Identification of lysophospholipase protein from *Spiroplasma eriocheiris* and verification of its function

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**Abstract**

* Spiroplasma eriocheiris is known to cause tremor disease in the Chinese mitten crab *Eriocheir sinensis*; however, the molecular characterization of this pathogen is still unclear. *S. eriocheiris* has the ability to invade and survive within mouse 3T6 cells. The invasion process may require causing damage to the host cell membrane by chemical, physical or enzymatic means. In this study, we systematically characterized a novel lysophospholipase (lysoPL) of *S. eriocheiris* TDA-040725-5†. The gene that encodes lysoPL in *S. eriocheiris* (SE-LysoPL) was cloned, sequenced and expressed in *Escherichia coli* BL21 (DE3). Enzymatic assays revealed that the purified recombinant SE-LysoPL hydrolysed long-chain acyl esters at pH 7 and 30 °C. SE-LysoPL was detected in the membrane and cytoplasmic protein fractions using the SE-LysoPL antibody in Western blot. The virulence ability of *S. eriocheiris* was effectively reduced at the early stage of infection (m.o.i.=100) by the SE-LysoPL antibody neutralization test. To the best of our knowledge, this is the first study to identify and characterize a gene from *S. eriocheiris* encoding a protein exhibiting lysoPL and esterase activities. Our findings indicate that SE-LysoPL plays important roles in the pathogenicity of *S. eriocheiris*.

**INTRODUCTION**

The Chinese mitten crab *Eriocheir sinensis* is an important species for freshwater aquaculture in southeast China. Tremor disease (TD) is one of the most devastating diseases of *E. sinensis* [1, 2]. *Spiroplasma eriocheiris* was previously identified as a causative pathogen of TD [3, 4] and as a novel pathogen in aquatic crustaceans [5]. Historically, spiroplasmas were associated with insects, ticks and plants; however, recent studies on crustaceans have changed our understanding of the host range of spiroplasmas [6].

* Spiroplasmas belong to the cell-wall-free class of bacteria called Mollicutes, and its surface proteins are of great importance in adherence, invasion and interaction with the host immune system. The surface proteins may perform structural, transport or enzymatic functions [7, 8]. In a previous study, we found that *S. eriocheiris* can invade *E. sinensis* haemocytes [9], mouse 3T6 cells [10] and embryonated chickens [3]. The agent was detected in the brain of embryonated chickens [3] and indicated that the agent represented a neurotropic characteristic in crab and embryonated chickens [11, 12]. When a high concentration of lipids [cholesterol (sterol)] was added in the culture medium, the growth rate of *S. eriocheiris* was better [4]. Phospholipids and proteins represent the major biomolecular constituents of the host cell envelope. Therefore, enzymes such as phospholipases and proteinases, which are capable of hydrolysing these biomolecules, are likely to be involved in the disruption of the membrane that occurs during host cell invasion. By cleaving phospholipids, phospholipases destabilize the membrane and cause cell lysis [13]. Furthermore, phospholipases may play fundamental roles in generating signals required for invasion as well as in producing arrays of metabolites with distinct biologic functions [14]. In *Mollicutes*, a *Mycoplasma* phospholipase C (PLC) can cleave membrane bilayer phospholipids and release diacylglycerol that activates protein kinases [14]. The activity of phospholipase A (PLA) can release free fatty acids that may perturb the host cell membrane and generate active metabolites [15, 16]. Phospholipase B/lysophospholipase (lysoPL), which
catalyses the hydrolysis of lyosphospholipids to yield a glycerolphosphate derivative and fatty acids, is one of the most important deacylating phospholipases. High concentrations of lyosphospholipids and free fatty acids have been shown to disturb membrane conformation, affect the activity of many membrane-bound enzymes and even cause cell lysis [17–19]. LysoPL is known to be an important virulence factor in Cryptococcus infections [20]. Furthermore, phospholipases, including lysoPL, have been shown to be associated with the pathogenesis of many bacteria and fungi [13, 21].

In the present study, we found the lysoPL gene of S. eriocheiris (SE-LysoPL). The aims of this study were to identify SE-LysoPL, to characterize its lysoPL and esterase activities and to verify the role of SE-LysoPL in the pathogenicity of S. eriocheiris.

**METHODS**

**Bacterial strains**

S. eriocheiris TDA-040725-5T was isolated from *E. sinensis* with TD using the methods described by Wang et al. [22] and cultured in R2 medium at 30 °C [23].

**DNA extraction, cloning of SE-LysoPL and site-directed mutagenesis**

Genomic DNA was extracted from *S. eriocheiris* using the DNeasy Tissue Kit (Qiagen), according to the manufacturer’s instructions. The full-length sequence of SE-LysoPL (GenBank Accession no. KP288667) was obtained from the genomic DNA of *S. eriocheiris* TDA-040725-5T. Primers (Table S1, available in the online Supplementary Material) were designed to amplify a mature peptide-coding gene by PCR. PCR products along with pET-30a vector were digested with restriction enzymes (*Kpn*1 and *Eco*RI) (TAKARA, Japan) and then ligated to generate pET-30a-SE-LysoPL. These plasmids were sequenced at Invitrogen. The reactions were terminated by adding 100 μl acetone. After site-directed mutagenesis, the product pET-30a-SE-LysoPL-M was sequenced to confirm that the mutation succeeded.

**Sequence analysis**

A similarity search was performed using BLAST (http://ncbi.nlm.nih.gov/blast/). The protein features were predicted by the Compute pl/Mw tool (http://www.expasy.ch/). The different domains of SE-LysoPL were detected using SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORM- MAL=1).

**Protein expression, purification, antibody preparation and ELISA**

*Escherichia coli* BL21 cells (DE3) (TransGen, China) were transformed with the pET-30a-SE-LysoPL-M plasmids for protein expression. Sample analysis was according to Meng *et al.* method [24]. High-Affinity Ni-NTA Resin (GE Healthcare, Sweden) was used to purify the recombinant proteins, according to the manufacturer’s instructions. The eluted protein was collected and dialysed against PBS (13.7 mmol l⁻¹ sodium chloride, 2.7 mmol l⁻¹ potassium chloride, 4.3 mmol l⁻¹ Na₂HPO₄ and 1.4 mmol l⁻¹ KH₂PO₄, at pH 7.0). The recombinant proteins were analysed by SDS-PAGE. Total protein was quantified using the Bradford method [25]. A detailed antibody preparation and ELISA method was performed according to a previous study [24]. *S. eriocheiris* was used as the antigen for preparing the immune serum, according to Wang’s protocol [26].

**Enzyme characterization**

Enzyme activity was analysed using a spectrophotometer (UltraSpec 2100 pro, GE Healthcare). The hydrolysis of p-nitrophenyl (p-NP) esters was carried out at 30 °C in 100 μl of 1× PBS buffer (pH=7.4) containing 0.5% Triton X-100 and a 5 mmol l⁻¹ solution of the corresponding p-NP ester. The reactions were terminated by adding 100 μl acetone. Enzyme activity was calculated from the changes in absorbance at 405 nm. The effects of temperature and pH were assessed by using p-NP palmitate as a substrate and followed Chu *et al.* methods [27]. To analyse thermal stability, the enzyme was incubated for 15 min at various temperatures in the 30–70 °C range. We also used 1, 2, 4, 8, 16 and 20 μg ml⁻¹ of anti-SE-LysoPL antibody to inhibit the enzyme activity and to test the sensitivity of the enzyme towards the antibody.

**Preparation of total protein and membranes of *S. eriocheiris***

*S. eriocheiris* was grown for 48 h at 30 °C in R2 medium. The cells were harvested at the mid-exponential phase of growth (when *A*₄₃₅=0.1–0.15 and *pH*=6.8) by centrifugation for 20 min at 12000 g. Membranes were isolated from the washed cells by ultrasonic treatment [28, 29]. Total protein from the cells and the membrane preparations was estimated by the Bradford method [25]. The total protein from the cells and membranes of *S. eriocheiris* was quantified by SDS-PAGE and Western blot.

**Cell culture and challenge**

Mouse 3T6 cells purchased from the Type Culture Collection cell bank of the Chinese Academy of Sciences Committee (Shanghai, China) were maintained in complete DMEM (WISENT, Canada) supplemented with 10% FBS, 0.15% NaHCO₃, 0.45% glucose, 4 mM L-glutamine (WISENT, Canada) and antibiotics (100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin) at a suitable *pH* of 7.20–7.40. The medium was sterilized by filtration through a sterile filter with a pore size of 0.22 μm (Millipore) in a Class II Biosafety Cabinet (ESCO). These cells were cultured in a 6-well tissue culture plate (Corning Costar, USA) and incubated at 37 °C with 5% carbon dioxide in a Model 4150C incubator (Contherm).

*S. eriocheiris* cells were collected during the exponential growth phase (10⁸ spiroplasmas ml⁻¹) by centrifuging at
12000 g at 4 °C for 30 min and resuspended in the same volume of DMEM without FBS. Once they had grown to a confluence of greater than 80%, mouse 3T6 cells were infected with S. eriocheiris (10^6 spiroplasmas ml^-1) in three replicate wells of the 6-well plate and the multiplicity of infection (m.o.i.=100) that followed Yao et al. methods [10].

**Antibody neutralization test and oxytetracycline protection assays infection rate**

An antibody neutralization test was used to investigate the effect of SE-LysoPL on S. eriocheiris infection of mouse 3T6 cells. The bacteria were pretreated by incubating with either specific anti-SE-LysoPL antibody or preimmune serum for 1 h at 37 °C. Pretreated bacteria were used to infect mouse 3T6 cells as described above. All experiments were repeated three times. The early stage of the cell culture and infection was performed using the methods described in the section 'Cell culture and challenge.' For the antibody neutralization test, mouse 3T6 cell samples were collected at 1, 6, 12, 24, 36, 48 and 72 h after infection of S. eriocheiris. Cell processing was in accordance with Yao’s method [10].

**Flow cytometric (FCM) analysis**

Apoptosis of the cells was assessed by FCM using Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions. Mouse 3T6 cells were plated at a density of 1×10^6 cells well^-1 into 6-well plates and precoated with poly-D-lysine. The treated mouse 3T6 cells were digested with 0.25% trypsin and EDTA (Sigma) to cleave the cells that were attached to the plate and centrifuged at 500 g for 10 min; the supernatant was then removed. The cells were washed twice with PBS and fixed using 70% ethanol. The cells were again centrifuged at 500 g for 10 min, washed with PBS twice and adjusted to a concentration of 1×10^6 cells well^-1. A 0.5 ml aliquot of RNase (1 mg ml^-1 in PBS) (Sigma, USA) was added to each 0.5 ml cell sample. After gentle mixing with 5 ml FITC Annexin V and 5 ml PI, the cells were collected by filtration and incubated in the dark at 4 °C for 30 min before FCM analysis.

**Immunofluorescence experiment**

During the antibody neutralization test, samples were collected and washed three times with PBS every 2 h between 30 and 48 h post-challenge. The mouse 3T6 cells were then fixed with 4% paraformaldehyde and permeated with PBS with 0.1% Triton X-100 for 30 min. After the cells were incubated with 3% BSA in PBST for 30 min, the polyclonal antibody against S. eriocheiris (1:1000) was immunoreacted with the infected cells. The cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:2000) (Invitrogen, USA). The cell morphology was examined using a Ti-s inverted phase-contrast microscope. A Nikon Intensilight C-HGFI (Nikon, Japan) UV fibre optic illuminator was used to obtain fluorescence microscopic data.

**Statistical analysis**

Data were analysed using the SPSS general linear models (GLM) procedure (SPSS 16.0, Chicago, IL, USA) to test for significant differences among treatments. If a significant (P<0.05) difference was found, a Duncan’s multiple range test [30] was used to rank the means. All data are presented as mean±SD of three replicates.

**RESULTS**

**Sequence analysis of lysoPL**

A DNA fragment of 927 bp encoding lysoPL was obtained by sequencing the genomic DNA of S. eriocheiris. The DNA sequence was found to encode 308 amino acid residues with a theoretical molecular mass of 35 kDa and pI of 9.64 (Fig. S1). The SE-LysoPL protein sequence exhibited the following degrees of similarity: (1) 59% identity similarity with lysoPLs of Spiroplasma chrysopilcola (WP_016399191.1) and Spiroplasma syphilitica (WP_016340977.1) [31], (2) 52% identity with Clostridium intestinale alpha/beta hydrolase (WP_021803979.1) and (3) 48% similarity with Turicibacter sanguinis putative alpha/beat hydrolase (WP_006783760.1). Based on alignment studies (Fig. S2), SE-LysoPL was predicted to be a member of the hydrolase-4 family (position: 14–98 amino acid), which is found in bacteria and eukaryotes and is approximately 110 amino acids in length. Many members of this family are annotated as lysoPLs [32]. The SE-LysoPL protein contains three hydrolase domains (abhydrolase-1, position: 14–98 amino acid; abhydrolase-5, position: 14–98 amino acid; and abhydrolase-6, position: 14–98 amino acid). This catalytic domain is found in a wide range of enzymes that do not share obvious sequence similarities. The alpha/beta hydrolase fold is found in proteases, lipases, peroxidases, esterases, epoxide hydrolases and dehalogenases [32]. The amino acid sequence of SE-LysoPL also includes an acylglycerol lipase domain (position: 13–252 amino acid). The SE-LysoPL protein sequence (Fig. 1) also contained the active-site serine motif (GXSXG) apart from the alpha/beta hydrolase fold, which are both characteristics of lipolytic enzymes. The catalytic triad in the alpha/beta hydrolase fold of SE-LysoPL can be assigned to Ser115, Asp254 and His288 [33].

**Expression, purification of SE-LysoPL and antibody preparation, antigen identification and localization**

After site-directed mutagenesis, the mutant gene was sequenced to verify the result. After being expressed, one protein with an apparent molecular mass of around 35 kDa was detected in the positive transformant with pET30a-SE-LysoPL-M by SDS-PAGE (Fig. 2a, lane 4) and Western blot (Fig. 2b, lane 3). This recombinant protein was purified by High-Affinity Ni-NTA Resin (Fig. 2a, lanes 6 and 7).

The titre of anti-SE-LysoPL in the serum was above 1:65336 and below 1:262144 as tested by ELISA (Fig. S3) and deduced to be approximately 1:70000. To determine the localization of SE-LysoPL, total proteins and the
Fig. 1. Multiple amino acid alignment of SE-LysoPL homologues. The proteins are lysoPLs from Spiroplasma chrysoplicola (SC-LysoPL, WP_016339119.1), Spiroplasma syrphidicola (SS-LysoPL, WP_016340977.1), Spiroplasma melliferum (SM-LysoPL, WP_004027650.1), Mesoplasma florum (MF-LysoPL, WP_011182996.1) and Mycoplasma leachii (ML-LysoPL, WP_013448130.1). Alpha/beta hydrolase from Clostridium intestinale (CI-alpha/beta, WP_021803979.1) and Turicibacter sanguinis (TS-alpha/beta, WP_006783760.1). Black background represents identical amino acids, while a grey background represents highly similar amino acids. Asterisk symbols indicate a conserved catalytic triad. Numbers at the end of each line on the right-hand side refer to the number of amino acid residues. Solid line shows the conserved GXSXG in the serine hydrolase.
membrane and cytoplasmic protein fractions from *S. eriocheiris* were prepared and reacted with the anti-SE-LysoPL serum. Western blot analysis showed that a band corresponding to the size of the SE-LysoPL protein (35 kDa) was detected in total protein, membrane protein as well as cytoplasmic protein fractions (Fig. 3).

**Characterization of the recombinant SE-LysoPL**

The optimum temperature of SE-LysoPL was investigated using *p*-NP palmitate as a substrate at pH 7 and was found to be 30 °C (Fig. 4a). The pH dependence of SE-LysoPL was studied using Good’s buffer for a pH range from 3.0 to 10.0 at 30 °C. The optimum pH of the protein was found to be 7.0 (Fig. 4b). Incubation experiments at various temperatures between 20 and 70 °C for 10 min at pH 7.0 showed that SE-LysoPL was stable in incubation temperatures from 20 to 40 °C (Fig. 4c) Hydrolysis of *p*-NP esters with different chain lengths by SE-LysoPL is shown in Table 1. SE-LysoPL showed a preference for long-chain fatty acid esters (C16 and C14) and did not react with the short-chain fatty acid ester (C4). We found that divalent metal ions (10 mmol l⁻¹), Ca²⁺, Fe²⁺, Hg²⁺ and Zn²⁺, significantly lowered the enzyme activity compared to that in the control (Fig. 4d). The enzyme activity was also found to be independent of the presence of metal ions such as Ca²⁺ and Mn²⁺. We used six various concentration of anti-SE-LysoPL antibody to inhibit the enzyme activity and found that 4 µg ml⁻¹ anti-SE-LysoPL antibody significantly (*P*<0.001 and *P*<0.05) inhibited enzyme activity compared to control and 1 and 2 µg ml⁻¹ treatments (Fig. 4e). The 16 and 20 µg ml⁻¹ groups exhibited the lowest enzyme activity (Fig. 4e).

**SE-LysoPL antibody neutralization test**

Oxytetracycline is rarely absorbed by or accumulated in eukaryotic cells. Thus, oxytetracycline can kill the extracellular spiroplasmas, but not the intracellular infective spiroplasmas. Therefore, the spiroplasma infection rates in host cells can be detected by using the oxytetracycline method [10]. In this study, we used oxytetracycline at a concentration of 200 µg ml⁻¹ [34]. With the same m.o.i. of 100 [10], the infection rate in the cell challenge group as well as the SE-LysoPL antibody neutralization test group was zero at 0 h. However, after 12 and 24 h, the infection rate of the cell challenge group and the neutralization test group treated with 2 µg ml⁻¹ anti-SE-LysoPL antibody was significantly higher (*P*<0.05) than that of the neutralization test group treated with 20 µg ml⁻¹ anti-SE-LysoPL antibody (Fig. 5). After 72 h, the infection rate of the neutralization test group was higher than the infection group (Fig. 5).

**SE-LysoPL antibody inhibits *S. eriocheiris*-induced apoptosis**

We found that mouse 3T6 cell cultures could be maintained in a relatively stable state, with continuous cell cultures remaining viable up to 12 days. A majority of mouse 3T6 cells in the culture appeared spherical or elliptical and they began to adhere to the substrate within 3 h of seeding. Mouse 3T6 cells from the cultures inoculated with the *S. eriocheiris* showed cytopathic effects 48 h post-inoculation (Fig. 6b). However, in the neutralization test group treated with 20 µg ml⁻¹ anti-SE-LysoPL antibody, the cell morphology was...
better maintained than that in the *S. eriocheiris* challenge group (Fig. 6c). Mouse 3T6 cells incubated with preimmune serum were used as the control group (Fig. 6a).

To determine whether anti-SE-LysoPL antibody (20 µg ml\(^{-1}\)) treatment inhibited *S. eriocheiris*-induced apoptosis of mouse 3T6 cells, we examined the extent of apoptosis using FCM analysis by double staining with FITC Annexin V and PI (Fig. 6d). Quantification revealed that anti-SE-LysoPL treatment inhibited *S. eriocheiris*-induced apoptosis of mouse 3T6 cells, as revealed by decreased Fluorescein isothiocyanate (FITC) Annexin V binding and increased Propidium iodide (PI) staining.

**Fig. 4.** Enzymatic characterization of SE-LysoPL. (a) Effect of temperature on esterase activity of the SE-LysoPL protein. The activity was measured by spectrophotometric method using p-NP palmitate as substrate at pH 7.0. Bars indicate standard errors. (b) Effect of pH on esterase activity of the SE-LysoPL protein. The enzyme reaction was carried out at 30 °C in Good's buffer using p-NP palmitate as substrate. Bars indicate standard errors. (c) Thermal stability of the SE-LysoPL protein. The residual activities after 10 min of incubation at different temperatures were measured using a spectrophotometric method with p-NP palmitate as substrate at 30 °C and pH 7.0. (d) Effect of metal ions on SE-LysoPL. Activities were measured using a spectrophotometric method with p-NP palmitate as substrate at 30 °C and pH 7.0 in PBS containing various metal ions at a concentration of 10 mmol l\(^{-1}\). The relative activities are presented as the ratios of enzyme reactions with different metal ions to those in the control that had no metal ions. (e) Effect of anti-SE-LysoPL on SE-LysoPL activity. The residual activities after 30 min of incubation at different concentrations of anti-SE-LysoPL were measured by using a spectrophotometric method with p-NP palmitate as substrate at 30 °C and pH 7.0. Bars indicate standard errors.

**Table 1.** Substrate specificity of SE-LysoPL in the hydrolysis of p-NP esters containing fatty acids of various chain lengths.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acyl group chain length</th>
<th>Relative activity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-NP palmitate</td>
<td>C16</td>
<td>100±6.11</td>
</tr>
<tr>
<td>p-NP myristate</td>
<td>C14</td>
<td>58.6±6.39</td>
</tr>
<tr>
<td>p-NP laurate</td>
<td>C12</td>
<td>23.2±1.52</td>
</tr>
<tr>
<td>p-NP butyrate</td>
<td>C4</td>
<td>6.8±1.30</td>
</tr>
</tbody>
</table>

*The highest activity of enzyme is denoted as 100% (for p-NP palmitate) as compared with other different substrates.

**Fig. 5.** Infection of mouse 3T6 cells by *S. eriocheiris* in oxytetracycline protection assays at different time points. Different letters above the bars denote significant differences between the groups at the *P*<0.05 level. Data are shown as the mean±SD of three independent experiments performed under the same condition.
antibody treatment significantly reduced the percentage of apoptosis of mouse 3T6 cells ($P<0.01$) (Fig. 6e). The number of FITC Annexin V-positive cells was significantly lower ($P<0.01$) among $S$. eriocheiris-treated mouse 3T6 cells (Fig. 6f) than among cells only infected with $S$. eriocheiris.

**SE-LysoPL antibody prevented $S$. eriocheiris invasion of cells**

The green fluorescent Alexa Fluor-488 was used to label the $S$. eriocheiris during a fluorescence immunoassay to study the adhesion to and infection of the host cell by spiroplasmas. The results showed that a large number of $S$. eriocheiris had infected the mouse 3T6 cells and that they were widely distributed in the cell interior (Fig. 7b). We also found that spiroplasmas formed aggregative bodies wrapped by membrane at 48h post-inoculation. These structures are inclusion bodies of $S$. eriocheiris and were probably formed owing to the $S$. eriocheiris proliferation in infected cells (Figs 7b and S4). The green fluorescent label in the neutralization test group treated with 20 µg ml$^{-1}$ anti-SE-LysoPL antibody showed that $S$. eriocheiris was initially distributed in the membrane and later became dispersed throughout the cell interior (Fig. 7c). The number of inclusion bodies observed in the neutralization test group was significantly lower than that in the $S$. eriocheiris challenge group. There was no evidence of the green fluorescent label in the control group (Fig. 7a). We analysed the total fluorescence intensities of these three groups by using ImageJ v1.48 (https://imagej.nih.gov/ij/). The fluorescence intensities in the $S$. eriocheiris challenge group were found to be higher than that in the anti-SE-LysoPL antibody group (Fig. S4a, c and d). However, the fluorescence signal in the control group was very weak compared to that in the other two groups (Fig. S4b–d).
DISCUSSION

Prevention and control of diseases are among the major priorities for the efficient development and maintenance of aquacultures. Although some aspects of aquaculture have been improved by better management strategies, TD remains a very serious disease of *E. sinensis* populations in China [24]. Detailed studies about the pathogen, *S. eriocheiris*, are required for controlling this disease. Earlier laboratory studies had shown that *S. eriocheiris* can invade *E. sinensis* haemocytes [9], mouse 3T6 cells [10] and embryonated chickens [3], and the results showed indicated that *S. eriocheiris* represented a neurotropic characteristic in crab and embryonated chickens [11, 12]. Many reports have shown that phospholipases play an important role as bacterial virulence factors [14–16, 29]. In this study, we identified a lysoPL from *S. eriocheiris* that shares structural and functional homology with prokaryotic lysoPLs. Moreover, the study found that, while SE-LysoPL could hydrolyse ester bonds with both short and long acyl chains, the optimal substrate was a long-chain fatty acid ester. The reason for this is that the amino acid sequence of the SE-LysoPL protein contains an acylglycerol lipase that hydrolyses glycerol monoesters of long-chain fatty acids [35]. The predicted protein sequence of SE-LysoPL contained the GXSXG sequence, which is conserved in the active sites of the serine proteases, lipases and esterases. The serine in the GXSXG motif was found to be essential for both PLA and lysoPL activities, indicating that these two activities were catalysed by the same active-site residues [36, 37]. For SE-LysoPL, the optimum pH and temperature to act on p-NP palmitate were around 7.0 and 30°C. The enzyme activity was not influenced by exogenous divalent cations (Ca²⁺, Mg²⁺ and Mn²⁺), similar to the activity of PLC in mycoplasmas [29].

In *Mycoplasma laidlawii*, the lysoPL enzyme appears to be localized at the membranes [38]. There have been reports that *Mycoplasma gallisepticum* strains also possess a membrane-bound lysoPL, which hydrolyses lysophospholipids generated in these membranes by recruiting an external phospholipase and removing one of their acyl groups [39]. In this study, Western blot analysis revealed that the anti-SE-LysoPL antibody not only responded effectively to the purified recombinant SE-LysoPLs but also specifically recognized native lysoPL from *S. eriocheiris* cytosolic protein fraction and outer membrane protein fraction. Phospholipases have been associated with multiple disease syndromes such as the massive tissue destruction related to gas gangrene, skin and lung infections caused by *Pseudomonas aeruginosa* and food-borne listeriosis [40]. Examples include PLA1, PLA2, phospholipase B and PLC in bacteria and fungi, which were all linked to the degradation of the phospholipid components of the mucosal barrier [13, 41]. To elucidate its possible role in the early events of *S. eriocheiris* infection, we performed oxytetracycline protection assays and indirect immunofluorescence assays.

The question of whether SE-LysoPL is an important virulence factor and thus has a direct relationship with *S. eriocheiris* pathogenicity is important and is still unresolved. Although some phospholipases have been identified as virulence factors in other bacteria and fungi, their functions in

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**Fig. 7.** Mouse 3T6 cells after *S. eriocheiris* challenge at 48 h. Alexa Fluor-488 was used to label *S. eriocheiris* in green. (a) *S. eriocheiris* was incubated with preimmune serum (control group); (b) *S. eriocheiris* challenge group; (c) *S. eriocheiris* was incubated with anti-SE-LysoPL antibody (20 µg ml⁻¹) before challenge. White arrows indicate *S. eriocheiris* inside cells, which formed inclusions and triangles indicate *S. eriocheiris* in cell membranes. Bars, 20 µm.
spiroplasmas are not very clear. In their study on the pathogenicity of spiroplasmas, Wang et al. found that, like Spiroplasma mirum, S. eriocheiris could also induce cataracts in mice. Yao et al. [10] successfully established an S. eriocheiris infection model for mouse 3T6 cells. In the cell challenge test (with m.o.i.=100), the infection rate of S. eriocheiris in the group treated with SE-LysoPL antibody (20 µg ml⁻¹) was significantly lower than that in the S. eriocheiris challenge test group. These results suggest that the anti-SE-LysoPL antibody could protect mouse 3T6 cells from S. eriocheiris infection.

In the inhibition experiments, we used the anti-SE-LysoPL antibodies to inhibit lysoPL activity in S. eriocheiris. The results showed that the variation in morphology of mouse 3T6 cells after inhibition was greater than that in the S. eriocheiris challenge group. The FCM analysis revealed that inhibition treatment significantly reduced the percentage of apoptotic mouse 3T6 cells. Through indirect immunofluorescence assays, we found that the anti-SE-LysoPL antibody could effectively prevent S. eriocheiris invasion of the cells 48 h post-inoculation. These results show that lysoPL plays an important role in the process of S. eriocheiris infection. SE-LysoPL may be an important virulence factor of S. eriocheiris. Similar results were obtained in an earlier study on the function of phospholipases in bacteria and fungi [13, 41].

In conclusion, our studies have clearly shown that SE-LysoPL exhibits phospholipase activity. Western blot analysis suggests that the SE-LysoPL is a membrane protein in S. eriocheiris. Through an antibody neutralization test, we also found evidence that SE-LysoPL may be an important virulence factor of S. eriocheiris and may also play an important role in pathogenicity and energy metabolism of S. eriocheiris. In order to further study this gene and its function, it would be necessary to establish a genetic map and design a gene knockout system of S. eriocheiris. By knocking out/silencing the SE-LysoPL gene, its potential function can be elucidated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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