant CDase from inclusion bodies were not affected by iodoacetic acid (1 mM), β-mercaptoethanol (0.1 mM), hexadecylsulfonylfluoride (50 μM), or PMSF (1 mM), suggesting that the CDase does not require sulfhydryl groups, disulfide bonds, or serine for its activity. The inhibitors of human acid CDase, D-*erythro*-MAPP (0.2 mM), and human alkaline CDase, *N*-oleoylethanolamine (0.5 mM), had no effect on the enzymatic activity of the *Pseudomonas* CDase. The D- and L-*erythro*-MAPP analogues L(−)- and D(−)-norephenedrin also had no effect on the CDase activity.

The CDase assays used during the cloning and purification steps of this study are qualitative (fluorescent C6-NBD-ceramidase assay, see Materials and methods) or require radioactive substrates. Both assays are discontinuous and, hence, are less suitable for proper kinetic studies. To overcome those drawbacks, we designed a continuous CDase assay, based on the increasing fluorescence which occurs when a quenched fluorescent ceramide analogue is hydrolyzed [22]. With the continuous assay it was shown that the recombinant enzyme from inclusion bodies has a broad alkaline pH optimum between 7.5 and 9.5 with maximum activity at pH 8.5. The recombinant CDase is inhibited by D/L-sphinganine (K_i 3.3 ± 0.3 μM) and by D-*erythro*-sphingosine (K_i 4.0 ± 0.4 μM). L-*erythro*-sphingosine is a less powerful inhibitor, which reduces only the CDase activity with approximately 20% at 20 μM. Phytosphingosine at 20 μM reduces the CDase activity with approximately 60%. The CDase activity is completely blocked by EDTA and EGTA, but is restored after the addition of Ca^{2+}. The ion-dependence of the CDase reaction was tested with Ca^{2+}, Ba^{2+}, Mg^{2+}, and Zn^{2+}. At pH 8.5, the apparent K_ds for Ca^{2+}, Ba^{2+}, and Mg^{2+} are 1.2 ± 0.4, 8.5 ± 0.2, and 9.0 ± 0.3 μM, respectively. The maximum activity with Mg^{2+} is only 36% of that in the presence of optimal Ca^{2+} or Ba^{2+} concentrations. The K_d of Zn^{2+} is 1.0 ± 0.1 μM and was determined at pH 6.5 because of the low solubility of Zn^{2+} at more alkaline pH. These findings show that the microbial enzyme probably has a different enzymatic mechanism as compared with its mammalian counterparts.

Discussion

CDases are an important class of enzymes. The ceramide hydrolysis products generated by CDase have important physiological functions and bacterial CDase may be a virulence factor in *P. aeruginosa* infections. CDase produces sphingosine and the latter lipid downregulates macrophages that are stimulated by bacterial lipopolysaccharides from Gram-negative bacteria [21] and, hence, the bacterium may escape the host defence system [22]. It is noteworthy in that respect that the *P. aeruginosa* PA01 SMase gene [31] is located 347 bp downstream of the CDase gene on the same DNA strand. SMase and CDase can ideally work in tandem when both enzymes are simultaneously expressed by *Pseudomonas*. It is, however, unlikely that the SMase and CDase genes are located in an operon, since there is a putative terminator sequence TTTGTTTTT starting 145 bp downstream of the CDase gene. Interestingly, CDase from *Pseudomonas* has been implicated to be involved in the ceramide deficiency in the horny layer of the epidermis of patients with atopic dermatitis suffering from bacterial infections [20].

CDases are interesting enzymes because they combine aspects of lipases, phospholipases, and proteases. CDases are lipolytic enzymes that act on the water-lipid interface, like lipases, but hydrolyze amide bonds like proteolytic enzymes. The availability of a good expression system and purification procedure to access sufficient quantities

of CDase will enable the study of the 3D structure of the protein. Understanding of the enzymatic mechanism may open ways to design inhibitors to treat infections.

Here, we describe an efficient CDase production system and purification procedure that enables the large-scale production of CDase. The expression of the complete CDase gene (i.e., including its signal peptide) with the pUC18 expression vector in *E. coli* DH5α or with the pT7-5 expression vector in *E. coli* BL21 (DE3) both produced active intracellular CDase. Subsequent purification was laborious, however, and provided only a low yield of active CDase (280 μg from a 3-L culture).

The expression of CDase without its signal peptide in *E. coli* BL21 (DE3) using the pT7-7/CDase expression plasmid yielded inactive CDase in inclusion bodies, but the subsequent refolding and purification of the active recombinant CDase were simple and yielded pure CDase in 25% recovery (1.8 mg from a 2.4-L culture).

Although more CDase activity is produced with the pUC18/CDase than with the pT7-7/CDase expression system (see Tables 1 and 2), we did not optimize the purification protocol for the former, since the CDase isolation from inclusion bodies is more convenient and can easily be scaled up.

Other expression systems like *P. pastoris* GS115 and *P. putida* KT2440 also produce active recombinant CDase, but the amounts were too low for efficient production of CDase.

The alignment of the amino acid sequence of CDase from *P. aeruginosa* with other prokaryotic and eukaryotic CDases did not give clues about the identity of the active site residues or other structural features of the enzyme. The use of inhibitors did also not provide any indication about the possible identity of active site residues. Like lipases, CDase is not inhibited by PMSF. In contrast to lipases, however, CDase is not inhibited by hexadecylsulfonylfluoride. This indicates that an active site serine can probably be discarded as the nucleophile involved in the mechanism of this ceramidase. Further studies are needed to substantiate this. Since iodoacetic acid did not influence the CDase activity, it is not likely that CDase is a cysteine hydrolase. The possibility that CDase is an aspartate- or a metallo hydrolase cannot be ruled out. This and the possibilities to crystallize CDase are currently under investigation.

Because the bacterial enzyme is the only Ca^{2+}-dependent CDase known to date, and because it is not inhibited by known mammalian CDase inhibitors, it is very likely that bacterial CDase has a enzymatic mechanism different than its mammalian counterparts. This offers the opportunity to develop highly specific inhibitors for bacterial CDase that do not interfere with the mammalian sphingolipid metabolism, and which may prove effective in the treatment of *Pseudomonas* infections.

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