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Purified Suilysin Secreted by *Streptococcus suis* Serotype 2 Induce Interleukin-6 by Rat Brain Microvascular Endothelial Cells

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***Streptococcus suis* (*S. suis*) serotype 2 is an important pathogen of pigs causing various diseases, including meningitis, and how the pathogen cross the blood-brain barrier (BBB) is unclear. As an important virulence factor of *S. suis*, suilysin is shown to be helpful for the *S. suis* crossing the BBB. The aim of this study was to investigate the ability of suilysin produced by *S. suis* serotype 2 strain CVCC606 to induce the release of pro-inflammatory cytokine by rat brain microvascular endothelial cells (RBMVECs), first suilysin was purified by using different filtration steps, fractional precipitation with ammonium sulfate and Sephadex G-100 column chromatography. Then RBMVECs were exposed to suilysin, intracellular lactate dehydrogenase (LDH) and interleukin (IL)-6 release assays were employed, a time- and dose-dependent cytotoxicity of suilysin to RBMVECs was observed, and high levels of IL-6 production by RBMVECs could be induced with suilysin. Our findings suggest that cell injury and pro-inflammatory cytokine release of RBMVECs induced with suilysin is contribute to increase BBB permeability, and suilysin may be involved in the pathogenesis of meningitis caused by *S. suis*.**

Key words: Suilysin; *Streptococcus suis*; purification; cell injury; cytokine; Microvascular endothelial cells

Streptococcus suis (S.*suis*) serotype 2 is an important pathogen of pigs responsible for arthritis, encephalitis, pneumonia, endocarditis, polyserositis, septicemia, miscarriage and meningitis [1-3]. 35 serotypes have been identified[4-6] and the serotype 2 is the most commonly associated with diseases in pigs and humans, and also the most frequently isolated strain from diseased pigs. Suilysin (SLY) was first characterized as a hemolysin in 1994 [7], and is considered as an important virulence factor of the pathogenesis of S.*suis* infection [8]. Suilysin, secreted by *S. suis*[7], which belongs to a family of cholesterol-binding cytolytic toxins [9], produced by some gram-positive bacteria [10]. The member of this family usually has been implicated as major virulence of several bacterial infection[7].

As a selective and hyposmotic barrier, blood-brain barrier (BBB) could restricted the free movement of most polar molecules and proteins [11], to protect the brain from most invasive pathogens. However, meningitis is still caused by

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some strains of *S. suis*, adhering to RBMVECs and easily invading the BBB, to enter the blood circulation as the permeability of BBB increased [12]. The breakdown of the BBB was always considered to be the consequence of the invasion of leukocytes releasing cytokines, reactive oxygen species and proteases, leading to an increase in cerebral microvascular permeability [13]. However, the mechanism of the pathogen crossing the BBB is still unclear. As an important component of BBB, specialized layer of brain microvascular endothelial cells (BMVECs) play a key role in many physiological and pathological processes of the BBB. Studies showed that most of the strains which cause damage to epithelial and endothelial cells, and macrophages were the SLY-positive *S. suis* [10,14,15].

It is hypothesized that suilysin can affect the barrier function of BMVECs, resulting in the increase of BBB permeability , which is contribute to the invasion of *S. suis* to BMVECs in bacterial meningitis (BM). Therefore, we first purified the suilysin by using different filtration steps, fractional precipitation with ammonium sulfate and Sephadex G-100 column chromatography. Then the cytotoxicity of suilysin to RBMVECs was investigated using the method of lactate dehydrogenase (LDH) release assay. Afterwards, the pro-inflammatory cytokine release was measured by BMVECs induced with suilysin by ELISA. Finally, the implication of the role suilysin playing in meningitis induced with *S. suis* was discussed.

MATERIALS AND METHODS

Cell culture

All experimental procedures were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Jilin University.

Brain microvascular fragments were isolated, and endothelial cells cultured, using a modified method introduced by Diglio CA [16], Gordon EL [17,] and Hee-Sang Lee [18]. Briefly, cerebral cortices of Sprague–Dawley (SD) rats (3-weeks-old) were used for the mircovessels isolation. After digestion with dispase II (Sigma), the homogenate was treated in the method of density gradient centrifugation using dextran (Sigma) and percoll (Pharmacia) respectively, between two operations of centrifugation, the sample was digested by collegnase/dispase (Roche). After washing and centrifuging twice, the microvessel fragments were suspended in DMEM culture medium (Gibco), supplemented with 20% heat-inactivated fetal bovine serum (FBS; Hyclone), 2 mM L-glutamine100 IU/ml penicillin (Sigma), 100µg/ml streptomycin(Sigma), 2.5µg/ml amphotericin B (Amresco) and 100µg/ml heparin(Sigma). 12-well tissue culture plates (Corning) were precoated with fibronectin (2 µg/cm^2, Roche) to support the cells. Cells were incubated at 37°C with 5% CO_2 in a humid atmosphere. Preparing for assay, RBMVECs were trypsinized by adding a 0.125% trypsin-0.02% EDTA solution (Sigma) and diluted in culture medium at 8×10^4, and the cell suspension was distributed in tissue culture plates and incubated until confluence was reached. Before the experiment, the medium was removed from the plates and was replaced by medium without antibiotics.

Bacterial strains and culture

S. suis serotype 2 strain CVCC606 was purchased from China Institute of Veterinary Drugs Control, which was isolated from a pig with meningitis in Shanghai, and the phenotype was MRP+EF+SLY+. 200µL of Todd-Hewitt broth (THB) was added into lyophilized powder of the bacteria to form a suspension, and 50 µl of the bacterial suspension was cultured on 7% sheep blood agar plates in a 5% CO_2 air incubator at 37°C for 18h. The isolated colonies were used as inocula for THB, which were incubated in the same condition described above. Working cultures of purified suilysin were produced by inoculating 400ml of these cultures in 20L of THB in a fermentor at 37°C for growth to mid-log phase to an optical density at 600 nm (OD600) of 0.4 (~10^8 CFU/ml).

Purification of suilysin

Suilysin was purified as described by Jacobs[7] and Gottchalk[9] with some modification. The culture supernatants (centrifuged at 15,000×*g* at 4°C for 30 min) of *S. suis* were filtered (0.22 µm pore size filters; Millipore, U.S.A.) for sterilization and precipitated with 40% of ammonium sulfate. The suspensions were centrifuged at 15,000×*g* at 4°C for 10 min. The supernatants were precipitated with 45% of ammonium sulfate and then centrifuged as described above. Pellets were resuspended with 5

ml 0.01 mM PB and centrifuged again, and the pellets were discarded. Supernatants were considered as crude purified suilysin, and marked as E1. In addition to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the hemolytic activity and the protein concentration of E1were measured [19]. Then the E1 was subjected to molecular sieve chromatography using a Sephadex G-100 column (Pharmacia, U.S.A.), carefully transferring the buffer solution of 10 mM PB (pH 7.2) into the column. Linear gradient elution was achieved at a flow rate of 0.5 ml/min. Fractions were collected every 5 ml, and the wavelength of detection was 280 nm. After collection, the samples were subjected to SDS-PAGE. The protein concentration and the hemolytic activity of each sample were measured, and the hemolytic specific activity was calculated. The fraction with the highest hemolytic specific activity was considered as purified suilysin and marked as E2.

Measurement of hemolytic activity

The hemolytic activity of the samples was measured using a modified method introduced by Jacobs[7], Gottchalk[9] and Kanclerski[18]. Briefly, twofold dilutions (99 µl) of test samples were prepared in polystyrene deep-well titer plates (Beckman, U.S.A.) with 10 mM PB (pH 7.4) as the diluent. We then added 2 µl of 0.5M dithiothreitol (DTT) to each well to restore the hemolytic activity of the samples that was reduced by exposing to atmospheric oxygen[10]. Subsequently, 99 µl of 1% porcine erythrocyte suspension in 10 mM PB was added to each well. The reference value for 50% lysis was obtained by mixing 50 µl of 1% erythrocyte suspension with 150 µl of diluting buffer. Controls included 200 µl of diluting buffer and 100 µl of diluting buffer mixed with 100 µl of 1% erythrocyte suspension, and 100 µl of deionized water mixed with 100 µl of 1% erythrocyte suspension. After the wells were sealed, the plates were incubated on a Coulter mixer for 30min at 37°C.The density of the remaining cells was measured in a vertical spectrophotometer at 620 nm. A hemolytic unit (HU) was defined as the amount of active hemolysin present in the dilution that resulted in lysis of 50% of the erythrocytes[18]. HU was calculated in the method of two-point interpolation, using the formula Titer (HU/ml) $=2^{\times}X$

$$= n + \frac{A(50) - A(n)}{A(n+1) - A(n)}$$

HSA = HU/mg

where

HU = hemolytic units

mg = total protein concentration

n = the last dilution number that resulted in lysis of less than 50%

$A(50)$ = 50% lysis reference absorbance value

$A(n)$ = absorbance value of the dilution number n

$A(n + 1)$ = absorbance value of the dilution number $n + 1$

HSA = hemolytic specific activity

The activity of a hemolysin preparation was then obtained through multiplication by the predilution factor.

LDH release assay for cytotoxicity

The RBMVECs monolayer model for cytotoxicity was exposed to purified suilysin by monitoring the release of LDH. The LDH activity in supernatants of RBMVECs was tested using the CytoTox 96 kit (Promega, U.S.A.), according to the manufacturer's instructions.

The confluent RBMVECs monolayers in 96-well plates and empty wells (containing 10 µl of 0.8% Triton X-100) were treated with 100 µl of the suilysin preparation in different concentrations (2, 4, 8, 16, 32, 64 µg/ml) and incubated for 2 h, 4 h, 8 h, 12 h or 24 h (37°C, 5% CO_2). Untreated cells, and suilysin in DMEM medium without cells were used as negative controls, whereas cells exposed to 0.8% Triton X-100 solution (added to the cells 45 min before termination of the incubation) served as a positive control (100% toxicity) After incubation, the plates were centrifuged at 250g for 4 min, 50 µl supernatants of the samples were transferred into an enzymatic assay plate. 50 µl of the reconstituted substrate mix was added to each well of the plate, and incubated at room temperature for 30 minutes, protected from light. Finally, 50 µl stop solution was added to each well, and the absorbance was measured at 492 nm (Thermo Multiskan Ascent, U.S.A).

Stimulation of cells

Subcultured RBMVECs monolayers in 48-well plates were used for stimulation assays. At confluence, 250 µl of the suilysin preparation (32 µg/ml in DMEM) was added in to replace the original medium. Cells incubated in medium alone served as controls for spontaneous cytokine

release. Plates were incubated at 37°C, 5% CO_2 in a humid atmosphere. At different time intervals (2h,4h,8h,12h,24h), culture supernatants were harvested from individual wells, and the supernatants aliquoted and frozen at -20 °C until cytokine determinations were performed. At the same time, measurement of cytokines release by RBMVECs induced with different concentration of suilysin was also carried out. The original culture medium was replaced with different concentrations (2, 4, 8, 16, 32, and 64 µg/ml) of suilysin, the blank control was as same as above. After incubated for 8h, culture supernatants were harvested and frozen for the cytokine determinations subsequently. Each test of RBMVECs stimulation was repeated at least three times.

Enzyme-linked immunosorbent assays (ELISA) for IL-6 release

IL-6 was measured by ELISA using rat-specific and pair-matched antibodies from R&D systems, U.S.A., according to the manufacturer's instructions. Twofold dilutions of recombinant rat IL-6 (4000-62.5 pg/ml) was used to generate standard curves. Standard and sample dilutions were added in duplicate wells to each ELISA plate, and all analysis were performed at least three times for each individual stimulation assay. 50 µl of standard, control, or sample were added to each well after 50 µl of assay diluent was added. The plate was covered with adhesive strip provided and incubated for 2h at room temperature. The solution in each well was aspirated and the wells were washed for 5 times with wash buffer. After the last wash, any remaining wash buffer must be removed for the accuracy of the results. 100 µl of IL-6 conjugate was added to each well, and then the plate were covered and incubated for 2h at room temperature. After washing each well for 5 times again, 100 µl of substrate solution was added to each well and then the plate was kept from light for incubation for 30min.The absorbance was measured at 450 nm (with wavelength correction of 540nm) following by 100 µl of stop solution was added. Results were derived from linear regression calculations and expressed in pg/ml of cytokine.

Statistical analysis

Statistical analysis was performed using the unpaired Student's t-test with $P \leq 0.05$ considered significant. The SPSS 18.0 software was used for calculations. Results were obtained as means ±S.E.M ($n = 3$ trials) in triplicates per group per trial, with the exception of the CE quantitation ($n = 1$).

RESULTS

Purificatin of suilysin and the measurement of hemolytic activity

After precipitation with 45% ammonium sulfate, the samples were subjected to SDS-PAGE. Three protein bands were observed following SDS-PAGE (Fig.1). The suilysin required purifying was located within these bands. The product was considered as crude purified suilysin and marked as E1.The protein concentration and the hemolytic activity of E1 were measured, and the hemolytic specific activity was calculated.

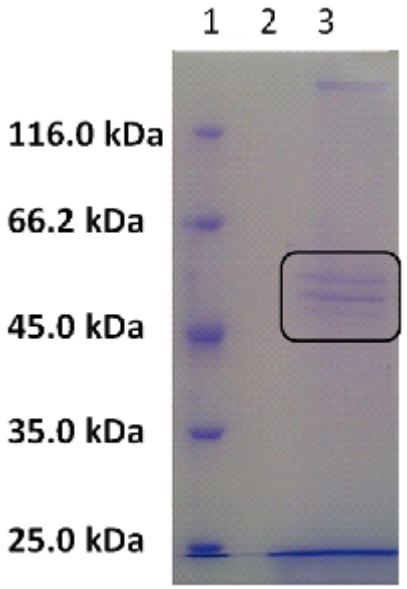

Fig. 1. SDS-PAGE of crude purified suilysin produced by *S. suis* serotype 2 strain CVCC606 using fractional precipitation with 45% of ammonium sulfate. Low-molecular-mass markers (Lane 1, sizes shown on the left); Blank lane(Lane 2); Crude purified suilysin (Lane 3, three protein bands were observed on SDS-PAGE in the position of the frame). The gel was stained with Coomassie brilliant blue.

The sample E1 was subjected to molecular sieve chromatography, fractions were collected and analyzed by SDS-PAGE, the result of gel electrophoresis showed that a single protein band at about 54kDa displayed from fractions 3 to 30

(Fig. 2A-2D). Most contaminants, including molecules with apparent molecular masses much smaller than 54kDa, eluted in fractions 18 to 40 (Fig. 2C-2E). The calculation of the hemolytic specific activity of each fraction showed that two peak of hemolytic specific activity eluted in fractions from 14 to 16, and from 20 to 21, interestingly, a valley of hemolytic specific activity was observed between two peaks from17 to 19 (Fig. 3A). In addition, a time-dependent total protein concentration of the fractions was observed (Fig. 3B). Based on the results of SDS-PAGE and the hemolytic specific activity, eluted fraction 21 was recognized as the purified suilysin. The pattern of SDS-PAGE indicated that a single protein band at about 54kDa represented the purified suilysin (Fig. 4).

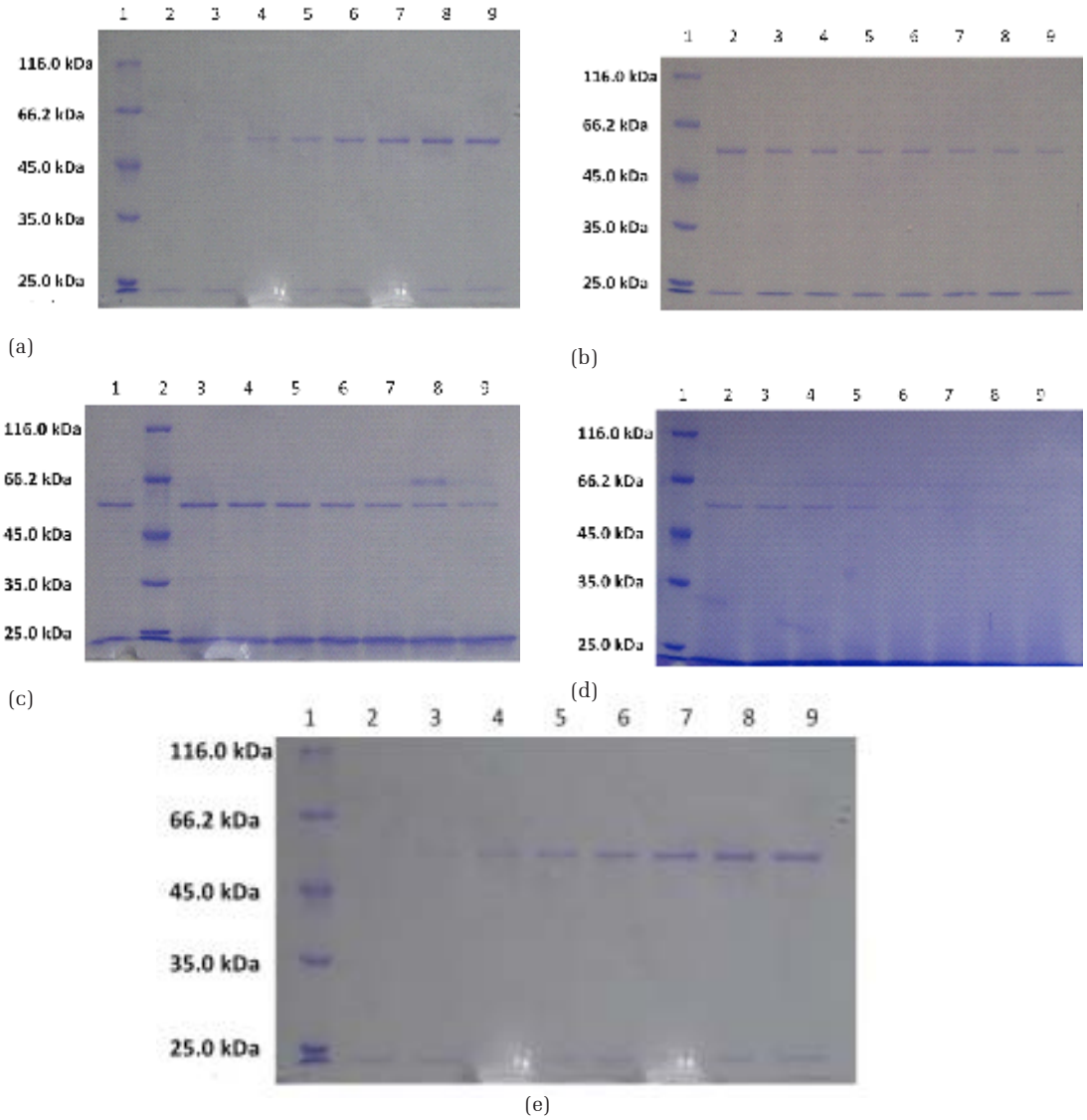

Fig. 2. SDS-PAGE of fractions eluted from Sephadex G-100 column chromatography. Low-molecular-mass markers (Lane 1 of A, B and D, Lane 2 of C and E, sizes shown on the left); Fractions 1-8(Lane 2-9 of A); Fractions 9-16(Lane 2-9 of B); Fractions 17-24(Lane 1 and Lane 3-9 of C); Fractions 25-32(Lane 2-9 of D); Fractions 33-40(Lane 1 and Lane 3-9 of E). A single protein band at about 54kDa displayed from fractions 3 to 30. Contaminants, whose molecular masses are much smaller than 54kDa, were eluted in fractions 18 to 40. The gel was stained with Coomassie brilliant blue

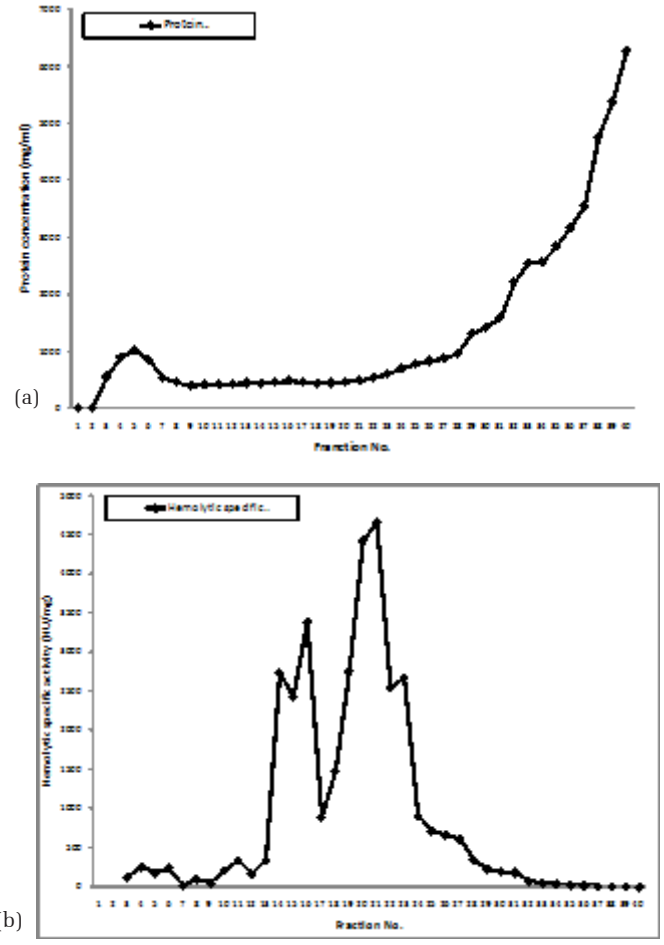

Fig. 3. Hemolytic specific activity of the fractions eluted from Sephadex G-100 column chromatography. Two peak of hemolytic specific activity eluted in fractions from 14 to 16, and from 20 to 21(A); interestingly, a valley of hemolytic specific activity was observed between the two peaks from17 to 19(A). A time-dependent protein concentration of the fractions was observed (B)

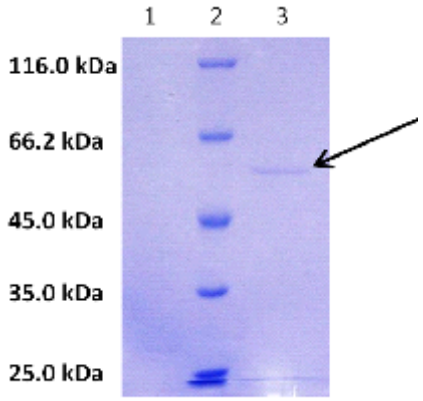

Fig. 4. SDS-PAGE of purified suilysin produced by *S. suis* serotype 2 strain CVCC606. Blank lane(Lane 1); Low-molecular-mass markers (Lane 2, sizes shown on the left); Purified suilysin (Lane 3, position that the arrow point to). The gel was stained with Coomassie brilliant blue

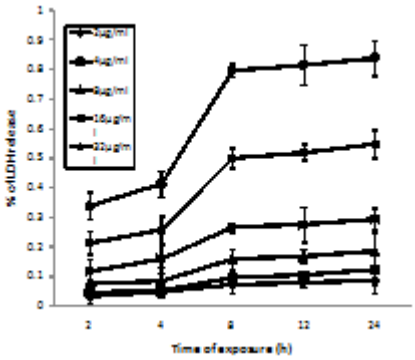

Fig. 5. Assessments of suilysin-induced LDH release by RBMVECs-. Exposure of RBMVECs to suilysin increased lactate dehydrogenase (LDH) release from the cells into the medium. A dose- and time-dependent cytotoxicity of suilysin to RBMVECs was observed. All data are expressed as means ± SD of three independent experiments

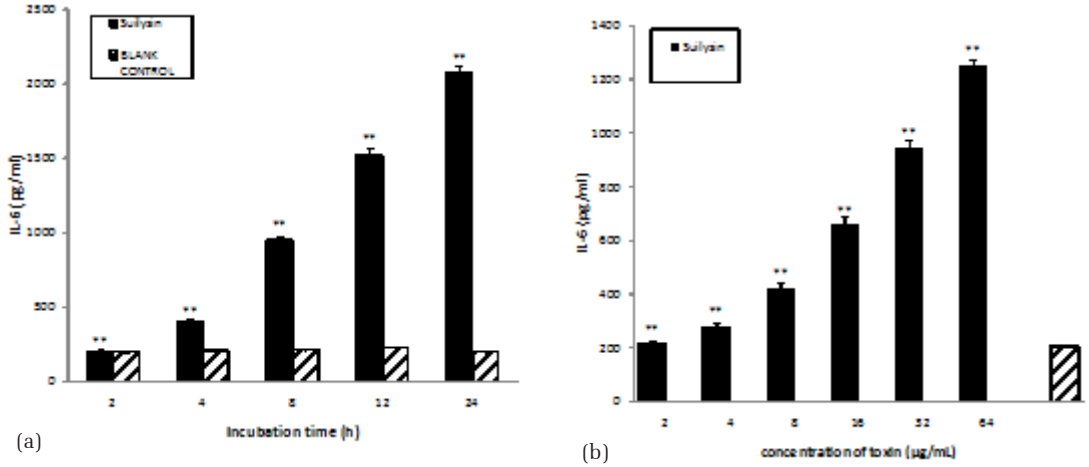

Fig. 6. Measurement of IL-6 release by RBMVECs with suilysin.. A time-dependent of IL-6 release by RBMVECs induced with suilysin was observed (A), suilysin showed a very significantly higher ability to induce the expression of IL-6 compared with spontaneous at each time point **($P < 0.01$); A dose-dependent of IL-6 release by RBMVECs induced with suilysin was observed, The level of IL-6 release induced with suilysin was very significantly higher than blank control group in all concentration (B) **($P < 0.01$). Data represent the means ± SD (in pg/ml)

Culture supernatants of *S. suis* serotype 2 strain CVCC606, product of fractional precipitation with 45% of ammonium sulfate, and suilysin obtained by means of molecular sieve chromatography were collected respectively. The parameters (such as volume, protein concentration, hemolytic activity, and so on) of these samples were measured and calculated, the results listed in Table 1. Results indicated that values of hemolytic specific activity of crude suilysin (E1) and purified suilysin (E2) were all significantly higher compared with the culture supernatants ($P < 0.01$). Values of E2 were significantly higher than that of E1 ($P < 0.01$), but the hemolytic activity of E2 was significantly lower than that of E1 ($P < 0.01$). The relatively low coefficient of recovery, and loss of a large amount of suilysin during molecular sieve chromatography might be responsible for the significant difference.

Cytotoxicity of suilysin to RBMVECs

To examine the cytotoxicity of suilysin, RBMVECs were exposed to different concentrations of suilysin solution for different time intervals and the levels of LDH in supernatants of RBMVECs were measured. A dose- and time-dependent cell lysis caused by suilysin was observed (Fig. 5). The cell lysis caused by 64, 32, 16, 8, and 4 μg/ml of suilysin reached plateau after 8h of exposure, which was significantly higher compared with 2 μg/ml of suilysin, respectively (P

Table 1. Purification of suilysin of *S. suis* serotype 2 strain CVCC606

Product name	Sample volume (ml)	Protein concentration (mg/ml)	Total protein content (mg)	Hemolytic activity (HU/ml)	Hemolytic specific activity (HU/mg)	Total hemolytic specific activity (HU*ml/mg)	Recovery rate (%)	fold of purification
Culture supernatants	20000	3.17	63400	$2^{4.29}$	6.17	391244.89	100	1
E1	50	96.76	4838	$2^{12.69b}$	68.29^{b}	330400.47	84.45	11.07
E2	5	0.48	2.42	$2^{11.14bB}$	4701.46^{bB}	11283.51	2.88	761.99

Note: $^{a}P < 0.05$,$^{A}P < 0.01$, vs. supernatants group; $^{b}P<0.05$,$^{B}P < 0.01$, vs. fractional precipitation group.

< 0.01). Different developments of the curves of cytotoxicity percentage were observed in different time intervals. Before 4h, the cytotoxicity of suilysin increased slowly, but the percentage of cytotoxicity entered log phase from 4h to 8h, and at 8h-24h, the growth rate of cytotoxicity markedly slowed down and reached a plateau (Fig. 5). At 8h, the percentage of cytotoxicity at 64, 32, 16, and 8 µg/ml of suilysin was significantly higher compared with 2 µg/ml, respectively (P < 0.01), but at 4h, only 64 µg/ml was still significantly higher compared with 2 µg/ml of suilysin (P < 0.01).

Suilysin induce high levels of IL-6 production

The stimulation of RBMVECs with suilysin in time- dependent made the IL-6 release reach a plateau after 24h of exposure (Fig 6A). Compared with spontaneous cytokine release at the same time point, suilysin showed significantly higher ability to induce the expression of IL-6 by RBMVECs (P < 0.01). The effect of suilysin doses on cytokine production was also measured, the results showed that cytokine induction varied directly with toxin doses, and a dose-dependent relationship was observed, which reached a plateau at 64 µg/ml (Fig 6B). The level of IL-6 release by RBMVECs induced with suilysin was significantly higher than spontaneous (P < 0.01) in each dose.

DISCUSSION

Suilysin, produced and secreted extracellularly by *S. suis*, which is only presented in culture supernatants. According to these features, we purified the suilysin from the culture supernatant by using different filtration steps, including selective ammonium sulfate precipitation and Sephadex G-100 chromatography. During the procedure of purification, culture of *S. suis*, products of ammonium sulfate precipitation, and fractions that eluted from chromatography were all collected and analyzed by SDS-PAGE, then the parameters (such as volume, protein concentration, hemolytic activity, and so on) of these collections were measured and calculated, respectively. The result indicated that a protein with molecular mass of 54kDa was the purified suilysin that secreted by *S. suis* serotype 2 strain CVCC606, which exhibited a specific activity of 4.7×10^4 HU/mg.

Currently, two kinds of the molecular mass of suilysin,54kDa[7] and 65kDa [8], have been reported

respectively. The diversity of the molecular mass of suilysin ,may be responsible for the purification method and be related to the different characteristic of the suilysin produced by different strain of *S. suis*. In this study, a simple two-step method was used and resulted in a high fold of purification (761.99).

Reports indicated that suilysin belongs to a family of cholesterol-binding cytolytic toxins with a multi-hit mechanism of action[8]. This family includes several hemolysins, such as pneumolysin, perfringolysin, listeriolysin O, and steptolysin O, which produced by *Streptococcus pneumoniae* , *Clostridium perfringens*, *Listeria monocytogenes*, and *Streptococcus pyogenes*, respectively [20]. These hemolysins have been involved in the pathogenesis of the diseases caused by each pathogen [21-24]. Besides serotype 2, Serotypes 1, 1/2, 4, 5, 14, 15, 17, 19, and 20 of *S. suis* also represented hemolytic activity [25].The hemolytic activity of suilysin was lost upon oxidation but restored after addition of reducing agents[7]. *In vitro* studies showed that suilysin also exhibits toxicity to various cells, including macrophages[15,26], epithelial cells[10,14], and BMVECs[7]. The cells mentioned above could be adhered or even lysed by SLY-positive strain of *S. suis*, similar to purified suilysin[14] or supernatants of SLY-positive strain of *S. suis*[10,15,27], which indicated that suilysin might be involved in the pathogenicity of *S. suis*.

The pathogenesis of *S. suis* infection is not fully cleared, especially the meningitis caused by *S. suis*. As known to us, BM is an inflammatory disease of the central nervous system (CNS) which occurs when bacteria gain entry to the subarachnoid space [27]. In order to reach the CNS and cause meningitis, circulating *S. suis* has to cross the BBB, and this is the key step in the pathogenesis of meningitis[8]. The BBB is responsible for maintaining the homeostasis within the CNS and is characterized by tight intercellular junctions that regulate the movement of cells, solutes, and macromolecules across the BBB [28]. As an important component of the BBB, BMVECs play a key role in many of the functions mentioned above. Reports showed that pneumolysin and beta-hemolysin, secreted by *streptococcus pneumonia* and *Group B Streptococci*, respectively, aslo could induce cell injury of BMVECs [16,29], and that damage to BMVECs from the effects of the hemolysin mentioned above could contribute to increasing the permeability of the BBB, and it is worth noting that pneumolysin was a toxin with strong sequence homology to suilysin[7].

It may be hypothesized that, after adherence of *S. suis* to BMVECs, toxic factors were secreted by bacteria could affecting the endothelial cells and lead to cell lysis, which could increase the BBB permeability and cause the development of cerebral oedema, increase intracranial pressure and cause cerebral blood flow blockage ,which are all the characteristics of bacterial meningitis[14]. Reports confirmed this assumptions, unlike other pathogens, *S. suis* serotype 2 only adhered to but not invaded Human BMVECs (HBMVECs), and it was shown that damage to BMVECs by suilysin of *S. suis* serotype 2 contributed to increased BBB permeability[16], eventually leading to the breakdown of BBB and meningitis by *S. suis*. One of the factors that cause cell injury is cytotoxicity of some pathogens or toxins, and the cell injury of BMVECs could lead to BBB permeability. In this study, the cytotoxicity of suilysin secreted by *S. suis* serotype 2 strain CVCC606 to RBMVECs were measured using LDH release assay, which is an accepted method to evaluate the viability, growth state, and cellular injury, especially used to test the integrity of cells, and evaluate invasion and lysis[31]. LDH was a marker enzyme presented in cytoplasm with stable chemical and biological properties. Generally, the intracellular leakage of LDH is rare, and LDH is released in case of increased permeability making damage to the cell membrane. Therefore, the release of LDH is an important enzymatic indicator of cellular injury. In this study, a dose- and time-dependent cytotoxicity of suilysin to RBMVECs was observed after determination of suilysin-induced LDH release. These data suggested that suilysin caused cell lysis, leading to LDH release, and dose- and time-dependent damage to RBMVECs. BMVECs was an important component of BBB. Since the suilysin synthesized by the bacteria triggered meningitis in SD rats (data unpublished), suilysin-induced BBB permeability might be an important pathogenic factor.

In addition to cytotoxicity to BMVECs, up-regulated expression of pro-inflammatory mediators and leukocyte trafficking may also contribute to increasing BBB permeability[32], and

inflammation to CNS seems to play a critical role in the pathogenesis of *S. suis* infection. IL-6, a multifunctional cytokine, which displays several pro-inflammatory properties, such as regulation of inflammation including the induction of the acute phase reaction, immune response and cellular differentiation, and so on[13]. It has been shown that several toxins can stimulate or modulate the inflammatory mediator cascade[33]. Interestingly, cholesterol-binding cytolysins, such as listeriolysin, pneumolysin, and streptolysin O, were recognized to stimulate inflammatory response[31]. In the present study of cytokine release assay, IL-6 production was strongly induced with suilysin by RBMVECs in a dose- and time-dependent manner, which was significantly higher than spontaneous cytokine release in each time point ($P < 0.01$) (Fig.6A). This result indicated that suilysin was contribute to the cytokine overproduction by endothelial cells after stimulation with S. *suis*, and suilysin might be involved in inflammation after adherence of *S. suis* to BMVECs, which might be result in the increase of the BBB permeability.

CONCLUSIONS

In summary, we successful obtained suilysin secreted by *S. suis* serotype 2 strain CVCC606 using a simple two-step method of purification, and assayed the cytotoxicity of suilysin to RBMVECs. Our findings indicate that suilysin induce cellular injury and pro-inflammatory cytokine response in RBMVECs, which lead to the increase of the BBB permeability. Therefore, suilysin may be involved in (or even play a key role in) the pathogenesis of meningitis caused by *S. suis*.

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