Brown recluse spider (*Loxosceles reclusa*) venom phospholipase D (PLD) generates lysophosphatidic acid (LPA)

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Envenomation by the brown recluse spider (*Loxosceles reclusa*) may cause local dermonecrosis and, rarely, coagulopathies, kidney failure and death. A venom phospholipase, SMaseD (sphingomyelinase D), is responsible for the pathological manifestations of envenomation. Recently, the recombinant SMaseD from *Loxosceles laeta* was demonstrated to hydrolyse LPC (lysophosphatidylcholine) to produce LPA (lysophosphatidic acid) and choline. Therefore activation of LPA signalling pathways may be involved in some manifestations of *Loxosceles* envenomation. To begin investigating this idea, we cloned a full-length cDNA encoding *L. reclusa* SMaseD. The 305 amino acid sequence of the *L. reclusa* enzyme is 87, 85 and 60 % identical with those of *L. arizonica*, *L. intermedia* and *L. laeta* respectively. The recombinant enzyme expressed in bacteria had broad substrate specificity. The lysophospholipids LPC, LPI (18:1-1-oleyol lysophosphatidylinositol), LPS, LPG (18:1-1-oleoyl-lysophosphatidylglycerol), LBPA (18:1-1-oleoyl-lysobisphosphatidic acid) (all with various acyl chains), lyso-platelet-activating factor ($C_{16:0}$), cyclic phosphatidic acid and sphingomyelin were hydrolysed, whereas sphingosylphosphorylcholine, PC (phosphatidylcholine; $C_{22:6}$, $C_{20:4}$ and $C_{6:0}$), oxidized PCs and PAF (platelet-activating factor; $C_{16:0}$) were not hydrolysed. The PAF analogue, edelfosine, inhibited enzyme activity. Recombinant enzyme plus LPC ($C_{18:1}$) induced the migration of A2058 melanoma cells, and this activity was blocked by the LPA receptor antagonist, VPC32183. The recombinant spider enzyme was haemolytic, but this activity was absent from catalytically inactive H37N ($His^{37} \rightarrow Asn$) and H73N mutants. Our results demonstrate that *Loxosceles* phospholipase D hydrolyses a wider range of lysophospholipids than previously supposed, and thus the term 'SMaseD' is too limited in describing this enzyme.

Key words: brown recluse spider, *Loxosceles* envenomation, lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), phospholipase D (PLD), sphingomyelinase D (SMaseD).

INTRODUCTION

Envenomation by the brown recluse spider (*Loxosceles reclusa*) can induce local dermonecrosis as well as systemic erythrocyte haemolysis, platelet aggregation and renal failure that, in rare cases, may lead to death [1]. Although the venom contains several proteins, the PLD (phospholipase D) 'SMaseD' (sphingomyelinase D) is responsible for dermonecrosis and complement-dependent haemolysis [2], blood vessel damage and fibrinogenolysis [3] as well as dysregulated endothelial cell-dependent neutrophil activation [4].

One of the products of *Loxosceles* SMaseD is C1P (ceramide-1-phosphate), which is known to be one component of the lipid bilayer but is not obviously a signalling molecule [5]. However, C1P is a bioactive lipid molecule capable of stimulating cell proliferation by increasing DNA synthesis, directly inhibiting acid sphingomyelinase and blocking ceramide synthesis and apoptosis, and, by directly activating phospholipase A_2, it induces prostanoid synthesis in cells (reviewed in [6]). Recently, Moolenaar's group showed that the recombinant SMaseD from *L. laeta* cleaved LPC (lysophosphatidylcholine) to LPA (lysophosphatidic acid) and choline [7]. LPA is known to induce various biological and pathological responses such as platelet aggregation, endothelial hyperpermeability and pro-inflammatory responses by signalling through three G-protein-coupled receptors [8,9]. Thus some of the pathology associated with the envenomation by the brown recluse spider might be due to aberrant LPA production and signalling.

To initiate a study of the role of LPA production and LPA signalling pathways in the manifestations of envenomation by *Loxosceles* species, we cloned SMaseD from *L. reclusa* and characterized the recombinant SMaseD regarding substrate specificity and enzyme function. The results of this initial characterization are reported herein. The *L. reclusa* PLD cDNA sequence has been deposited with GenBank® (accession no. AY862486). The sequence was determined by automated DNA sequencing using the cDNA isolated by the authors of this paper, and this sequence is reported for the first time in this paper.

EXPERIMENTAL

Materials and reagents

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Essentially fatty acid-free BSA, referred to as faf-BSA, peroxidase (EC 1.11.1.7; hydrogen-peroxide oxidoreductase), choline oxidase (EC 1.1.3.17), glycerol-3-phosphate oxidase (EC 1.1.3.21), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) and 3-α-hydroxysteroid dehydrogenase (EC 1.1.1.50) were purchased

Abbreviations used: ATX, autotaxin; C1P, ceramide-1-phosphate; DMEM, Dulbecco's modified Eagle's medium; faf-BSA, essentially fatty acid-free BSA; G3P, glycerol-3-phosphate; HRP, horseradish peroxidase; LBPA, 18:1-1-oleoyl-lysobisphosphatidic acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPE, 18:1-1-oleoyl-lysophosphatidylethanolamine; LPG, 18:1-1-oleoyl-lysophosphatidylglycerol; LPI, 18:1-1-oleoyl-lysophosphatidylinositol; LPS, 18:1-1-oleoyl-lysophosphatidylserine; PC, phosphatidylcholine; PLD, phospholipase D; lysoPLD, LPC-preferring PLD; ORF, open reading frame; PAF, platelet-activating factor; RACE, rapid amplification of cDNA ends; SMaseD, sphingomyelinase D; SPC, sphingosylphosphorylcholine, also known as lyso-sphingomyelin; TOOS, *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-*m*-toluidine; VBS^{2+}, veronal-buffered saline.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY862486.

from Sigma–Aldrich (St. Louis, MO, U.S.A.). Lysophospholipase (EC 3.1.1.5) was purchased from either Sigma–Aldrich or MP Biomedical (Irvine, CA, U.S.A.). A2058 (human melanoma) cells were a gift from Dr T. Clair (National Institutes of Health, Bethesda, MD, U.S.A.). S-protein–HRP conjugate (where HRP stands for horseradish peroxidase) was purchased from Novagen (San Diego, CA, U.S.A.).

Cell-culture conditions

The growth medium used for A2058 cells was DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) CD-FBS (charcoal dextran-stripped fetal bovine serum; Gemini Bioscience, Woodland, CA, U.S.A.) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B) (Invitrogen, Carlsbad, CA, U.S.A.).

Cloning of the SMaseD from venom glands of *L. recluse*

Total RNA was extracted from two pairs of *L. reclusa* venom glands purchased from SpiderPharm (Yarnell, AZ, U.S.A.) using the RNeasy kit (Qiagen, Valencia, CA, U.S.A.). A fragment [580–963 bp of the full translational ORF (open reading frame)] was amplified by using a degenerate primer set (sense, 5′-gggcatcccgagttaatgga-3′; anti-sense, 3′-ttaattcttgaatgtttccc-3′) designed from the nucleotide sequence of the related species, *L. arizonica*. The full mRNA sequence was identified by 3′- and 5′-RACE (3′- and 5′-rapid amplification of cDNA ends) using the Generacer kit (Invitrogen). The full ORF was subcloned DNA into pET30 (Novagen) for bacterial cell expression. The correctness of plasmid constructs was confirmed by automated DNA sequencing (Biomedical Research Core Laboratory, University of Virginia).

Introduction of point mutations into SMaseD

To construct mutant SMaseDs, the following primer sets were used for amplification of the plasmid construct. H37N ($His^{37} \rightarrow$ Asn) (sense, 5′-cctatatggatcatggggaacatggtcaacgctatttat-3′; antisense, 5′-ataaatagcgttgaccatgttccccatgatccatatagg-3′), H73N (sense, 5′-aatcctgaatacacgtataacggcgttccatgtgattgc-3′; antisense, 5′-gcaatcacatggaacgccgttatacgtgtattcaggatt-3′), H108N (sense, 5′-ccaggcgattccaagtataatgcaaaattagtgttagtt-3′; antisense, 5′-aactaacactaattttgcattatacttggaatcgcctgg-3′), H164N (sense, 5′-tccataccagaccttaacaattataaattaattactgga-3′; antisense, 5′-tccagtaattaatttataattgttaaggtctggtatgga-3′), H180N (sense, 5′-acgctcaaaagcgaggggaatcccgagttaatggacaaa-3′; antisense, 5′-tttgtccattaactcgggattcccctcgctttgagcgt-3′), H108A (sense, 5′-ccaggcgattccaagtatgctgcaaaattagtgttagtt-3′; antisense, 5′-aactaacactaattttgcagcatacttggaatcgcctgg-3′), T258V (sense, 5′-acagtggacaagcgcgcaacggttagagaggcactcgatgctgga-3′; antisense, 5′-tccagcatcgagtgcctctctaaccgttgcgcgcttgtccactgt-3′). Following amplification, the template DNA was digested with the restriction endonuclease DpnI and the plasmid was used to transform *Escherichia coli* TOP10F′ competent cells (Invitrogen). The correct mutations at the target sites were verified by automated DNA sequencing.

Purification of the recombinant SMaseD from bacteria

The SMaseD-pET30 plasmid was transformed into *E. coli* [strain: BL21(DE3)pLysS; Novagen.] and 1 mM isopropyl β-D-thiogalactoside was used for the induction of protein expression. The commercial bacterial cell lysis reagent (BugBuster; Novagen) including 25 unit/ml DNase I (Sigma, St. Louis, MO, U.S.A.) was used for the preparation of total cell lysates. Nickel-linked Sepharose was used for the purification of recombinant SMaseD from the bacterial cell lysates (Amersham Biosciences, Piscataway, NJ, U.S.A.) following the manufacturer's instructions. The SMaseD was found to be stable and resistant to bacterial protease; thus specific protease inhibitors were not used during the purification procedure. To increase the degree of purity, the S-protein–agarose (Novagen) was used as recommended by the manufacturer. The buffer used in the final elution was exchanged with PBS using a dialysis membrane (10 kDa cut-off) or a Centricon membrane (30 kDa cut-off). The concentration of the purified protein was determined by the FRETWorks S-Tag assay kit (Novagen) following the method recommended by the manufacturer.

Western blotting

Protein samples were subjected to SDS/PAGE (12–14 % polyacrylamide) and transferred on to nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using typical electrotransfer procedures. For detection of recombinant proteins encoded by SMaseD-pET30, the S-protein–HRP conjugate was used (Novagen). Bound S-protein was detected by using the Lightning Chemiluminescence reagent Plus kit (PerkinElmer, Boston, MA, U.S.A.).

Choline release assay

In some cases, the hydrolytic activity of the recombinant SMaseD protein was determined by measuring the amount of choline released from the phospholipid substrate [7]. Briefly, 1 mM substrate in Hepes-buffered saline (10 mM Hepes, 140 mM NaCl, 0.5 mM KCl, 0.1 mM $MgCl_2$ and 0.1 % faf-BSA, pH 7.4) was incubated with the recombinant enzyme for the indicated times (standard, 1 h) at 37 °C in a total volume of 0.1 ml. The same volume of enzyme cocktail {50 mM Tris/HCl, 2.7 mM TOOS [*N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl)-*m*-toluidine], 4.5 mM 4-aminoantipyrine, 47.7 units/ml peroxidase and 18 units/ml choline oxidase, pH 8.0} was added and incubated for a further 15 min at 37 °C. The amount of choline released from the reaction was calculated by measuring light absorbance A at 550 nm. In the case of SM (sphingomyelin), a 100 mM stock solution in 95 % ethanol was diluted to the required concentration in Hepes-buffered saline. Ethanol at concentrations below 1.0 % did not show significant inhibition of SMaseD enzyme activity or a decrease in K_m value.

LPA assay

To measure the hydrolysis of lysophospholipids with head groups other than choline, the amount of LPA released was measured by a colorimetric LPA assay [10]. Briefly, a 1 mM substrate was incubated with recombinant SMaseD for the indicated time (standard, 1 h) in a total volume of 0.1 ml of Hepes-buffered saline. The same volume of enzyme cocktail was then added to the reaction. The enzyme cocktail consisted of 20 units/ml lysophospholipase, 1.3 units/ml peroxidase, 100 units/ml glycerol-3-phosphate oxidase, 10 units/ml glycerol-3-phosphate dehydrogenase (added just before the assay), 10 units/ml 3-α-hydroxysteroid dehydrogenase, 0.01 mM NADH, 1.0 mM cholic acid, 0.5 mM TOOS, 1 mM 4-AAP (4-aminoantipyrine), 0.01 % Triton X-100 and 100 mM Hepes (pH 7.6). After incubation for 15 min to allow colour development, the light absorbance at 570 nm was measured and the amount of LPA was calculated from the standard curve generated using LPA18:1. The colour development was linear for more than 15 min using LPA18:1 concentrations up to 1 mM. To measure the level of contaminating G3P (glycerol 3-phosphate) in lipid substrates, the same enzyme cocktail without lysophospholipase was used and the amount of G3P was calculated from

the standard curve generated using G3P. The amount of contaminating G3P (generally $< 5\,\%$) was subtracted when constructing the LPA standard curve.

Migration assay

The lower surface of transwell membranes (8 μm pore size; 24 mm diameter; Corning Costar, Corning, NY, U.S.A.) was coated with 0.01 mg/ml matrigel (growth factor-reduced; BD Biosciences, Bedford, MA, U.S.A.) following the manufacturer's instructions. To prepare the cell suspension, 90 % confluent A2058 cell monolayers were detached by a brief treatment with 10× trypsin/EDTA solution (Invitrogen). Detached cells were washed once with normal growth media and one more time with the assay medium (DMEM without Phenol Red, 0.1 % faf-BSA, no serum), and finally resuspended in assay media at 1.5×10^6 cells/ml. Cell suspension (1 ml) was added to the upper chamber and 2 ml of the assay media was placed in the lower chamber. After waiting 1 min for volume equilibration between the chambers, 0.02 ml of compounds (100× stock in 3 % faf-BSA) was added to the lower chamber. After 4 h of incubation at 37 °C to allow cell migration, the assay media in both chambers were discarded and the cells from the lower surface of the membrane were detached by adding 0.8 ml of 10× trypsin/EDTA solution to the lower chamber. To confirm complete detachment of the migrated cells from the lower surface of the membrane, unmigrated cells were discarded by swabbing the upper membrane surface, and the lower surface of the membrane was examined by microscopy after staining with Diffquick staining kit (IMEB Inc., San Marcos, CA, U.S.A.). A 0.1 ml aliquot of the migrated cell suspension from the lower chamber was mixed with the same volume of 2× CyQuant assay solution (Molecular Probes, Eugene, OR, U.S.A.), and the cell mass was calculated by measuring the fluorescence at 480/520 nm using a FlexStation fluorimeter (Molecular Devices, Sunnyvale, CA, U.S.A.).

Haemolysis assay

Human erythrocytes (freshly donated) were washed three times with VBS^{2+} (veronal-buffered saline, pH 7.4; 10 mM sodium barbitone, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 145 mM NaCl) and resuspended in VBS^{2+} at 2 %. The cells were incubated with the recombinant enzyme for 30 min at 37 °C. Control samples were incubated with VBS^{2+} buffer alone. After washing five times with VBS^{2+}, the cells were resuspended to the original volume of VBS^{2+} and analysed for haemolysis [11]. Briefly, 0.1 ml of 2 % erythrocytes pretreated with purified toxin was mixed with 0.1 ml of autologous plasma. Background or total cell lysis was evaluated by incubation of erythrocytes with VBS^{2+} or water respectively. After incubation for 1 h at 37 °C, non-lysed cells were discarded by centrifugation; the light absorbance of the released haemoglobin was measured at 405 nm.

RESULTS

Cloning of the SMaseD from *L. reclusa*

The full sequence of mRNA encoding SMaseD was identified from cDNA made from the total RNA extracted from the venom glands of *L. reclusa* (Figure 1). The mRNA sequence includes a 918 bp full translational ORF, 34 bp of 5′-UTR (5′-untranslated region) and 181 bp of 3′-UTR. The conceptionalized peptide consisted of 305 amino acids (molecular mass, 34 kDa). Peptide sequence analysis showed one possible N-glycosylation site (Asn^{282}).

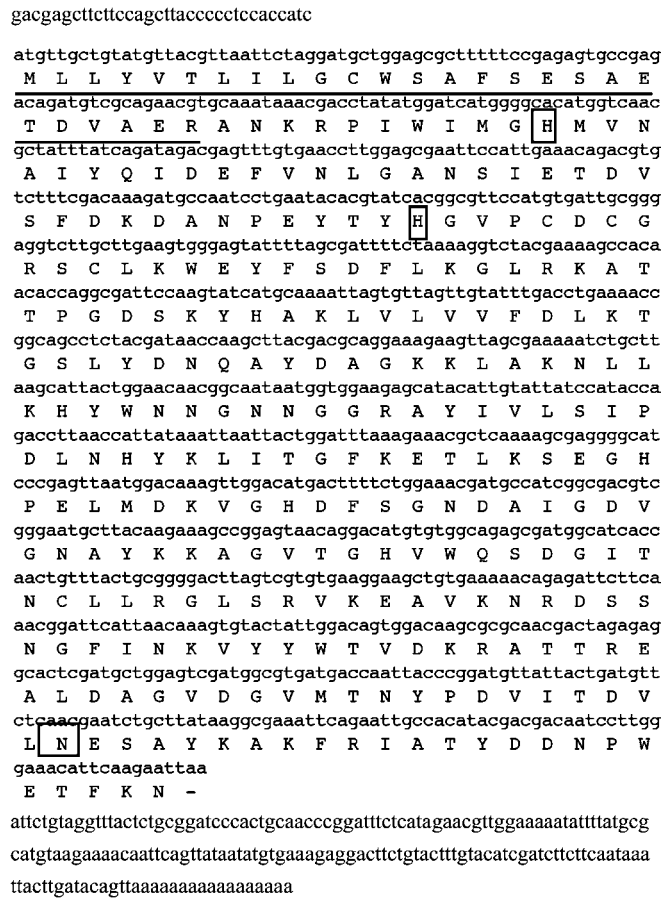

Figure 1 Complete cDNA and translated peptide sequence of *L. reclusa* SMaseD

Total RNA was extracted from the venom glands of *L. reclusa* (SpiderPharm). The degenerate primers designed from the sequence of SMaseD from related species, *L. arizonica*, were used for amplification of the initial fragment of SMaseD (580–963 bp of full ORF). The full cDNA sequence was identified by 3′- and 5′-RACE. Underlined, putative signal peptide; boxed, catalytic histidine residues, potential N-glycosylation site.

Recently, amino acid sequences of SMaseD from several *Loxosceles* species were reported [12–14]. Sequence alignment showed that the *L. reclusa* SMaseD shares 87 % (*L. arizonica*), 60 % (*L. laeta*), 85 % (*L. intermedia*) and 84 and 46 % (*L. boneti* SMaseD1 and SMaseD2) identical amino acids with SMaseD from these other *Loxosceles* species. The SMaseDs from *L. arizonica* and *L. intermedia* have longer signal peptide sequences than either *L. laeta* or *L. reclusa*. The location of the signal peptide in *L. reclusa* is inferred from the reported amino acid sequence of *L. reclusa* SMaseD protein purified from the venom [14]. The only other protein with significant amino acid sequence similarity to these spider enzymes is a bacterial (*Corynebacterium pseudotuberculosis*) PLD [7]. The alignment of these proteins is displayed in Figure 2.

Expression of the recombinant SMaseD in bacterial cells

Forced expression of epitope-tagged recombinant SMaseD in bacteria resulted in a soluble protein that migrated at a relative molecular mass of 42 kDa (Figure 3). We purified the recombinant SMaseD from bacterial cell lysates using sequential affinity columns of Ni-linked Sepharose resin and S-protein–agarose. This purified protein was used to examine substrate specificity and for further characterization.

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           1                                                  50
Intermedia ~~~~~~~~MA AAGIRFIMLP YIVLVLGCWS VLSQAAQTDD EERAGN..RR
   Reclusa ~~~~~~~~~~ ~~~~~~~MLL YVTLILGCWS AFSESAETDV AERA.N..KR
 Arizonica MSHSSTALLH PYVAARATEK FAPIYFFCHP L..QSAETDV AERA.N..KR
     Laeta ~~~~~~~~~~ ~~~~~~~MYA HLALILGCWT VVLQGAETDV GERADN..RR
     CP-PLD ~~~~~~~~~~ ~~~MREKFAL LLSIIMAIML PVGNAAAAPV VHNPASTANR
 Consensus ---------- ---------- YL-LIL-C-- ---Q-AETDV -ERA-N---R

           51                                                 100
Intermedia PIWIMGHMVN AIGQIDEFVN LGANSIETDV SFDDNANPEY TYH.GIPCDC
   Reclusa PIWIMGHMVN AIYQIDEFVN LGANSIETDV SFDKDANPEY TYH.GVPCDC
 Arizonica PIWIMGHMVN ANYQIDEFVN LGANSIETDV SFDSSANPEY TYH.GVPCDC
     Laeta PIWNLAHMVN AVAQIPDFLD LGANALEADV TFKGSV.PTY TYH.GTPCDF
     CP-PLD PVYAIAHRVL TTQGVDDAVA IGANALEID. .FTAWGRGWW ADHDGIPTSA
 Consensus PIW-M-HMVN A--QIDEFV- LGAN-IE-DV -F-----P-Y TYH-G-PCD-
                ●                                    ●
           101                                                150
Intermedia GRNCKKYENF NDFLKGLRSA TTPGNSKYQE KLVLVVFDLK TGSLYDNQAN
   Reclusa GRSCLKWEYF SDFLKGLRKA TTPGDSKYHA KLVLVVFDLK TGSLYDNQAY
 Arizonica RGWCKKWEYF NNFLKALRKA TTPGDSKYHE KLVLVVFDLK TGSLYDNQAY
     Laeta GRDCIRWEYF NVFLKTLREY TTPGNAKYRD GFILFVLDLK TGSLSNDQVR
     CP-PLD GATA..... EEIFKHIADK RKQG.....A NITFTWLDIK NPDYCRDARS
 Consensus G--C-KWE-F --FLK-LR-- TTPG--KY-- -LVL-V-DLK TGSL---Q--

           151                                                200
Intermedia DAGKKLAKNL LQHYWNNGNN GGRAYIVLSI PDLNHYPLIK GFKDQLTKDG
   Reclusa DAGKKLAKNL LKHYWNNGNN GGRAYIVLSI PDLNHYKLIT GFKETLKSEG
 Arizonica DAGKKLAKNL LQHYWNNGNN GGRAYIVLSI PNLAHYKLIT GFKETLKTEG
     Laeta PAGENVAKEL LQNYWNNGNN GGRAYVVLSL PDIGHYEFVR GFKEVLKKEG
     CP-PLD VCSINALRDL ARKYLEPA.. GVR...VL.. ..YGFYKTVG G...PAWKTI
 Consensus -AG---AK-L L--YWNNGNN GGRAYIVLSI P-L-HY--I- GFKE-L--EG

           201                                                250
Intermedia HPELMEKVGH DFSGN..... DDIGDVGK.. .AYKKAGITG HIWQSDGITN
   Reclusa HPELMDKVGH DFSGN..... DAIGDVGN.. .AYKKAGVTG HVWQSDGITN
 Arizonica HPELMEKVGY DFSGN..... DNIDQVAN.. .AYKKAGVTG HVWQSDGITN
     Laeta HEDLLEKVGY DFSGPYLPSL PTLDATHE.. .AYKKAGVDG HIWLSDGLTN
     CP-PLD TADLRDGEAV ALSGPAQDVL NDFARSGNKI LTKQKIADYG YYDINQGFGN
 Consensus H-ELMEKVG- DFSG------ --I------- -AYKKAGV-G H-W-SDGITN

           251                                                300
Intermedia C..LPRG.LS RVNAAVANRD SANGFINKVY YWTVDKRSTT R..DAL.DAG
   Reclusa C..LLRG.LS RVKEAVKNRD SSNGFINKVY YWTVDKRATT R..EAL.DAG
 Arizonica C..LLRG.LD RVRKAVANRD SSNGYINKVY YWTVDKRQST K..NAL.DAG
     Laeta F..SPLGDMA RLKEAIKSRD SANGFINKIY YWSVDKVSTT K..AAL.DVG
     CP-PLD CYGTWNRTCD QLRKSSEARD ..QGKLGKTF GWTIATGQDA RVNDLLGKAN
 Consensus C-----G-L- R---AV--RD S-NGFINKVY YWTVDK---T R---AL-DAG

           301                                                350
Intermedia VDGI.....M TNY...PDVI TDVLNEAAYK KK....FRVA TYDDNPWVTF
   Reclusa VDGV.....M TNY...PDVI TDVLNESAYK AK....FRIA TYDDNPWETF
 Arizonica VDGI.....M PNY...PDVI ADVPNESAYK AK....FRIA SYDDNPWETF
     Laeta VDGI.....M TNY...PNVL IGVLKESGYN DK....YRLA TYDDNPWETF
     CP-PLD VDGLIFGFKM THFYRHPDTE NSFKAIKRWV DKHSATHELA TVADNPW~~~
 Consensus VDGI-----M TNY---PDVI --V--E--Y- -K----FR-A TYDDNPW-TF

           351
Intermedia K~~
   Reclusa KN*
 Arizonica KN~
     Laeta KN~
     CP-PLD ~~~
 Consensus K--
```

Figure 2 Peptide sequence alignment of SMaseDs

Intermedia, *L. intermedia* SMaseD; Reclusa, *L. reclusa* SMaseD; Arizonica, *L. arizonica* SMaseD; Laeta, *L. laeta* SMaseD; CP–PLD, *C. pseudotuberculosis* PLD. The GCG program, PRETTY, was used for the analysis. Solid line box, putative signal sequences; ●, histidine residues essential for enzyme activity. The 305 amino acid peptide sequence of *L. reclusa* enzyme is 87, 85 and 60% identical with those of *L. arizonica*, *L. intermedia* and *L. laeta* respectively.

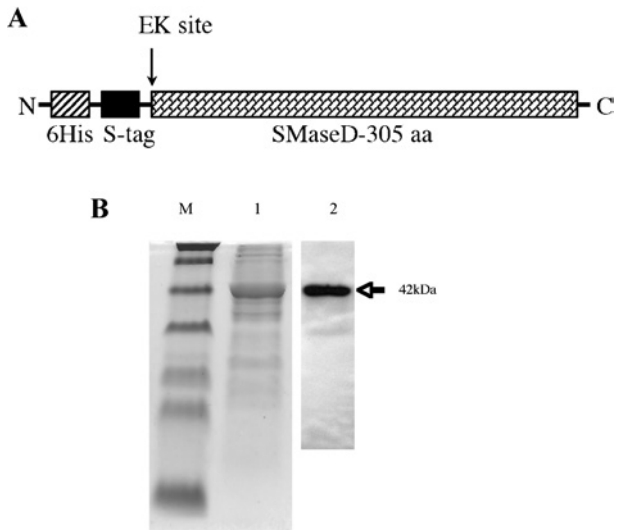


Figure 3 The expression of recombinant SMaseD in a bacterial expression system

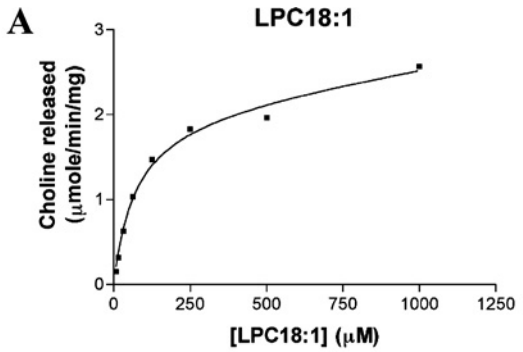
(**A**) Schematic diagram of recombinant SMaseD construct expressed in bacteria. EK, enterokinase cleavage site. (**B**) Coomassie Blue staining (lane 1, 2.4 μg of SMaseD loaded) and Western blotting with S-protein–HRP conjugate (lane 2, 0.1 μg of SMaseD loaded) of the SMaseD purified from the bacterial cell lysates by sequential affinity purification using Ni-Sepharose (Amersham Biosciences). Lane M, molecular mass standards.

Recombinant SMaseD has broad substrate specificity

As shown in Figure 4(A), the purified recombinant protein hydrolysed sphingomyelin as detected by the appearance of choline. Furthermore, sphingomyelin with artificially short fatty acids ($C_{2:0}$ and $C_{6:0}$) was also hydrolysed (results not shown). Thus, as expected, the cDNA that we cloned from venom gland RNA encodes a functional SMaseD. Also, as expected from a previous report using *L. laeta* SMaseD, LPC with various acyl chains ($C_{18:1}$, $C_{18:0}$, $C_{16:0}$, $C_{14:0}$ and $C_{12:0}$) was a substrate [7]. Compared with the previous kinetic values of mammalian lysoPLD (LPC-preferring PLD), ATX (autotaxin; $K_m = 250\ \mu$M and $V_{max} = 9.0\ \mu$mol $\cdot$ min^{-1} $\cdot$ mg^{-1} for LPC18:1) [15], the recombinant SMaseD showed approx. 3-fold lower K_m and V_{max} values. In contrast, PC (phosphatidylcholine) with various acyl chains ($C_{22:6}$, $C_{20:4}$ and $C_{6:0}$) did not show any detectable hydrolysis (results not shown). We conclude that the SMaseD from *L. reclusa*, like that of *L. laeta*, has intrinsic lysoPLD activity. Interestingly, the *L. reclusa* enzyme also hydrolysed lyso-PAF (where PAF stands for platelet activating factor) but not PAF, which has an acetyl group at the *sn2* position. We examined the hydrolysis of edelfosine (a PAF analogue) and miltefosine (an antimetastatic agent) [16,17] using the enzymatic choline detection method, but the purified enzyme did not release choline from either of these molecules (results not shown). To examine the hydrolysis of other lysophospholipids without a choline head group, we measured the amount of LPA released as described in the Experimental section [10]. As documented in Figure 4(B), LPI (18:1-1-oleoyl-lysophosphatidylinositol), LPG (18:1-1-oleoyl-lysophosphatidylglycerol), LPE (18:1-1-oleoyl-lysophosphatidylethanolamine), LBPA (18:1-1-oleoyl-lysobisphosphatidic acid) and LPS (18:1-1-oleoyl-lysophosphatidylserine) were all hydrolysed by the recombinant enzyme. At a concentration of 1 mM, the substrate preference was LPI > LPG > LPS > LPC $\gg$ LPE, LBPA. Furthermore, the recombinant enzyme also hydrolysed cyclic phosphatidic acid to LPA. In contrast with the mammalian lysoPLD (ATX) [18], we did not detect hydrolysis of SPC (sphingosylphosphorylcholine, also known as lyso-sphingomyelin) (results not shown). The SMaseD did not exhibit detectable $PLA_{1/2}$ activity, which was examined by using bis-BODIPY FL C_{11}-PC (Molecular Probes) as a potential substrate (results not shown).

His^{37} and His^{73} are essential for enzyme activity of the recombinant SMaseD

The peptide sequence analysis of the spider SMaseD suggested one putative GDPD (glycerol phosphodiester phosphodiesterase) domain (amino acids 246–276) and a potential HKD PLD catalytic domain (HXKXXXD, amino acids 108–117) [19]. However, the point mutation of amino acids in these consensus sequences (T258V, H108N and H108A) did not abolish enzyme



Substrate	Km (μM)	Vmax (μmole/min/mg)
LPC12:0	436±95	0.47±0.05
LPC14:0	219±42	2.97±0.26
LPC16:0	220±48	1.18±0.10
LPC18:0	104±30	2.37±0.43
LPC18:1	98±15	2.62±0.41
LysoPAF	56±15	1.00±0.05
Sphingomyelin	396±23	2.95±0.78

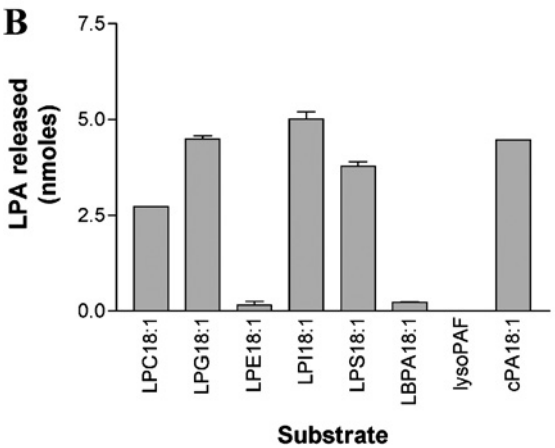


Figure 4 Substrate specificity of the purified recombinant SMaseD

(**A**) LysoPLD activity was assayed by measuring hydrolysis of various lysophospholipids with choline head groups. Briefly, the substrate in Hepes-buffered saline was incubated with SMaseD (125 ng) for 1 h at 37 °C in a total volume of 0.1 ml. The amount of choline released from the reactions was measured as described in the Experimental section. K_m and V_{max} values were calculated using the Michaelis–Menten equation. Results shown are from a representative experiment of at least three independent assays. (**B**) Hydrolysis of non-choline lysophospholipids and cyclic phosphatidic acid by SMaseD was measured. The LPA produced from the reaction of 1 mM substrate with SMaseD (1 ng) was measured by direct LPA assay described in the Experimental section. LPA18:1 was used as a positive control, whereas lysoPAF was used as negative control for the assay. (Although SMaseD hydrolyses lysoPAF, the product, ether LPA, cannot be detected because lysophospholipase in LPA assay enzyme cocktail does not cleave ether-linkage.) The results shown are the means ± S.E.M. for triplicate experiments.

activity (Figure 5). Because previous studies of the catalytic mechanism of PLD suggested the involvement of histidine residue(s) [20], we systematically mutated histidine residues conserved among *Loxosceles* SMaseDs (Figure 5A). The H37N and H73N mutations abolished detectable hydrolysing activity using LPC18:1 as a substrate (Figure 5B). Likewise, the H37N and H73N mutant proteins did not hydrolyse SM (results not shown). We could not find any peptide domain previously reported that has significant similarity to the peptide sequence of amino acids 37–73 of the spider enzyme.

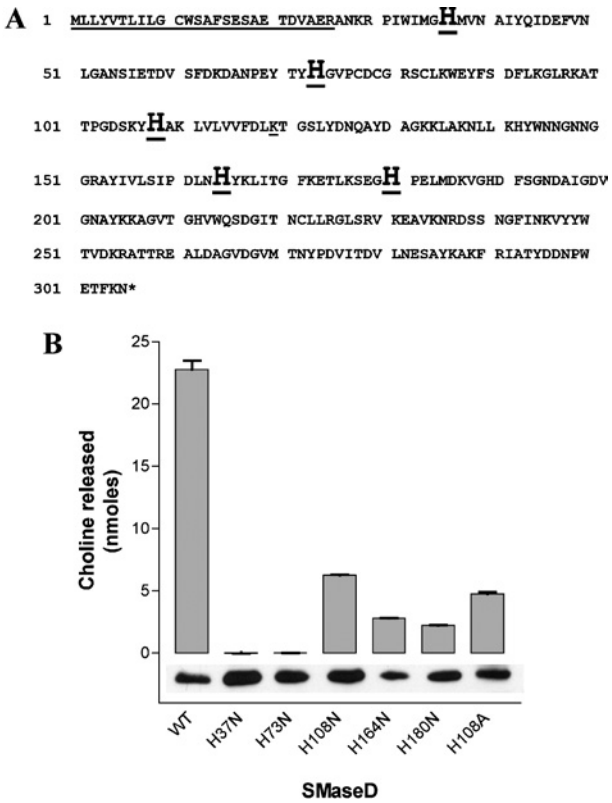


Figure 5 His[37] and His[73] are required for enzyme activity of the recombinant *L. reclusa* SMaseD

(**A**) The mutated amino acids are indicated by large bold letters. Potential signal peptide is underlined. (**B**) LysoPLD activity of the wild-type and mutant SMaseD proteins (2 ng/reaction) expressed in bacteria. Briefly, 1 mM LPC18:1 in Hepes-buffered saline was incubated with SMaseD for 1.5 h at 37 °C in a total volume of 0.1 ml. The amount of choline released from the reactions was measured as described in the Experimental section. The presence of the wild-type and mutant proteins (100 ng/lane) was confirmed by Western blotting using S-protein–HRP conjugate. The results shown are the means ± S.E.M. for triplicate experiments.

The recombinant SMaseD induced the migration of human melanoma A2058 cells through LPA receptor signalling

LPA is known to induce the chemotactic migration of the human A2058 melanoma cells via the LPA_1 receptor [21–23]. ATX, which is a recently identified mammalian lysoPLD, evokes A2058 cell migration by releasing LPA from LPC [23]. Initially, we were unable to observe migration when recombinant spider SMaseD and LPC were added to the lower chamber of the migration well without pre-incubation. However, when the enzyme and substrate were pre-incubated, migration was observed and this migration was sensitive to the LPA_1 receptor antagonist, VPC32183 (Figure 6). This result suggests that the migration assay buffer (DMEM + 0.1 % faf-BSA) might not be optimal for LPA generation by the *L. reclusa* SMaseD.

The purified recombinant SMaseD induced the erythrocyte haemolysis

Among the effects of *Loxosceles* PLD is erythrocyte haemolysis [11]. As shown in Figure 7(A), treatment of the human erythrocytes with purified recombinant of SMaseD from *L. reclusa* also induced haemolysis in a dose-dependent manner. This activity was not observed with the catalytically inactive (H37N and H73N) SMaseD protein (Figure 7B).

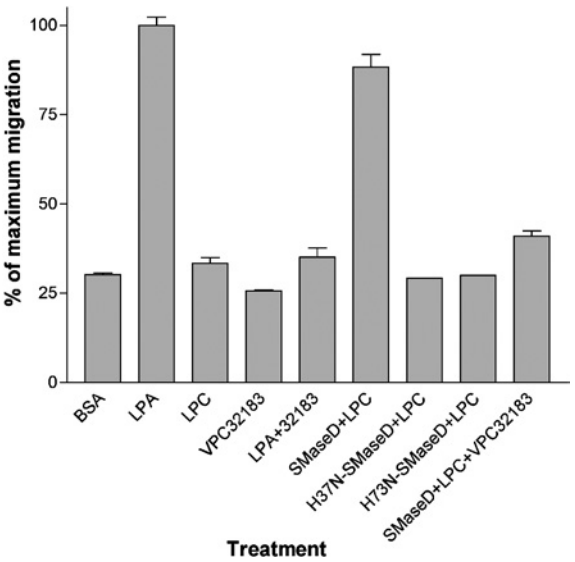


Figure 6 Recombinant *L. reclusa* SMaseD induced the migration of human melanoma A2058 cells

Purified wild-type, H37N and H73N recombinant SMaseDs were used for the induction of A2058 cell migration as described in the Experimental section. Lipid concentrations were: LPA18:1, 50 nM; LPC18:1, 2.5 μM; and VPC32183, 10 μM. WT and mutant SMaseDs (5 ng) and LPC18:1 (1 mM) were pre-incubated for 2 h at 37 °C in a total volume of 20 μl after which 5 μl of the final reaction mixture was added to the 2 ml of assay buffer in the lower chamber of transwell. The results shown are the means ± S.E.M. for duplicate experiments.

Edelfosine inhibited the lysoPLD enzyme activity of the recombinant SMaseD

Finally, we examined each lipid that was not a substrate for inhibitory activity. In doing so, we found that edelfosine inhibited the enzyme activity of recombinant *L. reclusa* SMaseD (Figure 8).

DISCUSSION

Using several *Loxosceles* sp. SMaseD DNA sequences as a guide, we cloned a similar cDNA from RNA of *L. reclusa* venom glands. As expected, the *L. reclusa* protein is closely related (60–87 % identical amino acids) to the SMaseD enzymes of other *Loxosceles* species and we found that the recombinant *L. reclusa* protein releases choline from sphingomyelin, i.e. it has 'SMaseD' activity. We confirmed also for *L. reclusa* SMaseD the seminal finding of Moolenaar's laboratory that *L. laeta* SMaseD is a lysoPLD [7]. We extended that finding by demonstrating that lysophospholipids with a variety of headgroups are hydrolysed by the *L. reclusa* enzyme to release LPA. Thus this enzyme, as well as presumably related proteins from other *Loxosceles* sp., is both an SMaseD and a broad-spectrum lysoPLD. Like the *L. laeta* enzyme, the *L. reclusa* enzyme displays K_m values for albumin-presented LPC that are well within the range of LPC levels in blood [24].

Through site-directed mutation of histidine codons, we identified two histidine residues (His^{37} and His^{73}) that, when changed to asparagine residues, resulted in catalytically inactive proteins. While this manuscript was being revised, Murakami et al. published a high-resolution structure obtained from *L. laeta* SMaseD crystals [25]. We were gratified to find that the histidine residues identified as essential for catalysis by our mutation studies (i.e. His^{37} and His^{73}) were found to be integral to the active site predicted by the crystal structure. A distantly related bacterial

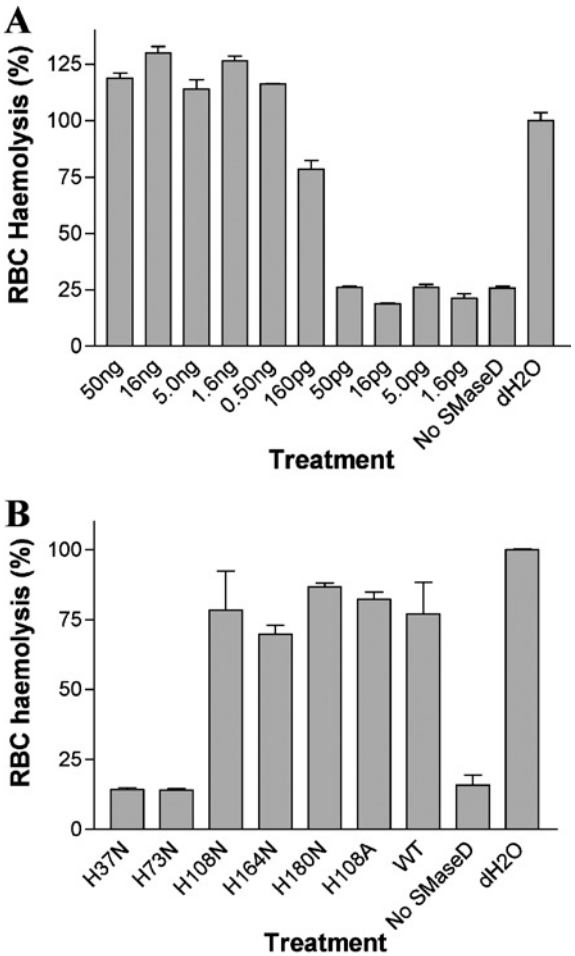


Figure 7 The purified recombinant *L. reclusa* SMaseD showed dose-dependent erythrocyte lysis

Human erythrocytes (freshly donated) were washed three times with VBS^{2+} and resuspended in the same buffer at 2 %. The cells were incubated with the purified recombinant SMaseDs [indicated amounts of wild-type SMaseD (**A**) and 2 ng of wild-type and mutant SMaseD (**B**)] for 30 min at 37 °C. Control samples were incubated with VBS^{2+} buffer alone. After washing with VBS^{2+}, the cells were resuspended to the original volume of VBS^{2+} (0.1 ml) and mixed with 0.1 ml of autologous plasma. After incubation for 1 h at 37 °C, non-lysed cells were discarded by centrifugation (1000 ***g*** and 1 min); the light absorbance of the released haemoglobin was measured at 405 nm. Background or total cell lysis was evaluated by incubation of erythrocytes with VBS^{2+} (no SMaseD) or distilled water (dH_2O) respectively. The results shown are the means ± S.E.M. for duplicate experiments.

PLD from *C. pseudotuberculosis* has a similar substrate pattern [7]. Mutation of His^{44} of the bacterial enzyme, which corresponds to His^{37} in *L. reclusa* (Figure 2), results in loss of enzyme activity [26]. This concordance strengthens the argument that the spider and bacterial enzymes are related. The mutant proteins allow testing of the presumption, widely held but heretofore not verified, that catalytic activity is necessary for the pathological activities ascribed to *Loxosceles* PLD. Indeed, in one assay of biological activity, haemolysis, we found that catalytic activity is required.

We discovered that the PAF analogue, edelfosine, is a low-affinity inhibitor of the enzyme. Although a variety of glycerol-containing phospholipids are hydrolysed by the *L. reclusa* enzyme, our data suggest a strict preference for a lyso structure, i.e. an hydroxyl at the *sn2* position of the glyceryl moiety. This sensitivity was demonstrated most directly with the ether lipid series where lysoPAF (*sn2* hydroxyl) is a substrate while PAF (*sn2* acetyl) is not and edelfosine (*sn2* methoxy) is an inhibitor.

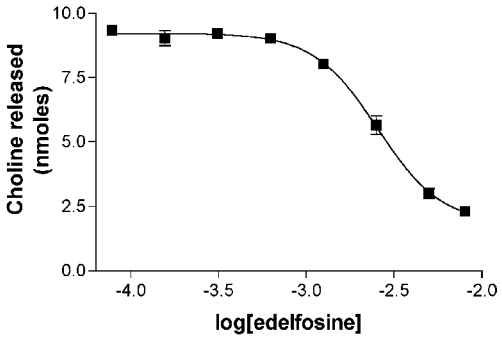


Figure 8 Edelfosine inhibits lysoPLD activity of the recombinant *L. reclusa* SMaseD

Wild-type SMaseD (1 ng) was incubated with 1 mM LPC18:1 and various concentrations of edelfosine for 1 h at 37 °C in a total volume of 0.1 ml of Hepes-buffered saline. The amount of choline released from the reactions was measured by the enzymatic choline detection assay as described in the Experimental section. The results shown are the means ± S.E.M. for triplicate experiments.

A curious feature of the *Loxosceles* SMaseD enzymes is that, whereas only glycerol lysophospholipids are recognized, sphingoid lipids are not hydrolysed as the lyso form (e.g. SPC). Rather, sphingolipids are substrates only when the nitrogen at the second carbon is in an amide linkage, i.e. sphingomyelin (ceramide-1-phosphorylcholine). It would be interesting to know whether the SM analogues with different head groups (e.g. dihydroceramide-1-phosphorylinositol from yeast) are substrates of the spider enzyme.

Finally, we endorse Moolenaar's suggestion that the name 'SMaseD' is overly restrictive and thus not accurate. The 'lysoPLD' designation, although broader, is also not fully accurate. Although 'SMaseD/lysoPLD' is accurate, it seems rather too cumbersome. We suggest that the *Loxosceles* enzyme be referred to simply as 'PLD' to avoid the connotations inherent in other names.

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