Characterization of *Streptococcus suis* capsular type 2 haemolysin

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The production of a haemolysin by *Streptococcus suis* capsular type 2 was investigated. Human group O erythrocytes were the most susceptible, followed by horse, sheep, cow and pig red blood cells, which exhibited similar susceptibilities; rabbit erythrocytes were the least susceptible. The haemolysin was produced at the end of the exponential growth phase. The toxin described in this paper was purified by affinity chromatography using a thiopropyl-Sepharose 6B column. It is an extracellular protein with a molecular mass of 65 kDa. The haemolysin belongs to the family of toxins known as antigenically related cholesterol-binding cytolytic toxins, since it shares common characteristics with other members of this family, such as sensitivity to oxygen and oxidizing agents, activation by reducing agents, inhibition by low concentrations of cholesterol, formation of transmembrane pores and a 'multi-hit' mechanism of action. In addition, anti-streptolysin antibodies inhibited the haemolytic activity caused by the *S. suis* haemolysin. Antibodies against the haemolysin could not be detected in pigs experimentally infected with a haemolytic positive strain of *S. suis* capsular type 2. To our knowledge, this is the only Lancefield group D *Streptococcus* producing a haemolysin with these characteristics. The role of this haemolysin in the pathogenesis of *S. suis* infections remains to be investigated.

Keywords: *Streptococcus suis*, haemolysin

INTRODUCTION

Streptococcus suis capsular type 2 is an important swine pathogen responsible for a variety of infections such as septicaemia, meningitis, arthritis, endocarditis and pneumonia (Higgins *et al.*, 1992). It may also be the cause of serious infections in man, with various degrees of complication (Arends & Zanen, 1988). So far, 29 capsular types have been described and capsular type 2 is more frequently isolated from diseased pigs than any other capsular type (Gottschalk *et al.*, 1989, 1991a). Pathogenesis of these infections is still unclear and different bacterial components, such as capsular polysaccharides and some cell wall proteins, have been reported as virulence factors (Vecht *et al.*, 1992; Gottschalk *et al.*, 1992). It has been shown that *S. suis* capsular type 2 isolates recovered from diseased animals possess a larger amount of capsular material than those recovered from nasal cavities or tonsils of clinically healthy animals (Gottschalk *et al.*, 1991b); in addition, studies using intra-

peritoneal chambers have shown that virulent strains possess a thicker capsule than non-virulent strains (Quessy *et al.*, 1994). Finally, a non-capsulated mutant obtained from a highly virulent strain was demonstrated to be totally avirulent (Gottschalk *et al.*, 1992).

Vecht and co-workers have recently reported that two proteins were detectable only in virulent strains of *S. suis* capsular type 2 (Vecht *et al.*, 1992, 1993). The first protein, which is known as muramidase release protein (MRP), is a cell-wall-associated protein. The second one, called extracellular factor (EF), is an extracellular protein. Three different phenotypes were recognized with these proteins: MRP⁺ EF⁺, MRP⁺ EF⁻ and MRP⁻ EF⁻. Most isolates from diseased pigs were MRP⁺ EF⁺, whereas most human isolates were MRP⁺ EF⁻ (Vecht *et al.*, 1992). However, when culture supernatants of MRP⁺ EF⁻ strains were tested by immunoblotting using specific monoclonal antibodies raised against EF, related proteins of variable size (EF*) and with a higher molecular mass were recognized (Vecht *et al.*, 1993). More recently, Quessy *et al.* (1993) described a new phenotype, MRP⁻ EF⁺.

Different streptococci groups produce haemolysins which

Abbreviations: EF, extracellular factor; HU, haemolytic units; MRP, muramidase release protein; RBC, red blood cells.

have been shown to be involved in the pathogenesis of infection. For example, Lancefield group A *S. pyogenes*, Lancefield group B streptococci, Lancefield group C streptococci, Lancefield group G streptococci and *S. pneumoniae* (Alouf, 1980; Tapsall & Phillips, 1991; Alouf & Geoffroy, 1991). *S. suis* is known to be α -haemolytic on blood agar plates; however, some authors have reported that β -haemolysis can be observed with some strains, especially when cultured on horse blood agar (Estoepongastie & Lämmler, 1993; Hommez *et al.*, 1986). Lee *et al.* (1984), who studied cross-reactivities between non-groupable streptococci and pneumococcal group 19 polysaccharides, reported that the reference strain of *S. suis* capsular type 8 produces a lysis which is different from that of *S. pneumoniae*. Jacobs *et al.* (1994) have recently reported the presence in some strains of *S. suis* of a thiol-activated haemolysin, based on its inhibition by cholesterol. The aim of this study was to characterize a haemolysin produced by *S. suis* capsular type 2.

METHODS

Bacterial strains and growth conditions. The reference strain of *S. suis* capsular type 2 (S735) was obtained from Dr J. Henrichsen, Pneumococcus Department, Statens Serum Institut, Denmark. A *S. pneumoniae* serotype 5 (Ambrose) strain, also provided by Dr J. Henrichsen, was used as a positive control. Inoculum was prepared by inoculating two to three colonies from a blood-agar plate into 5 ml Todd-Hewitt broth (THB; Difco). After 18 h incubation, 0.5 ml of this culture was used to inoculate 50 ml fresh, pre-warmed THB. Culture samples were taken at different times of incubation and haemolysin activity was evaluated as described later. The number of colony-forming units (c.f.u.) was calculated by viable cell count. Occasionally, shaking cultures were assayed. The bacterial sediment obtained after centrifugation was washed twice, sonicate five times for 30 s each (Vibra Cell; Sonics and Materials) and also tested for haemolytic activity.

Haemolysin assay. The haemolysin assay was performed by preparing twofold dilutions (from 1:2 to 1:256) of the supernatant in a solution of 0.145 M NaCl and 7 mM Na_2HPO_4 , pH 7.2. An equal amount of horse red blood cells (RBC), washed twice in the solution mentioned above, was added to 0.5 ml of each dilution (final concentration of RBC: 1.4%). Following incubation for 1 h at 37 °C, the mixtures were sedimented by centrifugation (1500 g for 10 min) and supernatants transferred to polystyrene microplates (Dynatech). Following corrections for controls, which lacked either haemolysin preparation or erythrocytes, the OD_{540} of the supernatant fluid of each dilution was read on a spectrophotometer (Titertek Multiskan, Flow Laboratories). To study the kinetics of haemolysin-induced erythrocyte lysis at different temperatures, experiments were performed essentially as described above and samples of the haemolysin-erythrocyte suspension were obtained at different time intervals.

A standard curve of haemolysis was obtained using a methodology previously described (Palmer, 1981). The reciprocal of the highest dilution of a given cell-free supernatant fluid sample that exhibited at least 50% of RBC lysis was taken as the titre, in haemolytic units (HU), of the haemolysin in that sample. Occasionally, the activity was also recorded as direct spectrophotometric readings of a 1:32 dilution of the supernatant. Values expressed in Results are the mean of at least three

independent assays. In addition to horse RBC, the activity of the haemolysin was also determined on RBC from pig, rabbit, sheep, cow and human (group O). The haemolysin test was performed as described above.

Effect of different treatments on the haemolytic activity. For the characterization of the haemolysin, supernatants from a 15 h culture were used. Culture supernatant from the haemolytic positive strain was tested for its haemolytic activity after being heated at 55 °C, 58 °C and 60 °C for 1 h, and boiled for 30 min. The effect of pH on the haemolytic activity was tested by modifying the pH of a saline solution from 3 to 11 by the addition of HCl or NaOH. The supernatant was also treated for 1 h at room temperature with 5 mM dithiothreitol (DTT; Sigma) (pH previously adjusted to 7.6); with 100 μg and 5 mg of ethanol-soluble and water-soluble cholesterol ml^{-1} respectively, and with 1 mM and 5 mM sodium periodate. The supernatant was also treated with 100 μg proteinase K ml^{-1} (Sigma), and incubated at 55 °C for 1 h. In addition, the effect of CaCl_2 and MgCl_2 (0.1 and 0.01 M), ZnCl_2 (0.1 and 0.05 mM) and EGTA (2.5 mM) on the haemolysin was studied. After incubation of the supernatant with each of these compounds for 1 h at room temperature, haemolytic activity was then evaluated as described above. Finally, the effect of oxygen on haemolytic activity was determined by bubbling oxygen gas at a flow rate of 100 ml min^{-1} through 5 ml supernatant for 90 min. For all the studies, the haemolytic activity of the treated supernatant was measured and compared with that of a non-treated supernatant which was evaluated at the same time. It has to be noted that, under the above conditions, none of the products used induced erythrocyte lysis.

The haemolysin was tested in inhibition assays using human anti-streptolysin immunoglobulins (WBAG Resources). Two-fold dilutions of the IgG were made and incubated for 1 h at 37 °C with the supernatant containing the haemolysin. The mixtures were then incubated with RBC and the haemolysis test was performed as described above. The titre was evaluated as the highest dilution which inhibited 50% of the haemolytic activity. In addition, the production of antibodies against *S. suis* haemolysin *in vivo* in infected animals was tested using sera from six pigs experimentally inoculated with the reference strain of *S. suis* capsular type 2. Six-week-old specific-pathogen-free piglets were inoculated intravenously with a suspension containing 10^8 c.f.u. All animals presented severe clinical signs and they had to be treated with a single dose of antibiotics. Animals received a second challenge with a similar number of c.f.u. one month after the first inoculation. Animals were bled once a week for six weeks (until two weeks after the second inoculation). The inhibition assay was performed as described above.

Osmotic protection of erythrocytes. The protective effect of high molecular mass compounds against lysis of erythrocytes was evaluated using hydrophilic molecules of increasing diameter (Scherrer & Gerhardt, 1971). The following colloids were tested (molecular diameter in nm): xylose (0.62), raffinose (1.14), polyethylene glycol (PEG) 1450 (2.40), inulin (2.80), PEG 3350 (3.80), PEG 8000 (6.40) and PEG 10000 (7.20). The colloids were mixed with RBC and a 1:32 dilution of the supernatant containing the haemolysin was added. The final concentration of colloids was 30 mM, with the exception of inulin and PEG, which were used at 15 mM because of their solubility. The mixtures were incubated at 37 °C for 60 min before reading haemolytic activity.

Purification of the haemolysin. As complete media such as THB should be avoided for purification purposes because of the presence of large molecular mass peptides (Alouf, 1980), a beef and yeast extract diffusate medium was used. The medium was

prepared by dissolving 100 g beef extract and 100 g yeast extract (Difco) in 1 litre distilled water by moderate heating (45–50 °C). This concentrate was dialysed against 5 l distilled water for 72 h at 4 °C. The dialysis sacs and contents were discarded and the diffusate was adjusted with distilled water to 25 l and sterilized. Prior to inoculation, 500 ml 10% (w/v) sodium bicarbonate in distilled water and 1% (w/v) glucose were added. After 18 h incubation, the culture was centrifuged for 30 min at 10000 g, filtered (0.2 µm pore size filters; Millipore) and precipitated with 50% (v/v) (NH₄)₂SO₄. The precipitate was ultrafiltered using an Amicon system YM 30 membrane. The unfiltered material (> 30 kDa) was applied to an affinity column consisting of a thiopropyl-Sepharose 6B column (Pharmacia) at a flow rate of about 6–8 ml h⁻¹. The column was washed with PBS (pH 6.8) and eluted with PBS (pH 7.5) containing 5 mM DTT. The material was collected in different fractions and monitored for haemolytic activity. The haemolytic fractions were pooled and subjected to SDS-PAGE (20 µl loading volume; a total of 15 µg protein) in 7.5% (w/v) polyacrylamide slab gels as described by Laemmli (1970) and stained using silver nitrate.

RESULTS

General characteristics of the haemolysin produced by *S. suis* capsular type 2

The haemolysin was not cell-bound, since the bacterial sediment obtained after centrifugation of cultures (regardless of cultural conditions) did not lyse erythrocytes, even after sonication of bacterial cells. Haemolytic activity was always observed in whole cell culture, culture supernatants and culture filtrates. On the other hand, the positive control (*S. pneumoniae*, Ambrose strain) gave a positive haemolytic reaction with both supernatant and washed sonicated cells. A lower haemolytic activity was obtained with shaking (32 HU) than with stationary (64 HU) cultures.

The haemolysin of *S. suis* capsular type 2 was produced at the end of the exponential phase of the growth cycle (Fig. 1). Haemolysin production was preceded by a latent period (about 8 h) where no haemolytic activity could be detected. Maximum haemolytic activity was observed between 15 and 24 h incubation. After 24 h incubation, the haemolysin detected in the supernatant decreased, and almost completely disappeared after 48 h incubation.

Relatively similar haemolytic kinetics were observed when different temperatures of incubation were used for the haemolytic assay, the highest haemolytic activity being observed at 37 °C and the lowest at 4 °C. In all cases, erythrocyte lysis was preceded by a pre-lytic phase of approximately 10 min for the incubation at 37 °C; 15 min for the incubation at 25 °C; and 20 min for the incubation at 4 °C. After 120 min, the activity almost levelled off for the three incubation temperatures (Fig. 2).

Human group O RBC were the erythrocytes most susceptible to the haemolysin; horse, pig, cow, and sheep RBC presented similar susceptibilities whereas rabbit RBC were the most resistant to haemolysis. When various amounts of erythrocytes were incubated with the haemolysin, maximum lysis was observed when a 2% (v/v) RBC suspension was used (Fig. 3).

Finally, haemolysin activity in the cell-free supernatant disappeared after 1 and 4 d storage at 37 °C and 25 °C, respectively; at 4 °C, some activity was still present after 6 d storage. Activity was conserved better still at –20 °C. Integral haemolytic activity was observed after at least 1 year of storage at –70 °C.

Effect of different treatments on haemolytic activity

Results of the effect of different treatments on haemolytic activity are shown in Table 1. Temperature affected haemolytic activity. Incubation of the supernatant at 60 °C for 1 h or boiling for 30 min completely abolished haemolytic activity. When the haemolysin was incubated

Fig. 1. Bacterial growth and haemolysin production by *S. suis* capsular type 2, strain S735. Bacterial cells were grown in THB and haemolysin assayed using horse RBC. ■, Bacterial growth; ♦, haemolytic activity in culture supernatant.

Fig. 2. Kinetics of the haemolytic reaction. Mean OD₅₄₀ corresponds to a 1:32 dilution of the supernatant of *S. suis* capsular type 2, strain S735. Haemolysin assay was performed using horse RBC. ■, Incubation at 37 °C; ♦, incubation at 25 °C; ●, incubation at 4 °C.

Fig. 3. Effect of erythrocyte concentration on haemolytic activity. Results are expressed as the mean OD₅₄₀ of a 1:32 dilution of the supernatant of *S. suis* capsular type 2, strain S735. The haemolysin was assayed using horse RBC.

Table 1. Effect of different treatments on haemolytic activity of *Streptococcus suis* capsular type 2, strain S735

The activity of untreated supernatant was 32 HU. One HU is defined as the reciprocal of the haemolysin titre, which was calculated as the highest dilution of the supernatant which caused at least 50% haemolysis.

Treatment	HU
Heating at:	
55 °C for 60 min	16
58 °C for 60 min	4
60 °C for 60 min	0
100 °C for 30 min	0
Proteinase-K (100 µg ml ⁻¹)	0
DTT (5 mM)	128
Cholesterol (water soluble; 5 mg ml ⁻¹)	64
Cholesterol (ethanol soluble; 100 µg ml ⁻¹)	0
Sodium periodate (1 and 5 mM)	4
CaCl ₂ (0.01 and 0.1 M)	32
MgCl ₂ (0.01 and 0.1 M)	32
ZnCl ₂ (0.05 and 0.10 mM)	32
EGTA (2.50 mM)	32
Oxygen (100 ml min ⁻¹)	16

at 55 °C or 58 °C for 1 h, a partial reduction of haemolytic activity was observed. Haemolytic activity was also completely abolished following the addition of proteinase K. Addition of CaCl₂, MgCl₂, ZnCl₂ and EGTA did not influence the activity of *S. suis* capsular type 2 haemolysin. Ethanol-soluble cholesterol (100 µg ml⁻¹) completely inhibited haemolytic activity, whereas concentrations of water-soluble cholesterol to 5 mg ml⁻¹ did not have any effect.

Haemolytic activity was significantly increased when the supernatant was incubated with DTT. Treatment of the

Fig. 4. The inhibitory effects of colloidal protectants on *S. suis* capsular type 2 haemolysin, strain S735 (A, none; B, xylose; C, raffinose; D, PEG 1450; E, inulin; F, PEG 3350; G, PEG 8000; H, PEG 10000). Mean OD₅₄₀ corresponds to a 1:32 dilution of the supernatant. The haemolysin was assayed using horse RBC.

cell-free supernatant with oxygen reduced haemolytic activity from 32 to 16 HU. When this supernatant was treated with DTT, haemolytic activity was totally restored (data not shown). Addition of sodium periodate significantly reduced activity (Table 1). Results obtained by modifying the pH of the solutions showed that maximal activity was present between pH 4 and 8, with a peak at pH 5. A slight decline was observed below pH 4 and above pH 8.

Finally, the haemolysin shares common epitopes with streptolysin, since a 12800-fold dilution of anti-streptolysin IgG inhibited 50% of haemolytic activity. In addition, a positive reaction was also obtained with the purified haemolysin by dot-ELISA (results not shown).

Production of haemolysin *in vivo*

Most sera from pigs experimentally infected with the reference strain of *S. suis* capsular type 2 did not show a significant anti-haemolysin titre, even after two challenges. Only one animal significantly increased its titre from 3200 to 25600 2 weeks after the second infection. Interestingly, animals presented a mean titre of 3200 prior to inoculation, which probably means that cholesterol present in normal sera highly inhibited the action of the haemolysin and high dilutions should be used to test anti-haemolytic activity.

Osmotic protection of erythrocytes

Erythrocytes were suspended in PBS containing various colloid protectants. The inhibitory effects were dependent on the molecular diameter of the colloids. As shown in Fig. 4, most of the protectants used did not prevent haemolysis. However, PEG 8000 and PEG 10000 (molecular diameter of approximately 6.40 and 7.20 nm, respectively) significantly protected the RBC. When the

Fig. 5. SDS-PAGE of the crude culture supernatant (lane A) and a pool of haemolytic fractions after purification using affinity chromatography on a thiopropyl-Sepharose 6B column (lane B). Arrows on the left indicate molecular mass markers in daltons. The gel was stained with silver nitrate.

cells were subsequently washed twice in PEG-containing buffer and resuspended the third time in PBS (without PEG), complete haemolysis occurred immediately. These results indicated that PEG did not primarily inhibit the binding of *S. suis* haemolysin to RBC, but acted as an osmotic protectant by increasing the colloid osmotic pressure in the extracellular milieu.

Purification of the haemolysin

The ratio of HU to total protein was significantly higher using the beef and yeast extract diffusate medium than with the THB. However, even in beef and yeast extract diffusate medium numerous protein bands were observed upon SDS-PAGE. After precipitation with ammonium sulfate and ultrafiltration, purification was achieved using the thiopropyl-Sepharose 6B column. This procedure is based on thiol-disulfide exchange chromatography for the selective purification of various thiol proteins. A single protein band at 65 kDa representing the purified haemolysin was observed after affinity chromatography (Fig. 5).

DISCUSSION

This investigation was initiated by the observation of narrow zones of β -haemolysis around the colonies of some strains of *S. suis*, especially when cultured on horse blood agar plates. Although *S. suis*, a Lancefield group D *Streptococcus*, is known as an α -haemolytic *Streptococcus*, several authors previously reported the presence of β -haemolytic strains as well as a synergistic, CAMP-like haemolytic reaction with staphylococcal β -lysin (Estoepeangestie & Lämmler, 1993; Hommez *et al.*, 1986).

The results of the present investigation showed that *S. suis* capsular type 2 produced an extracellular haemolysin in THB. The haemolytic activity was higher in stationary cultures than in shaking cultures, probably due to the adverse effect of oxygen.

Most haemolysins possess a first temperature-independent-step mechanism of action, as revealed by the fact that at 4 °C no haemolytic activity is observed (Honda & Miwatani, 1992; Hossain *et al.*, 1993; Shinoda *et al.*, 1985). This does not seem to be the case for *S. suis* haemolysin, since it was active at 4 °C, although a longer lag time was observed. The greatest haemolytic activity was observed at 37 °C, whereas an intermediate activity was observed at 25 °C.

S. suis haemolysin is completely inhibited by free cholesterol, as is the case with other haemolysins (Alouf & Geoffroy, 1991). Results presented in this study indicated that testing a lipid in aqueous solution for interaction can lead to erroneous results due probably to micellar formation. These erroneous results were also reported for *Aeromonas hydrophila* haemolysin (Kozaki *et al.*, 1987). The haemolytic differences observed with erythrocytes from different animal species and *S. suis* haemolysin had previously been demonstrated for other haemolysins (Hossain *et al.*, 1993; Kozaki *et al.*, 1987). Bernheimer *et al.* (1975) reported that the erythrocyte membranes from different animal species contained a variable amount of phosphatidylcholine, which could explain the different susceptibilities.

The haemolysin was significantly activated by DTT. As mentioned by Alouf (1980), the effect of DTT was only observed after a previous increase in pH (results not shown). As is the case for the streptolysin O, periodate oxidation caused complete and irreversible loss of activity (Alouf, 1980). On the other hand, divalent cations did not affect haemolytic activity, as is the case with other haemolysins (Miyake *et al.*, 1989; Frey & Nicolet, 1988).

The finding that colloidal compounds such as PEG 8000 and PEG 10 000 can inhibit erythrocyte lysis suggests that *S. suis* haemolysin induces osmotic lysis of erythrocytes by forming transmembrane pores of approximately 7 nm diameter. A similar mechanism has been reported in studies of, for example, *Escherichia coli* haemolysin (Bhakdi *et al.*, 1986), staphylococcal toxin (Bhakdi *et al.*, 1984) and streptolysin O (Alouf & Geoffroy, 1991). In addition, it would seem that a multi-hit lytic system is involved: more than one haemolysin molecule seems to be required to lyse a single erythrocyte since a decrease in haemolysis was obtained by increasing the concentration of erythrocyte suspension (Inoe *et al.*, 1976; Hossain *et al.*, 1993).

The haemolysin of *S. suis* capsular type 2 is a protein of 65 kDa, as shown by SDS-PAGE and silver staining. Jacobs *et al.* (1994) reported that a haemolysin produced by the P1/7 strain had a molecular mass of 54 kDa. Data concerning the molecular mass of streptolysin O also varied considerably, depending on the purification method (Alouf, 1980). The haemolysin protein is not the EF or EF* factor reported by Vecht *et al.* (1992, 1993), since the

molecular masses of the latter are much higher. In addition, we did not find any correlation between EF, EF* and/or MRP and haemolysin production (unpublished observations).

Finally, the production of haemolysin *in vivo*, by measuring the production of anti-haemolysin antibodies after an experimental inoculation, was studied in pigs. Even though challenged pigs were extremely ill, most of them did not produce antibodies against the haemolysin. It is possible that the haemolysin is produced *in vivo* in low quantities in infected pigs and only a weak immunologic reaction is generated.

In conclusion, the haemolysin produced by *S. suis* capsular type 2 belongs to the family of toxins known as thiol-activated toxins or, as more recently named, antigenically related cholesterol-binding cytolytic toxins (Alouf & Geoffroy, 1991), since it shares common characteristics with other members of this family, such as sensitivity to oxygen and oxidizing agents, activation by reducing agents, inhibition by low concentrations of cholesterol, formation of transmembrane pores and a 'multi-hit' mechanism of action. In addition, anti-streptolysin antibodies inhibited haemolytic activity. To our knowledge, *S. suis* is the only Lancefield group D *Streptococcus* which produces a haemolysin with these characteristics. The role of this haemolysin in the pathogenesis of *S. suis* infections remains to be investigated.

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