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Identification, Purification, and Characterization of a Thiol-Activated Hemolysin (Suilysin) of *Streptococcus suis*

ANTON A. C. JACOBS,* PETER L. W. LOEFFEN, ANTON J. G. VAN DEN BERG, AND PAUL K. STORM

Bacteriological Research Department, Intervet International B.V., 5830 AA Boxmeer, The Netherlands

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The present report describes the identification, purification, and characterization of a hemolysin produced by *Streptococcus suis* type 2. The hemolysin was purified from the culture supernatant by using different filtration steps, Superose-12 column chromatography, and selective $(NH_4)_2SO_4$ precipitation. The purified hemolysin, designated suilysin, had an apparent molecular mass of 54,000 Da and exhibited a specific activity of 0.7×10^6 hemolytic units per mg. Suilysin appeared to belong to a family of toxins known as the thiol-activated toxins, with which it had several characteristics in common: loss of activity upon oxidation, reactivation upon reduction, and inhibition of activity by small amounts of cholesterol. The N-terminal amino acid sequence of suilysin showed many similarities with parts of the deduced N-terminal amino acid sequences of perfringolysin O, streptolysin O, listeriolysin O, alveolysin, and pneumolysin. Mice immunized with a vaccine containing purified suilysin appeared to be completely protected against a lethal *S. suis* type 2 challenge, indicating that suilysin is an important factor and that the neutralization of this single factor is sufficient to protect mice against the detrimental effects of an *S. suis* type 2 infection. Most of the different (serotype) strains appeared to secrete hemolytic activity which was biochemically and immunologically indistinguishable from suilysin into the culture supernatant in vitro, indicating that suilysin might be a cross-protection factor.

Streptococcus suis has been identified as a major cause of contagious disease in pigs. The disease is characterized by arthritis, septicemia, meningitis, pericarditis, endocarditis, polyserositis, and/or pneumonia (4, 5, 15, 28, 32). Occasionally, the organism is also associated with disease in other animal species and humans (1, 5, 12, 18).

So far, 29 capsular types of *S. suis* have been identified, and most of them have been associated with various pathological conditions (10, 12, 14). Except in the Scandinavian countries and Australia, where types 7 and 9, respectively, are the most prevalent, type 2 is the most frequently isolated from diseased pigs worldwide (2, 9).

Little is known about the pathogenesis, virulence factors, or protective antigens of *S. suis*. Whole-cell vaccines seem to induce significant protection in pigs against homologous challenge (16). However, the protection induced by whole-cell vaccines is probably type specific (20), implying that such vaccines should contain many serotypes if broad protection is desired.

Potential virulence factors, i.e., hemagglutinins and fimbriae, have been described, but their function in pathogenesis is not known and the respective molecules or proteins were not identified (13, 19). Gottschalk et al. (11) proposed that a 44-kDa protein which was absent in a totally avirulent mutant strain could act as a virulence factor. Furthermore, Vecht et al. (29, 30) described two proteins, a 110-kDa extracellular protein termed EF and a 136-kDa cell wall-associated protein termed MRP. Both proteins appeared to be present in pathogenic type 2 strains, the 110-kDa protein appeared to be absent in strains of low pathogenicity, and both proteins appeared to be absent in apathogenic strains and therefore were thought to be virulence factors. In addition, Holt et al. (17) found that rabbit serum directed against a 94-kDa protein protected mice against challenge with a pathogenic strain of *S. suis* type 2. However, in all three studies, the function in pathogenesis of the respective proteins remained unclear, and the proteins were only shown to be present in type 2 strains.

Hemolysins have often been implicated as virulence factors in various kinds of infections, e.g., those caused by clostridia, *Escherichia coli*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Pasteurella haemolytica*, and *Actinobacillus pleuropneumoniae*. The observation that many *S. suis* strains show narrow zones of hemolysis on blood agar plates led us to the supposition that a hemolysin could be involved in the pathogenesis of one or more of the different manifestations of *S. suis* infections. The present report describes the identification, purification, and characterization of a thiol-activated hemolysin, designated suilysin, of *S. suis*.

MATERIALS AND METHODS

Bacterial strains. *S. suis* type 2 strains P1/7 and 688/9 were provided by T. Alexander, University of Cambridge. Type 2 strains 4005, D282, 3921, 3977, 3889, and T15 were obtained from U. Vecht, Central Veterinary Institute, Lelystad, The Netherlands. Type 7 strains 10681, 10727, and 14391 were provided by B. Nielsen, Intervet Scandinavia, Copenhagen, Denmark. Reference strains 1 through 22 were obtained from J. Henrichsen, Statens Serum Institute, Copenhagen, Denmark. Strains NV92109 (serotype 8), 220891KM (serotype 9), and 220891GV (serotype 14) are recent field isolates from diseased pigs.

Bacterial cultures. Bacterial strains were streaked on sheep blood agar and cultured for 24 h at 37°C. For the determination of hemolysin production in batch cultures, a few colonies were inoculated into 100 ml of Todd-Hewitt broth (Difco) and cultured at 37°C until the end of the exponential growth phase (usually 5 to 6 h). Subsequently, the cells were removed by

* Corresponding author. Mailing address: Intervet International B.V., P.O. Box 31, NL-5830 AA Boxmeer, The Netherlands. Phone: (31)8855-87586. Fax: (31)8855-87490.

centrifugation at 10,000 × *g* for 10 min, and the supernatant was stored at −20°C until use.

Titration of hemolytic activity. Serial twofold dilutions (150 μl) of test samples were prepared in polystyrene deep-well titer plates (Beckman) with 10 mM Tris-buffered saline (pH 7.4) as the diluent. Subsequently, 150 μl of a 2% (washed) horse erythrocyte suspension in 10 mM Tris-buffered saline was added to each well. After the wells were sealed, the plates were incubated on a Coulter mixer for 2 h at 37°C. After unlysed erythrocytes were allowed to pellet overnight at 4°C, 150-μl portions of the supernatant fluids were transferred to a polystyrene flat-bottomed microtiter plate and measured at 540 nm with a microELISA (enzyme-linked immunosorbent assay) reader (Titertek Multiskan Plus type 314; Flow Laboratories). The titer was defined as the reciprocal of the highest dilution of hemolysin inducing at least 50% lysis of erythrocytes.

Hemolysin inhibition test. For determination of the hemolysin inhibition titer of sera, serial twofold dilutions (75 μl) of test sera were prepared in polystyrene deep-well titer plates (Beckman) with 10 mM Tris-buffered saline (pH 7.4) as the diluent. Subsequently, 75 μl of a hemolysin solution containing 2^5 hemolytic units was added to each well. After incubation at 20°C for 10 min, 150 μl of a 2% (washed) horse erythrocyte suspension was added to each well, and the test was completed as described above for the titration of hemolytic activity. The titer was defined as the reciprocal of the highest dilution resulting in at least 50% inhibition of hemolysis.

The capacity of specific pig antiserum P399 against purified suilysin (derived from *S. suis* type 2) to inhibit hemolytic activity produced by different (serotype) strains was tested in a single well, with 75 μl of 1:128 diluted serum P399 and 75 μl of undiluted culture supernatants of the different strains. The test was then completed as described above. Preimmune serum, also diluted 1:128, was used as the control (maximal hemolysis). Cross-neutralization was apparent if serum P399 inhibited hemolysis by more than 50% compared with inhibition by the preimmune serum. Samples with a hemolysin titer of $<2^4$ give no detectable result in this test because the maximal hemolysis (preimmune serum) does not reach two times the background (serum P399).

Purification of hemolysin. A 14-liter Biostat E fermentor filled with 12 liters of Todd-Hewitt broth was inoculated with 250 ml of an overnight culture of strain P1/7 in the same medium. After anaerobic growth for 6 h at 37°C and pH 7.4, the culture was cooled to 4°C, and all subsequent steps were carried out at 4°C. The cells were removed by continuous-flow centrifugation. The supernatant was passed through a ceramic 0.8-μm-pore-size filter (Millipore) to remove most of the remaining cells and then concentrated to 150 ml on 10,000-nominal-molecular-weight-limit filters (PTCG; Minitan; Millipore). Subsequently, the preparation was sterilized by filtration through 0.2-μm-pore-size filters (Falcon), and 1-ml portions were applied to a Superose-12 gel filtration column (FPLC; Pharmacia) and eluted in 40 mM phosphate-buffered saline (pH 7.2) supplemented with 0.5 M NaCl. Fractions (0.5 ml) were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Hemolytic activity was tested in the hemolysin assay after the addition of 0.1% β-mercaptoethanol. Hemolytic fractions (no. 35 to 45) were pooled and selectively precipitated with 50% $(NH_4)_2SO_4$ for 3 h. After centrifugation at 10,000 × *g* for 20 min, the pellet was resuspended in 20 ml of 40 mM phosphate-buffered saline (pH 7.2). Column fractions 19 to 31, containing most of the other extracellularly produced antigens, were also pooled, precipitated with 60% $(NH_4)_2SO_4$, centrifuged, and resuspended in 20 ml of 40 mM phosphate-buffered saline (pH 7.2). Both preparations were stored at −20°C until use.

Hemolytic activity after different treatments. For all treatments, 0.5-ml portions of purified hemolysin with a titer of 2^7 in 40 mM phosphate-buffered saline (pH 7.2) were used.

The effect of different temperatures was measured in the hemolysin assay after incubation of the hemolysin at −20, 4, 20, 37, and 100°C.

To test the effect of proteinase K treatment, 5 μl of a concentrated enzyme solution (2 mg/ml) was added to 0.5 ml of hemolysin solution and incubated for 10 min at 20°C. Subsequently, any residual hemolytic activity was measured in the hemolysin assay.

To test the effect of reduction by β-mercaptoethanol, 5 μl of a 10% (vol/vol) β-mercaptoethanol solution was added to 0.5 ml of hemolysin solution and incubated for 10 min at 20°C, after which the activity was measured in the hemolysin assay.

The effect of alkylation of any thiol groups with TLCK (*N*-α-*p*-tosyl-L-lysine chloromethylketone; Sigma) was determined by the addition of 5 μl of a 10% (wt/vol) TLCK solution to 0.5 ml of hemolysin solution followed by incubation at 20°C for 10 min. Subsequently, any residual activity was measured in the hemolysin assay.

To test the effect of oxidation by H_2O_2, 5 μl of a 10% H_2O_2 solution was added to 0.5 ml of hemolysin solution and incubated at 20°C for 10 min. Subsequently, any residual activity was measured in the hemolysin assay.

The effect of cholesterol was tested by the addition of 10 μl of 5% cholesterol (in 10% ethanol) to 0.5 ml of hemolysin solution followed by incubation at 20°C for 10 min, after which any residual activity was measured in the hemolysin assay.

The reversibility of some treatments by reduction was tested by the addition of excess β-mercaptoethanol (2% final concentration) to part of the reaction mixtures, followed by incubation at 20°C for 10 min and titration of hemolytic activity.

Control tests were carried out with duplicate samples from which the respective reagents were left out. Furthermore, any effect of the reagents on erythrocytes in the hemolysin assay was tested by omitting hemolysin from the reaction mixtures.

Susceptibility of erythrocytes from different species. The susceptibility of erythrocytes from different species was tested with a reduced (0.1% β-mercaptoethanol) preparation of purified hemolysin which had a titer of 2^8. The hemolysin test was carried out as described above except that horse erythrocytes were replaced by erythrocytes from human, bovine, turkey, pigeon, mouse, chicken, ovine, guinea pig, rabbit, cat, dog, and pig sources.

Vaccines. Three vaccines based on purified hemolysin, concentrated culture supernatant, or pooled Superose-12 column fractions 19 to 31 and a placebo vaccine were prepared as follows.

Purified hemolysin (40 μg of protein per ml) was mixed 1:1 with Diluvac Forte adjuvant until a homogeneous formulation was obtained. This vaccine was termed VAC-SLY.

For the vaccine containing concentrated culture supernatant, part of the PTCG concentrate (1.9 mg of protein per ml) was mixed 1:1 with Diluvac Forte adjuvant until a homogeneous formulation was obtained. This vaccine was termed VAC-CCS.

The third vaccine was prepared by mixing pooled and concentrated Superose-12 column fractions 19 to 31 (containing 2 mg of protein per ml) 1:1 with Diluvac Forte adjuvant until a homogeneous formulation was obtained. Column fractions 19 to 31 contained most of the extracellularly produced proteins of *S. suis* P1/7 but were essentially free of hemolysin. This vaccine was termed VAC-SCF.

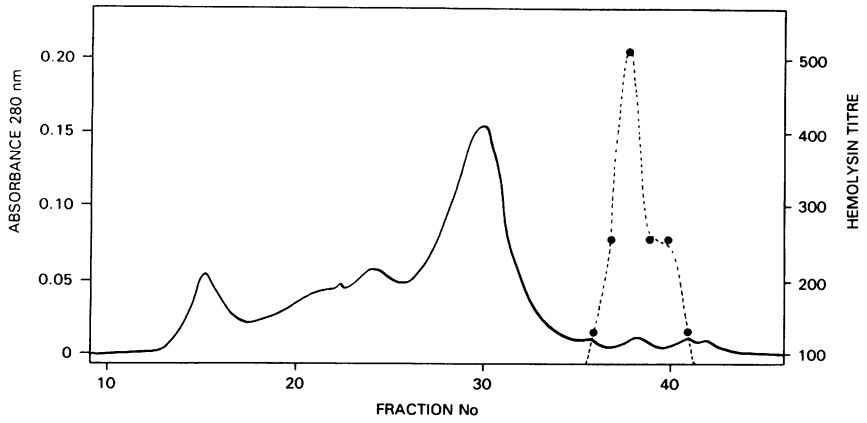

FIG. 1. Elution profile of Superose-12 chromatography of concentrated culture supernatant of *S. suis* type 2 strain P1/7. Solid line, A_{280}; dashed line, hemolysin reciprocal titer.

Placebo vaccine was prepared as described above except that the antigen solution was replaced by 40 mM phosphate-buffered saline (pH 7.2).

Antisera. Polyclonal rabbit serum R2089 to the crude culture supernatant of strain P1/7 was obtained as follows. A New Zealand White rabbit (3 kg) was immunized subcutaneously with 1 ml of vaccine VAC-CCS. Four weeks after priming, the rabbit was given a booster by using the same (amount of) vaccine and vaccination route. Two weeks after the booster, the rabbit was bled, and the serum was stored at −20°C until use.

Specific polyclonal mouse serum M189 to purified hemolysin was obtained as follows. Ten 4-week-old BALB/c mice (Iffa Credo) were immunized subcutaneously with 0.5 ml of vaccine VAC-SLY. Two weeks after priming, the mice were given a booster by using the same (amount of) vaccine and vaccination route. Two weeks after the booster, the mice were bled, and the sera were pooled and stored at −20°C until use.

Specific polyclonal pig serum P399 to purified hemolysin was obtained as follows. A 4-week-old pig was immunized intramuscularly (neck) with 2 ml of vaccine VAC-SLY. Two weeks after the priming, the pig was given a booster by using the same (amount of) vaccine and vaccination route. Two weeks after the booster, the pig was bled, and the serum was stored at −20°C until use.

Mouse protection test. Four-week-old BALB/c mice were divided into four groups and vaccinated subcutaneously with 0.5 ml of VAC-CCS, VAC-SCF, VAC-SLY, or placebo vaccine. Two weeks after priming, the mice were given a booster by using the same vaccine and vaccination route. Two weeks after the booster, the mice were challenged intraperitoneally (0.5 ml) with a 6-h culture of strain P1/7 in Todd-Hewitt broth containing 4×10^9 CFU/ml. After challenge, mortality was recorded for 7 days.

Protein determination. Protein concentrations were measured by the method of Lowry et al. (23) with bovine serum albumin as the standard.

SDS-PAGE and Western blotting (immunoblotting). SDS-PAGE in 9% polyacrylamide slab gels and sample preparation were done essentially as described by Laemmli (22). After electrophoresis, polypeptides were stained with Coomassie brilliant blue R250 or electroblotted onto an Immobilon polyvinylidene difluoride membrane. Blots were probed with polyclonal rabbit serum R2089 or polyclonal mouse serum M189 (see above). After washing, bound antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin or goat anti-mouse immunoglobulin, respectively, and diamino-benzidine as the substrate.

N-terminal amino acid sequence determination. The first 16 amino acids of purified hemolysin were determined by Eurosequence B.V., Groningen, The Netherlands, by sequence analysis on an automated sequenator (model 477A; Applied Biosystems) equipped with an on-line high-pressure liquid chromatograph (HPLC; model 120A; ABI) in accordance with the instructions of the manufacturer.

RESULTS

Identification of hemolytic activity. After aerobic growth for 24 h at 37°C on sheep blood agar, many but not all *S. suis* strains produced narrow zones of complete or partial hemolysis. Hemolysis was slightly enhanced after anaerobic growth. Compared with other hemolytic streptococci and many other hemolytic bacteria, *S. suis* appears to be poorly hemolytic on blood agar plates. However, when tested in the hemolysin assay as described in Materials and Methods, culture supernatants of many but not all strains appeared to induce complete hemolysis, with titers of up to 2^7. Furthermore, hemolytic activity in the culture supernatants appeared to be heat labile and susceptible to proteinase K treatment. These findings indicated the presence of a soluble proteinaceous hemolysin in the culture supernatants.

Purification of hemolysin. Strain P1/7 was grown for 6 h in 12 liters of Todd-Hewitt broth, after which the cells were removed by continuous-flow centrifugation. The culture supernatant was passed through a 0.8-μm-pore-size filter and then concentrated to 150 ml on 10,000-nominal-molecular-weight-limit filters. Subsequently, 1.0-ml portions were applied to a Superose-12 gel filtration column (FPLC; Pharmacia) and eluted in 40 mM phosphate-buffered saline (pH 7.2) containing 0.5 M NaCl. Fractions (0.5 ml) were collected and analyzed by SDS-PAGE, immunoblotting, and the hemolysin test. Peak hemolytic activity eluted in fractions 35 to 45 (Fig. 1). Analysis of the different column fractions by SDS-PAGE and immunoblotting showed that the hemolytic activity had comigrated with a single antigen at about 54 kDa (Fig. 2). Hemolytic fractions 35 to 45 were pooled, and minor contaminants were removed by selective precipitation with 50% $(NH_4)_2SO_4$ at 4°C for 3 h. After centrifugation, the pellet was resuspended in 20 ml of 40 mM phosphate-buffered saline (pH 7.2). This final

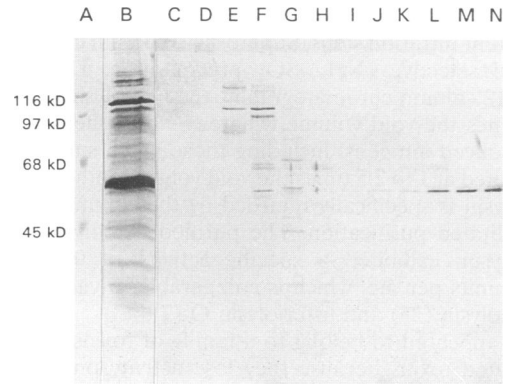

FIG. 2. Western blot of marker proteins (lane A), concentrated culture supernatant of strain P1/7 (lane B), and Superose-12 column fractions (lanes C to N): lane C, fraction 15; lane D, fraction 18; lane E, fraction 20; lane F, fraction 23; lane G, fraction 26; lane H, fraction 28; lane I, fraction 30; lane J, fraction 32; lane K, fraction 34; lane L, fraction 36; lane M, fraction 38; lane N, fraction 40. Marker proteins (lane A) were stained with Coomassie brilliant blue; sizes are shown on the left. *S. suis* antigens (lanes B to N) were probed with rabbit serum R2089 and then stained with goat anti-rabbit immunoglobulin conjugate and diamino benzidine as the substrate.

preparation appeared as a single polypeptide species at about 54 kDa in SDS-PAGE after staining with Coomassie brilliant blue (Fig. 3) and had a specific activity of about 0.7×10^6 hemolytic units per mg after reduction with β-mercaptoethanol. Most contaminants, including molecules with apparent molecular masses much smaller than 54 kDa, eluted in fractions 19 to 31 (Fig. 1 and 2). These column fractions were also pooled, precipitated with 60% $(NH_4)_2SO_4$, centrifuged, and resuspended in 20 ml of phosphate-buffered saline (pH 7.2) as described above. Both preparations were stored at −20°C until use.

Effects of different treatments on hemolytic activity. The effects of different treatments on hemolytic activity are shown in Table 1. The results show that the hemolysin is heat labile and susceptible to proteinase K digestion, oxidation by H_2O_2, and alkylation with TLCK. Furthermore, hemolytic activity is inhibited by cholesterol. Increased activity was found after incubation with β-mercaptoethanol, and addition of excess β-mercaptoethanol to the H_2O_2-oxidized preparation restored hemolytic activity. Addition of excess β-mercaptoethanol to the TLCK-treated preparation resulted in partial restoration of hemolytic activity. This partial restoration might be explained by the presence of oxidized thiol groups which had not reacted with TLCK. The effects of temperature and cholesterol could not be reversed by the addition of β-mercaptoethanol.

Duplicate samples from which the various reagents or the hemolysin was left out showed no effects of the different treatments on hemolytic activity or effects of the different reagents on erythrocytes, respectively.

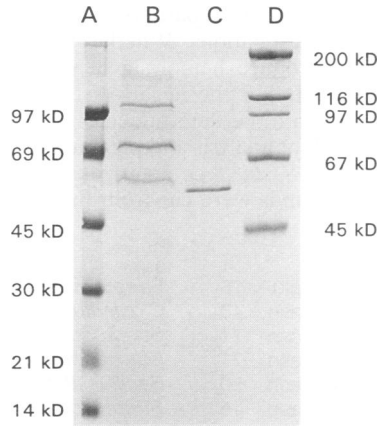

FIG. 3. SDS-PAGE of low-molecular-mass markers (lane A; sizes shown on the left), concentrated culture supernatant (lane B), purified suilysin (lane C), and high-molecular-mass markers (lane D; sizes shown on the right). The gel was stained with Coomassie brilliant blue.

Susceptibility of erythrocytes from different species. The susceptibility of human, bovine, turkey, pigeon, mouse, chicken, ovine, guinea pig, rabbit, cat, dog, and pig erythrocytes was tested with a preparation of purified hemolysin with a titer of 2^8. All of the erythrocytes appeared to be about equally susceptible to the hemolysin. The titers varied from 2^7 (mouse, cat, and turkey) to 2^{10} (human) (data not shown).

N-terminal amino acid sequence. The first 16 amino acid residues of purified suilysin were determined by automated Edman degradation (Fig. 4). Alignment of this sequence with those of perfringolysin O (27), streptolysin O (21), listeriolysin O (6, 24), alveolysin (8), and pneumolysin (31) revealed many similarities. Depending on the criteria applied, even more similarities can be found if structurally and/or functionally identical amino acids are considered. In particular, the isoleucine at position 6, leucine at position 13, and tyrosine at position 15 appear to be highly conserved. In this context, it is interesting that the valine at position 6 and methionine at position 13 in the pneumolysin sequence can be regarded as being isofunctional with isoleucine and leucine, respectively.

Prevalence of suilysin-like molecules in different *S. suis* strains. All of the strains available, including serotype strains 1 through 22, were cultured in Todd-Hewitt broth until the end of the exponential growth phase (5 to 6 h), after which the cells were removed by centrifugation. The presence of suilysin-like molecules in the culture supernatant was determined in the hemolysin assay and by immunoblotting. Many but not all strains appeared to produce various amounts of hemolytic activity in the culture supernatant (Table 2). Except for strains 4417 and 14636, a hemolytic activity titer of $>2^4$ corresponded with the presence of a 54-kDa antigen band in an immunoblot made with specific mouse serum M189. Samples with a hemolysin titer of 2^5 gave only faint 54-kDa bands in the immunoblot, and for most samples with a titer of $<2^5$, no staining was observed. Apparently, immunoblotting is not sensitive enough to detect the 54-kDa antigen in samples with lower activity.

To further characterize the hemolytic activity produced by the different (serotype) strains, all hemolytic samples were tested in the hemolysin inhibition test with specific pig serum P399 and preimmune serum. In the hemolysin inhibition test, negative pig sera appeared to have aspecific titers of 2^4 to 2^5, presumably due to inhibiting substances in pig sera, such as cholesterol. Serum P399 had a hemolysin neutralization titer of 2^9, whereas preimmune serum had a titer of 2^5. For these reasons, the hemolytic samples of the different strains were tested for inhibition with 1:128 diluted sera. The hemolytic activity of all samples with a titer of $>2^4$ and that of half of the samples with a titer of 2^4 were completely inhibited by specific pig serum P399 but not by preimmune serum. Samples with lower activity gave no results because the maximal hemolysis (preimmune serum) was not different from or did not reach two times the background value (serum P399).

Because immunoblotting and the hemolysin inhibition test appeared not to be sensitive enough to characterize hemolytic

TABLE 1. Hemolytic activity of purified suilysin after different treatments

Treatment	Treatment time and temp	Hemolysin titer (log_2)	
		No further treatment	Addition of 2% β-mercaptoethanol
Temp (°C)			
−20	7 days	7	ND[a]
4	7 days	6	ND
20	7 days	3	ND
37	7 days	0	0
100	5 min	0	0
20	10 min	7	ND
Proteinase K (20 μg/ml)	10 min, 20°C	0	ND
β-Mercaptoethanol (0.1%)	10 min, 20°C	12	ND
H_2O_2 (0.1%)	10 min, 20°C	0	12
TLCK (0.1%)	10 min, 20°C	2	6
Cholesterol (0.1%)	10 min, 20°C	0	0

[a] ND, not determined.

samples with lower activity, all hemolytic samples were also tested for inhibition by 0.1% cholesterol and 0.1% H_2O_2. All hemolytic samples, including samples with low activity, appeared to be inhibited by cholesterol and H_2O_2. Furthermore, the addition of excess β-mercaptoethanol to the H_2O_2-oxidized preparations resulted in complete restoration of hemolytic activity.

In summary, most of the strains tested appear to secrete a hemolytic activity into the culture supernatant which is immunologically and biochemically related to suilysin, and no indications of hemolysins other than suilysin were found.

Immunogenicity in mice. After challenge, 10 of 10 placebo-vaccinated mice died within 3 days, whereas 9 of 9 mice vaccinated with VAC-CCS and 10 of 10 mice vaccinated with VAC-SLY appeared to be completely protected. VAC-SCF induced only partial protection (6 of 10 mice survived). This vaccine contained most of the extracellularly produced antigens of strain P1/7 but was essentially free of hemolysin, as determined by SDS-PAGE and immunoblotting with specific mouse serum M189 (data not shown). Furthermore, in an immunoblot with the whole culture as the antigen, sera from VAC-SCF-vaccinated mice, taken on the day of challenge, reacted with several antigen bands, including a very faint 54-kDa band, whereas with sera from VAC-SLY-vaccinated mice obtained on the day of challenge, only a strong 54-kDa band was stained (data not shown).

DISCUSSION

The present report describes the purification and characterization of a hemolysin produced by *S. suis* type 2 strain P1/7. The hemolysin was purified from the culture supernatant by using different filtration steps, Superose-12 column chromatography, and selective $(NH_4)_2SO_4$ precipitation. During the Superose-12 column chromatography, the hemolysin eluted at about 3 times the void volume, whereas most other extracellularly produced antigens, including those much smaller than 54 kDa, eluted at 1 to 2.5 times the void volume, indicating that the hemolysin is specifically retarded by the column material which facilitated purification. The purified hemolysin, designated suilysin, exhibited a specific activity of 0.7×10^6 hemolytic units per mg, which is comparable to values found for pneumolysin (25) and listeriolysin O (7).

Suilysin appeared to belong to a family of toxins known as thiol-activated toxins because they lose activity on oxidation but regain full activity after addition of reducing agents (3). Thiol-activated toxins are produced by four genera of gram-positive bacteria and have an assumed common mode of action on mammalian cells. The presence of cholesterol in membranes appears to be a critical determinant of the susceptibility of cells, and free cholesterol is a potent inhibitor of cytolytic activity (3). The use of an aspecific receptor such as cholesterol was also indicated by the finding that all erythrocytes tested appeared to be about equally susceptible to suilysin. The apparent molecular mass of 54 kDa found for suilysin fits well within the range of 52 to 60 kDa described for other thiol-activated toxins (3). In addition, the N-terminal amino acid sequence of suilysin showed many similarities to parts of the deduced N-terminal amino acid sequences of perfringolysin O (27), streptolysin O (21), listeriolysin O (6, 24), alveolysin (8), and pneumolysin (31). In particular, the isoleucine at position 6, leucine at position 13, and tyrosine at position 15 in the suilysin sequence appear to be highly conserved, indicating an essential role for these residues in a common function, such as receptor recognition, lytic activity, or structural integrity. Export to the environment is not a common characteristic of all these toxins because pneumolysin is produced without a signal sequence in the cytoplasm (3).

Most of the different (serotype) strains tested appeared to secrete hemolytic activity which was biochemically and immunologically indistinguishable from suilysin into the culture supernatant in vitro, and no indications of hemolysins other than suilysin were found. Unfortunately, immunoblotting appeared not to be sensitive enough to confirm the identity of suilysin in the culture supernatants of all of the hemolysin-producing strains, especially strains with low activity. We did not test more concentrated preparations, but this could be a subject for future investigations. Whether the ability of the different strains to produce hemolysin in vitro is correlated with virulence and whether the negative strains produce hemolysin in vivo remain to be determined.

By using Western blot analysis and pig immune sera, Holt et al. (17) identified five *S. suis* type 2 antigens of 44, 54, 78, 86, and 94 kDa. These bands were cut from the gel and used to

SLY	1	asp	ser	lys	gln	asp	ile	asn	gln	tyr	phe	gln	ser	leu	thr	tyr	glu	16	*Streptococcus suis*
PFO	33	asp	lys	asn	gln	ser	ile	asp	ser	gly	ile	ser	ser	leu	ser	tyr	asn	48	*Clostridium perfringens*
SLO	56	asp	his	thr	glu	glu	ile	asn	asp	lys	ile	tyr	ser	leu	asn	tyr	asn	71	*Streptococcus pyogenes*
LLO	56	lys	his	ala	asp	glu	ile	asp	lys	tyr	ile	gln	gly	leu	asp	tyr	asn	71	*Listeria monocytogenes*
ALV	35	thr	glu	pro	asn	asp	ile	asp	met	gly	ile	ala	gly	leu	asn	tyr	asn	50	*Bacillus alveoli*
PLY	1	met	ala	asn	lys	ala	val	asn	asp	phe	ile	leu	ala	met	asn	tyr	asp	16	*Streptococcus pneumoniae*

FIG. 4. Alignment of the N-terminal amino acid sequence of suilysin (SLY) with parts of the predicted N-terminal amino acid sequences of perfringolysin O (PFO), streptolysin O (SLO), listeriolysin O (LLO), alveolysin (ALV), and pneumolysin (PLY). The sequences of PFO, SLO, LLO, and ALV are preceded by amino acid stretches which have all the features of a signal sequence (6, 8, 21, 24, 27, 31). Amino acids identical to those found in SLY are shaded.

TABLE 2. Prevalence of suilysin-like molecules in culture supernatants of different *S. suis* strains

Strain	Serotype[a]	Phenotype[b]	Hemolysin titer (log_2)	Inhibition of hemolysin by 0.1% cholesterol	Reversible inactivation by oxidation/reduction	Inhibition of hemolysin by serum P399	54-kDa protein present in immunoblot
S428	1 (R)		5	+	+	+	+
RS 2651	1/2 (R)		6	+	+	+	+
P1/7	2	MRP^+ EF^+	8	+	+	+	+
688/9	2	MRP^+ EF^+	6	+	+	+	+
4005	2	MRP^+ EF^+	5	+	+	+	+
D282	2	MRP^+ EF^+	7	+	+	+	+
R735	2 (R)	MRP^+ EF^-	5	+	+	+	+
3921	2	MRP^+ EF^-	4	+	+	NR[c]	−
3977	2	MRP^+ EF^-	2	+	+	NR	−
3889	2	MRP^- EF^-	6	+	+	+	+
T-15	2	MRP^- EF^-	6	+	+	+	+
4961	3 (R)		0	ND[d]	ND	ND	−
6407	4 (R)		4	+	+	+	−
11538	5 (R)		4	+	+	NR	−
2524	6 (R)		2	+	+	NR	−
8074	7 (R)		0	ND	ND	ND	−
10681	7		0	ND	ND	ND	−
10727	7		0	ND	ND	ND	−
14391	7		2	ND	ND	ND	−
14636	8 (R)		5	+	+	+	−
NV92109	8		3	+	+	NR	−
22083	9 (R)		0	ND	ND	ND	−
220891KM	9		3	+	+	NR	−
4417	10 (R)		2	+	+	NR	+
12814	11 (R)		2	+	+	NR	−
8830	12 (R)		0	ND	ND	ND	−
10581	13 (R)		2	+	+	NR	−
13730	14 (R)		6	+	+	+	+
220891GV	14		6	+	+	+	+
T639	15 (R)		6	+	+	+	+
2726	16 (R)		0	ND	ND	ND	−
93A	17 (R)		4	+	+	+	−
NT77	18 (R)		6	+	+	+	+
42A	19 (R)		3	+	+	+	+
865192	20 (R)		0	ND	ND	ND	−
14A	21 (R)		0	ND	ND	ND	−
88/1861	22 (R)		2	+	+	NR	−

[a] R, reference strain.
[b] For the type 2 strains, the phenotypes described by Vecht et al. (29, 30) are presented.
[c] NR, no result because hemolytic activity was too low (see text).
[d] ND, not determined.

raise antisera in rabbits. Only rabbit serum against the 94-kDa antigen appeared to confer passive protection on mice. The 54-kDa antigen described by these authors most probably was not suilysin, because the antigens were derived from the cell pellet, whereas suilysin is produced extracellularly in the culture supernatant. Furthermore, in our experiments, SDS-PAGE of whole cells or concentrated culture supernatant revealed at least three antigen bands at about 54 kDa, differing slightly in molecular mass. Moreover, in an immunoblot made with specific mouse serum M189, no bands were stained if a cell lysate was used as the antigen, whereas a single 54-kDa band was stained if the whole culture was used as the antigen (data not shown).

Vecht et al. (29, 30) described two proteins, a 110-kDa extracellular protein termed EF and a 136-kDa cell wall-associated protein termed MRP. In an intranasal challenge model with pigs preexposed to *Bordetella bronchiseptica*, *S. suis* strains expressing both proteins appeared to be pathogenic, strains lacking the 110-kDa protein appeared to be slightly pathogenic, and strains lacking both proteins appeared to be nonpathogenic. It was suggested that MRP and EF could act as virulence factors and that EF could have a role in the invasion process. In the present study, strains lacking only EF appeared to produce lower amounts of hemolysin in vitro than strains expressing EF and MRP and strains lacking both proteins. To study further the significance of MRP, EF, and hemolysin in pathogenesis, it would be interesting to compare these strains in an intravenous pig challenge model.

A vaccine containing purified suilysin appeared to be protective in mice, indicating that suilysin is an important factor and that neutralization of this single factor is sufficient to protect mice against the detrimental effects of an *S. suis* type 2 infection. In addition, pigs vaccinated with a vaccine containing purified suilysin appeared to be significantly protected against (intravenous) challenge with strain P1/7 (unpublished data). In an immunoblot with the whole culture as the antigen, sera from VAC-SLY-vaccinated mice reacted only with a 54-kDa band, whereas sera from VAC-SCF-vaccinated mice reacted with several antigen bands, including a very faint 54-kDa band. The partial protection found with VAC-SCF could be due to very low amounts of suilysin and/or to one of the other antigens present in this preparation.

Whole-cell vaccines appeared to induce significant protection in pigs against homologous challenge (reference 16 and unpublished results). However, the protection induced is probably type specific (20), implying that such vaccines should contain many serotypes if broad protection is desired. Furthermore, potential virulence factors or immunogens, e.g., a 44-kDa protein described by Gottschalk et al. (11), the 110- and 136-kDa proteins described by Vecht et al. (29, 30), and a 94-kDa protein described by Holt et al. (17), were shown to be present only in type 2 strains. In contrast, suilysin was shown to be produced by most of the strains tested and thus could be an important cross-protection factor. In this context, it is interesting that experiments with pneumolysin showed that this toxin confers limited protection on mice in a serotype-independent manner (25, 26). The possible role of suilysin as a cross-protection factor will be investigated further in an intranasal as well as intravenous pig challenge model and with different serotype challenge strains.

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