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1 **Identification and characterization of *Laodelphax striatellus* (Insecta:**
2 **Hemiptera: Delphacidae) neutral sphingomyelinase**

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Abstract: The neutral sphingomyelinase (nSMase) 1 homologue gene *LsSMase* was cloned from *Laodelphax striatellus*, a direct sap-sucker and virus vector of gramineous plants, and expressed via a Bac to Bac baculovirus expression system. The LsSMase-EGFP fusion protein was located in the endoplasmic reticulum in a similar manner to mammalian neutral sphingomyelinase 1. The biochemical properties of LsSMase were determined in detail. The optimal pH and temperature for recombinant LsSMase was 8 and 37 °C respectively. LsSMase was an Mg^{2+} or Mn^{2+} dependent enzyme with different concentration requirements. The activity of LsSMase was significantly stimulated by EGTA, whereas it was inhibited by EDTA. Millimolar concentrations of Zn^{2+} completely inhibited LsSMase. The reducing agents dithiothreitol and β -mercaptoethanol varied in their effects on activity. Phospholipids were not found to stimulate LsSMase.

Key words: sphingomyelinase; *Laodelphax striatellus*; sphingolipid; insect

Introduction

Sphingolipids are a class of bioactive lipids with diverse structures and functions and includes sphingomyelin and ceramide. Sphingomyelin largely exists in the plasma membrane (Milhas *et al.*, 2010) and is a major component of lipid raft (de Almeida *et al.*, 2003; Lingwood & Simons 2010). Furthermore, sphingomyelin can be converted to ceramide, an important bioactive molecule that mediates cellular responses to stressors. Ceramide is also a precursor molecule to other signaling lipids. Ceramide can be recycled back to sphingomyelin and therefore, the balance between sphingomyelin and ceramide serves as a signaling switch to regulate signal transduction.

Sphingomyelinase (EC 3.1.4.12) is the enzyme which hydrolyzes sphingomyelin to form ceramide and phosphorylcholine. This reaction is the main way which ceramide is generated under stress (Sawai & Hannun 1999). Depending on their pH optimum *in vivo*, sphingomyelinases are divided into three groups including acid

sphingomyelinase, neutral sphingomyelinase (nSMase) and alkaline sphingomyelinase (Clarke *et al.*, 2011b; Schuchman 2010; Zhang *et al.*, 2011). All types of sphingomyelinases were found to play anti-stress roles in mediating cell progress. Acid sphingomyelinase, a lysosomal enzyme, was the first known sphingomyelinase reported by Kanfer *et al.*, in 1966 (Kanfer *et al.*, 1966). Its mutation results in Niemann-Pick disease (Jenkins *et al.*, 2009). Four nSMase proteins are found in mammals and are encoded by four different genes. nSMase1 (neutral sphingomyelinase 1), nSMase2 (neutral sphingomyelinase 2) and nSMase4 (neutral sphingomyelinase 4) share some amino acid similarities and belong to the DNaseI superfamily (Clarke *et al.*, 2006). nSMase3 (neutral sphingomyelinase 3) is not an apparent homologue to the other three nSMase, but belongs to a distinct family (Krut *et al.*, 2006). These plural nSMases have different functions depending on their intracellular localization, cell type, or organ in response to different signals (Clarke *et al.*, 2011a). Alkaline sphingomyelinase shares no structural similarities with either acid sphingomyelinase or nSMase. It belongs to the ectonucleotide/pyrophosphatase/phosphodiesterase family and is predominately located in the digestive system (Duan *et al.*, 1996).

In *Drosophila*, several sphingomyelinase homologous genes were identified several years ago. However, it was demonstrated that *Drosophila* does not synthesize sphingomyelin but produces the sphingomyelin analogue ceramide phosphoethanolamine (Rao *et al.*, 2007; Vacaru *et al.*, 2013). The biochemical properties and function of the sphingomyelinase homologue gene of *Drosophila* has not been reported yet. Herein, we report an nSMase from *Laodelphax striatellus*. It is the homologous gene of mammalian nSMase1. *L. striatellus* is an economically important pest of rice in Asia. It causes rice yield loss not only by sucking phloem sap, but also by transmitting the rice stripe virus (Zebol *et al.*, 2009). To our knowledge, this is the first case in Insecta for which the biochemical properties of an nSMase are reported.

Results

82 *L. striatellus* nSMase cDNA cloning and sequence analysis

83 The complete *L. striatellus* nSMase (*LsSMase*) open reading frame (ORF) was
84 assembled from the transcriptome database (GenBank accession number KX013771).

85 The *LsSMase* ORF sequence was validated by PCR product sequencing. Figure 1
86 shows the complete ORF of *LsSMase*. The *LsSMase* ORF contained 1278 base pairs
87 which encoded 426 amino acids with two putative transmembrane domains in the
88 C-terminal. Compared with mammalian nSMase 1, some of amino acids were highly
89 conserved. These highly conserved amino acids, Asn 15, Glu 49, Asp 178, Asn 80,
90 Asp 270 and His 271, are potential divalent cation binding sites (Ago *et al.*, 2006)
91 (Figure 1). The conserved domain in *LsSMase* protein is an
92 exonuclease/endonuclease/phosphatase (EEP, Pfam accession number PF03372). To
93 identify other sphingomyelinase homologous genes, the proteins containing the
94 PF03372 domain were identified in different insect species, and a phylogenetic tree
95 was constructed. In contrast to mammals, it is clear that there is only one neutral
96 sphingomyelinase homologue gene in insects (Figure S1). From the phylogenetic tree
97 analysis, nSMase can be clustered into four groups (Figure 2). The first is the nSMase
98 I group, which contains nSMase 1 homologue genes including *LsSMase*. The second
99 is the nSMase II group. It contains nSMase 2 and nSMase 4 homologue genes. The
100 nSMase III group contains the nSMase 3 homologue genes. And the nSMase IV group
101 contains the bacterial nSMase.

102 *Tissue-specific expression of LsSMase*

103 It is reported that the disruption of sphingolipid metabolism elicits reproductive
104 defects in *Drosophila* (Phan *et al.*, 2007; Guo *et al.*, 2014). Whether the *LsSMase* was
105 highly expressed in reproductive organs needed to identify. The tissue-specific
106 expression of *LsSMase* was examined by quantitative real time PCR (Figure 3).
107 *LsSMase* mRNA expression could be observed in all investigated tissues. The
108 expression of *LsSMase* was highest in the ovary in female adults. However, there was
109 not significant difference among different tissues.

110 *LsSMase* encodes a protein with *in vitro* sphingomyelinase activity

111 The enzymatic assay of the LsSMase protein was performed by transient
112 expression in High Five cells. The microsome of *Autographa californica nuclear*
113 *polyhedrosis* virus infected cells were prepared for western blot assay. Figure 4 shows
114 that the recombinant protein of LsSMase was expressed successfully in High Five
115 cells. Membrane extracts from BAC-LsSMase infected cells showed a strong increase
116 of sphingomyelinase activity compared with BAC-HTB infected cells at pH 7.4
117 (Figure 4). This demonstrated that LsSMase is a sphingomyelinase.

118 *Subcellular location of LsSMase*

119 To examine the subcellular localization of LsSMase, the endoplasmic reticulum
120 membrane dye ER Tracker and Golgi-specific lipid probe BODIPY TR ceramide were
121 employed for co-localization studies. The endoplasmic reticulum and Golgi of High
122 Five cells were stained respectively. The localization of eGFP-fusion protein was
123 viewed as green fluorescence. The specific location of the endoplasmic reticulum
124 using ER Tracker and the specific location of the Golgi using BODIPY TR ceramide
125 were observed as red fluorescence. Overlapping of the fluorescent images arising
126 from LsSMase-eGFP and ER Tracker showed an extensive yellow image, revealing
127 the co-localization of these two probes (Figure 5). This suggested that LsSMase was
128 localized at the endoplasmic reticulum. The fluorescence of LsSMase-eGFP was not
129 co-localized with the Golgi marker suggesting that LsSMase was not localized to the
130 Golgi.

131 *Biochemical characterization of LsSMase*

132 The LsSMase activity was analyzed after incubating the reaction mixture for 10
133 min, 20 min, 30 min, 45 min, 60 min and 120 min respectively (Figure S2). The
134 generation of C6-NBD-ceramide was linear with time for first 30 min. Thus all the
135 enzyme reactions in our study were incubated for 30 min. The optimum pH of
136 LsSMase was determined first. As shown in Figure 6A, LsSMase was strictly
137 dependent on a slightly alkaline environment with almost no detectable substrate
138 cleavage under acidic conditions. The highest sphingomyelinase activity was around
139 pH 8. The sphingomyelinase activity at pH 7.5 was only about 50% compared with

140 the activity at pH 8. At pH 8, the highest activity was found at 37°C (Figure 6B).

141 The metal ion dependence of LsSMase was analyzed as shown in Figure 6C, D.

142 LsSMase required Mg^{2+} and Mn^{2+} , but the concentration needed was different. The
143 lower concentration of Mn^{2+} could stimulate the LsSMase activity (Figure 6D). No
144 significant effect of Mn^{2+} was found at a higher concentration like 5 mM (Figure 6C,
145 D). In contrast, the activity increased with increasing Mg^{2+} concentrations (Figure
146 6D). However, the stimulatory effect of Mg^{2+} was absent at 50 mM (Figure 6D).
147 LsSMase activity was totally inhibited by Zn^{2+} at 0.1 mM Zn^{2+} and at concentrations
148 higher than this (Figure 6D).

149 At a concentration of 5 mM, the metal-chelating reagent EDTA also showed a
150 significant inhibitory effect on LsSMase activity (Figure 6C). However, the
151 subsequent addition of Mg^{2+} not only rescued the LsSMase activity, which was
152 inhibited by EDTA, but also resulted in a better stimulatory effect compared with
153 Mg^{2+} alone (Figure 6F). A low concentration of EGTA also stimulated LsSMase
154 activity in a manner similar to Mn^{2+} (Figure 6D). The combination of Mg^{2+} and EGTA
155 was the optimal choice to stimulate LsSMase activity (Figure 6F).

156 The addition of dithiothreitol (DTT), which is a reducing agent, enhanced LsSMase
157 activity. A further study revealed that the reducing agent β -mercaptoethanol inhibited
158 its activity (Figure 6E). The combination of Mg^{2+} and DTT increased LsSMase
159 activity better than Mg^{2+} alone (Figure 6F).

160 Phospholipids have been described to stimulate the Mg^{2+} dependent nSMase (Liu *et*
161 *al.*, 1998). Thus the effect of phospholipids on LsSMase activity was investigated
162 (Figure 6G). Different phospholipids including phosphatidylserine (PS), phosphatidic
163 acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin
164 (CL) were each added directly into the reaction mixture at a concentration of 100 μ M.
165 None of these phospholipids was found to have a stimulatory effect on the LsSMase
166 activity. CL, PA and PE were found to significantly inhibit LsSMase activity.

167 Oxidized glutathione and GW4896 were previously reported as inhibitors of
168 mammalian neutral sphingomyelinase (Liu and Hannun 1997; Luberto *et al.*, 2002).
169 Accordingly, their inhibition effect on LsSMase was assayed. As shown in Figure 7A,

oxidized glutathione significantly inhibited LsSMase. Half of LsSMase activity could be inhibited by 5 mM oxidized glutathione and 10 mM oxidized glutathione almost completely inhibited LsSMase. The mammalian nSMase 2 specific inhibitor GW4869 also efficiently inhibited LsSMase. At a concentration of 100 μ M, GW4869 inhibited LsSMase activity by 80% (Figure 7B). However, the inhibition effect of GW4869 was more significant toward mammalian nSMase, which was completely inhibited by 10 μ M GW4869 (Luberto *et al.*, 2002).

Discussion

In recent years, increased attention has focused on nSMase and its functional roles in cellular stress response, such as cell growth arrest, oxidative stress, and apoptosis (Wu *et al.*, 2010). Four nSMase were identified in mammals. Among them, nSMase 1, nSMase 2 and nSMase 4 have some similar amino acids and they belong to the DNAase I superfamily (Clarke *et al.*, 2006). Their hydrolyzed domain, the exonuclease – endonuclease - phosphatase domain, is highly conserved. Although *in vitro* activity has been demonstrated for nSMase 1, in mammals, nSMase 1, it lost *in vivo* activity (Zumbansen & Stoffel 2002). nSMase 2 represents the primary sphingomyelinase activity *in vivo* (Marchesini *et al.*, 2003). Deficiency of nSMase 2 may reduce ceramide formation under environment stress to protect the cell from apoptosis induced by ceramide. Although the gene encoding nSMase has been reported in mammals since 1998 (Tomiuk *et al.*, 1998), no information on the genes encoding nSMase and their properties in insects have been reported. As the only gene which belongs to the DNAaseI superfamily in *L. striatellus*, LsSMase may be the primary *in vivo* sphingomyelinase of *L. striatellus*. It mediates the relative levels of sphingomyelin and ceramide.

The mammalian nSMase 1 was reported to be primarily located at the endoplasmic reticulum (Tomiuk *et al.*, 2000). It was also found partially located at the Golgi (Tomiuk *et al.*, 2000). In our study, LsSMase protein was located at the endoplasmic reticulum and no Golgi localization was found. LsSMase only showed high activity *in vitro* from pH 8 to pH 8.5. The nSMase reported in mammals showed the highest

activity around pH 7.5 (Bernardo *et al.*, 2000; Chatterjee *et al.*, 1999; Wu *et al.*, 2010). In contrast, compared with the LsSMase activity at pH 8, only 50% activity was present at pH 7.5. *L. striatellus* neutral ceramidase also had the highest activity at pH around 8 (Zhou *et al.*, 2013). It is presumed that an *in vivo* environment of around pH 8 in *L. striatellus* was suitable for neutral enzymes related to ceramide metabolism.

The biochemical properties of LsSMase are quite different from other nSMase 1 enzymes. LsSMase was dependent on Mg^{2+} or Mn^{2+} for its activity, similar to what has been reported for murine nSMase 1 (Tomiuk *et al.*, 2000). However, in *Plasmodium falciparum*, the malaria parasite, Mn^{2+} could not act as a substitute for Mg^{2+} to activate nSMase (Hanada *et al.*, 2000). Loss of LsSMase enzyme activity was observed in the presence of EDTA treatment, indicating its activity was dependent on the presence of metal ions. The activity of EDTA-inactivated LsSMase was completely restored by Mg^{2+} , which suggested this divalent cation may be required in the protein active site for activity. Interestingly, a stimulatory effect of EGTA was observed at a low concentration. This characteristic distinguishes LsSMase from other neutral sphingomyelinases. The activation with EGTA has not been reported for any mammalian nSMase enzymes to date. Bernardo *et al.* previously found that 10 μ M EGTA had no significant effect on bovine nSMase (Bernardo *et al.*, 2000). One possible model to explain the EGTA activation of LsSMase was that EGTA chelated an inhibitory cation of LsSMase such as Zn^{2+} resulting in an increase of LsSMase activity. EGTA is a potent Zn^{2+} chelator (Brown *et al.*, 2000), but its affinity for Mg^{2+} is weaker. The present study showed that Zn^{2+} completely inhibited LsSMase activity. EGTA chelated Zn^{2+} selectively to restore LsSMPD activity whereas EDTA chelated all the cations including Mg^{2+} and thus, it inhibited LsSMase activity.

Besides cation dependence, LsSMase showed other unique properties. Firstly, mammalian nSMase 1 activity was not affected by treatment with DTT or β -mercaptoethanol (Tomiuk *et al.*, 1998). In *L. striatellus*, LsSMase is significantly stimulated by DTT and slightly inhibited by β -mercaptoethanol. Secondly, phospholipids did not affect LsSMase activity as observed for mammalian nSMase (Bernardo *et al.*, 2000; Marchesini *et al.*, 2003; Wu *et al.*, 2010). Third, GW4869

efficiently inhibited LsSMase activity. GW4869 was identified as an nSMase2 inhibitor both *in vitro* and *in vivo* (Luberto *et al.*, 2002; Marchesini *et al.*, 2003). In a previous report, GW4869 could only inhibit 18% of the activity of human nSMase 1 (Luberto *et al.*, 2002). In our study, LsSMase was effectively inhibited by GW4869. Although the gene for LsSMase is homologous to nSMase 1, 10 μ M GW4869 inhibited 50% of the activity of LsSMase. Therefore, the biochemical properties of LsSMase and its mammalian homologue gene are likely different.

In conclusion, we identified a neutral sphingomyelinase from *L. striatellus* and described its biochemical properties. It is clear that LsSMase is a cation dependent enzyme. LsSMase has a distinct pH optimum and metal dependence. An efficient inhibitor of mammalian neutral sphingomyelinase 2, GW4869, also inhibited the activity of LsSMase at a low concentration. None of the tested phospholipids were found to stimulate LsSMase activity. Oxidized glutathione was found to significantly inhibit LsSMase activity.

Experimental procedures

Materials

NBD-C6-ceramide and NBD-C6-sphingomyelin were purchased from Life Technologies (Carlsbad, CA, USA). Different cations and phospholipids, dithiothreitol, β -mercaptoethanol, oxidized glutathione and GW4869 were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Acetonitrile and phosphoric acid were purchased from Merck (Darmstadt, Germany). *Laodelphax striatellus* colonies were reared in a rice seedling at 25°C under a 16:8 h (day:night) photoperiod with constant humidity in separated cages in an insectary. The High Five insect cell line was grown in TNM-FH medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Life Technologies, Carlsbad, CA, USA) at 28 °C in a non-CO₂ incubator.

cDNA cloning

The complete open reading frame (ORF) information of the *L. striatellus* nSMase 1 homologue gene *LsSMase* was obtained by sequence assembly from the transcriptome

database of *L. striatellus*. Total RNA was isolated from 6 adult *L. striatellus* with Trizol (Life technologies, Carlsbad, CA, USA). The first strand cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Dalian, China). The *LsSMase* ORF was amplified with the forward primer GGATCCATGAGTACACAATCAGAC and reverse primer ACTAGTTCAACTCTCAATGAAAGG using one cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min 30 s. The resulting PCR product was cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced to confirm the correctness of the *LsSMase* ORF. The homologue genes of neutral sphingomyelinase in different species were searched in NCBI database with human nSMase1, human nSMase 2, human nSMase 3, human nSMase 4 and *Bacillus cereus* neutral sphingomyelinase. The neighbor-joining tree was generated using MAGE 5 software (Tamura *et al.*, 2011).

LsSMase overexpression in High Five Cells

The *LsSMase* open reading frame was subcloned into the pFast-HTB vector with XhoI and SpeI restriction sites. pFast-HTB/*LsSMase* was transformed into *Escherichia coli* DH10Bac. The bacmid of a positive clone was prepared and used to transfect High Five cells with lipofectamine 2000 (Life technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. After amplification for five generations, High Five cells that were infected by *Autographa californica nuclear polyhedrosis* virus (AcNPV) for 60 h were used for preparing the microsome.

Subcellular localization study in High Five cells

The pFast-HTB-eGFP-*LsSMase* vectors were constructed to directly express the eGFP-tagged *LsSMase*. High Five cells were seeded on cover glass (Fisher Scientific, Pittsburgh, PA, USA). After infection for 20 h, cells were washed with HBSS twice. For the endoplasmic reticulum (ER) staining, the ER was stained with ER-Tracker Red (Beyotime, Jiangsu, China) at 28°C for 15 min. The Golgi was stained with Golgi-Tracker Red (Beyotime, Jiangsu, China) at 4°C for 30 min. After staining, cells were washed with HBSS twice. The fluorescence was examined using a confocal

286 fluorescence microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany).

287 *Microsome preparing and western blotting*

288 The microsome was prepared and western blotting was analyzed according to our
289 previous study (Zhou et al. 2010).

290 *Assay for LsSMase activity*

291 A total of 50 µg microsome and 20 µM C6-NBD-sphingomyelin were used for each
292 *in vitro* activity reaction. The fluorescence of reaction product C6-NBD-ceramide is
293 easy to detect by HPLC. The final volume was adjusted to 100 µl. For the pH
294 optimum determination, the following buffers were used: 100 mM acetate buffer (pH
295 6), 100 mM Tris buffer (pH 7 to 8), and 100 mM glycine-NaOH buffer (pH 8.5 to 10).
296 To determine the cation and reducing reagent effects, various concentrations of EDTA,
297 EGTA, ZnCl₂, MnCl₂, MgCl₂, FeCl₂, CaCl₂, dithiothreitol (DTT) and
298 β-mercaptoethanol were added directly into the 100 mM Tris buffer. For the inhibitor
299 assay, GW4869 and oxidized glutathione were pre-incubated with enzyme for 15 min.
300 After incubation for 30 min, the reaction was stopped by adding 1 ml of
301 chloroform/methanol (2:1) followed by 450 µl of water. The lower organic phase was
302 collected, and the solvent was evaporated by N₂. The sphingomyelinase activity was
303 determined by HPLC using a C18 reverse-phase column connected to a fluorescence
304 detector (excitation 455 nm, emission 530 nm). The mobile phase was a mixture of
305 acetonitrile /water/phosphoric acid (850:150:1.5 by volume).

306 *Quantitative real time PCR (qRT-PCR)*

307 To obtain different organs, an *L. striatellus* adult colony was dissected on ice, and
308 the total RNA was isolated immediately after dissection. The quality of the isolated
309 RNA was analyzed by agarose gel electrophoresis and Nanodrop (Thermo Fisher
310 Scientific, Waltham, MA, USA). Genomic DNA was removed by incubating with
311 gDNA Eraser (Takara Bio, Dalian, China) for 2 min at 42°C. A total of 10 µl cDNA
312 was synthesized using 500 ng of total RNA with the PrimeScript™ RT reagent Kit
313 (Takara Bio, Dalian, China) according to the manufacturer's instructions. The reverse
314 transcription condition was 37°C for 15 min and 85°C for 5 s with RT primer Mix

provided by manufacture. The sense primer for *LsSMase* was GTCTCTGTACATCTCCACCAAT, and the antisense primer was CCGTCTGCCGTCAATCGTA. *Actin* was used as an internal standard with a sense primer of CCGCCTCCTCCAGTTCAC and an antisense primer of TGTCCACGTCGCACTTCAT (He *et al.*, 2014). The primer efficiency of *Actin* is 94%, the primer efficiency of *LsSMase* is 90%. Reactions were performed using the SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus) (Perfect Real Time) (Takara Bio, Dalian, China) in a 50 µl volume containing 25 µl SYBR[®] Premix Ex Taq[™] II (2×), 0.4 µM of each specific sense and antisense primer, and 1 µl ROX Reference Dye (50×). A 2 µl aliquot of a 10 fold dilution of the template was used for the 50 µl PCR reaction. The qRT-PCR was carried out on an Applied Biosystems 7500 Fast Real-Time System (Applied Biosystems, Foster City, CA, USA) using one cycle of 95°C for 30 s, 40 cycles of 95°C for 3 s, and 60°C for 34 s. A melt curve was performed at the end of each reaction to verify the specificity of the PCR product. The $2^{-\Delta\Delta Ct}$ method was used to analyze the result. Changes in the mRNA level of *LsSMase* for each treatment were normalized to that of *Actin*.

Statistical analysis

Statistical analysis was performed using SPSS software, version 18.0. One-way ANOVA was performed by following Duncan's multiple comparison tests. Two-way ANOVA was used to determine the differences among the groups influenced by two factors. Statistically significant differences ($P < 0.01$) are indicated in the figures.

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Figure captions

Figure 1. The amino acid sequence alignment.

The Amino acid sequences of the magnesium-dependent nSMase from different species were aligned by program T-Coffee (Notredame *et al.*, 2000). LsSMase: *Laodelphax striatellus* nSMase (KX013771); Dm-SMase: *Drosophila melanogaster* nSMase (AAF47741); Li-SMase: *Listeria ivanovii* sphingomyelinase (WP_014092638); Bc-SMase: *Bacillus cereus* sphingomyelinase (AAS39678); hmSMase1: human nSMase 1 (NP_003071); hmSMase2: human nSMase 2 (NP_061137). ▼ shows the predicted Mg^{2+} binding sites.

Figure 2. Phylogenetic analysis of nSMase.

Classification of various nSMase was based on amino acid sequence similarities. Neighbor-joining tree was generated using MAGE 5 software. Bootstrap values from 1000 replicates were used to assess the robustness of the trees.

Figure 3. Tissue specific expression of LsSMase.

cDNA was synthesized from 500 ng of total RNA isolated from various *L. striatellus* tissue. (A) mRNA levels of LsSMase in different tissues of female adult *L. striatellus*. (B) mRNA levels of LsSMase in different tissues of male adult *L. striatellus*. *Actin* was used as an internal reference control gene. Transcript abundance was calculated based on difference in the threshold cycle (C_t) values between *LsSMase* and *actin* transcripts. The relative mRNA levels were calculated based on the normalized relative quantification $2^{-\Delta\Delta C_t}$ method. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate. The statistical significance was determined by Duncan's multiple comparison tests. Different letters above bars indicate significant differences ($P < 0.01$).

Figure 4. LsSMase showed sphingomyelinase activity.

(A) Microsomes were prepared from pFastBac HTB/LsSMase (BAC-LsSMase)

and pFastBac HTB (BAC-HTB) cells and were subjected to Western Blot analysis with the anti-His $\times$ 6 antibody (1:1,000). (B) The microsomes were assayed for sphingomyelinase activity at pH 7.4. The standard reaction contained 100 mM Tris, 5 mM MgCl₂, 5 mM DTT, 20 μ M C6-NBD-sphingomyelin, 50 μ g enzyme in volume of 100 μ l.

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Figure 5. LsSMase was located in endoplasmic reticulum.

High Five cells were infected with LsSMase-EGFP expression vector. After 20 h, the cells were co-stained with endoplasmic reticulum or Golgi specific dye. (A, D) LsSMase-EGFP fusion protein was observed as green under confocal fluorescence microscope. (B) Endoplasmic reticulum was stained by ER-Tracker Red. (C) Merged. Extensive co-location signal of LsSMase-EGFP with endoplasmic reticulum was observed as yellow. (E) Golgi was stained by Glogi-Tracker Red. (F) Merged. Co-location of LsSMase-EGFP with Golgi was not observed. Scale bars:10 μ m.

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Figure 6. Biochemical properties of LsSMase.

(A) The LsSMase activity in microsomes of pFast-HTB/LsSMase and pFast-HTB cells was assayed at different pH values. The pH was adjusted by adding the following buffer: NaAc (pH 6), Tris-HCl (pH 7-8), Glycine (pH 8.5-10). (B) Temperature dependence of LsSMase. The above microsomes were assayed for sphingomyelinase activity at indicated temperature. (C) Cation dependence of LsSMase. The sphingomyelinase activity was assayed with adding 5mM indicated cation directly in reaction buffer. The control reaction contained 100 mM Tris, 20 μ M C6-NBD-sphingomyelin, 50 μ g enzyme in volume of 100 μ l. (D) The LsSMase activity was measured at various concentration of indicated cation. (E) Effect of reducing agents on LsSMase activity. The sphingomyelinase activity was assayed with adding 5 mM dithiothreitol or β -mercaptoethanol directly. The control reaction contained 100 mM Tris, 20 μ M C6-NBD-sphingomyelin, 50 μ g enzyme in volume of 100 μ l. (F) Combination of two factors stimulated LsSMase better than Mg²⁺ alone. The indicated cation or dithiothreitol was added directly to reaction buffer. (G) Effect

of phospholipids on LsSMase activity. LsSMase activity was assayed in the presence of 100 μ M indicated phospholipids. PS: phosphatidylserine, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, CL: cardiolipin. Sphingomyelinase activity of the recombinant LsSMase was computed by subtracting sphingomyelinase activity in pFast-HTB microsomes from that in pFast-HTB/LsSMase microsomes. The highest activity in each biochemical properties assay was set as 100%, and others was expressed as % of the maximal activity. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate. The statistical significance (Figure 6A, 6B, 6C, 6E, 6F) was determined by Duncan's multiple comparison tests. Different letters above bars indicate significant differences ($P<0.01$). The statistical significance of Figure 6D was determined by two-way ANOVA analysis. $F(\text{cation})=161.6364$, $P<0.01$; $F(\text{concentration})=225.798$, $P<0.01$; $F(\text{cation} \times \text{concentration})=90.8332$, $P<0.01$.

Figure 7. LsSMase activity was inhibited by oxidized glutathione and GW4869. Oxidized glutathione or GW4869 was pre-incubated with enzyme for 15 min. (A) LsSMase activity was measured *in vitro* in the presence of the indicated concentrations of oxidized glutathione. (B) LsSMase activity was measured *in vitro* in the presence of indicated concentration of GW4869 with 10 μ M or 20 μ M C6-NBD-sphingomyelin respectively. Sphingomyelinase activity of the recombinant LsSMase was computed by subtracting sphingomyelinase activity in pFast-HTB microsomes from that in pFast-HTB/LsSMase microsomes. The activity without oxidized glutathione or GW4869 was highest and was set as 100%, others was expressed as % of the maximal activity. Data represent the mean value $\pm$ SE of three independent experiments performed in duplicate. The statistical significance of Figure 7A was determined by Duncan's multiple comparison tests. Different letters above bars indicate significant differences ($P<0.01$). The statistical significance of Figure 7B was determined by two-way ANOVA analysis. $F(\text{substrate concentration})=72.489$, $P<0.01$; $F(\text{GW4869 concentration})=92.644$, $P<0.01$; $F(\text{substrate concentration} \times \text{GW4869 concentration})=8.377$, $P<0.01$.

149x192mm (300 x 300 DPI)

Figure 2

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Figure 3

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Figure 4

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Figure 5

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Figure 6

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Figure 7

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