Purification and partial characterization of a cohaemolysin (CAMP-factor) produced by Streptococcus canis

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1. SUMMARY

A cohaemolysin from the culture supernate of a canine pathogenic group G streptococcus (S. canis) was purified to electrophoretic homogeneity. The purification procedure involved ammonium sulphate precipitation, ultrafiltration, gel filtration and preparative isoelectric focusing. The cohaemolysin consisted of a single polypeptide chain, 18.6 kDa, with an isoelectric point at pH 5.1. The protein reacted with an homologous antiserum, appeared to be trypsin-sensitive and relatively heat-stable. The cohaemolysin did not show any non-specific IgG binding activities.

2. INTRODUCTION

In 1944, Christie et al. [1] described an extracellular product of serological group B streptococci which synergistically lysed sheep erythrocytes in the diffusion zone of the Staphylococcus aureus β-lysin, a sphingomyelinase. This product, now known as the CAMP-factor, is generally considered to be highly specific for group B streptococci and serves for their presumptive identification [2]. The CAMP-protein was purified by Bernheimer et al. [3] and characterized as a thermostable protein with a molecular mass of 23 500 and an isoelectric point at pH 8.3. Jürgens et al. [4] described the CAMP-protein as a polypeptide with a molecular mass of 25 000 and an isoelectric point at pH 8.9. A comparable molecular mass of the CAMP-protein was obtained by cloning the genetic determinants and expression of the CAMP-factor in E. coli [5]. Previously, Jürgens et al. [6] described the non-specific reactions of the group B streptococcal CAMP-protein with immunoglobulins. As with the protein A of S. aureus, the binding property seemed to be mediated by the Fc portion of the immunoglobulin. Therefore, group B streptococcal CAMP-factor was designated protein B [6]. Similar synergistic haemolytic activities to the CAMP-reaction of serological group B streptococci were observed with Streptococcus uberis [7], S. suis [8], groups U and V streptococci [9] and with a number of other bacterial species [10,11].

In a previous study, CAMP-like activities were observed with group G streptococci isolated from...
dogs (S. canis) [12]. The aim of the present work was to purify and further characterize the cohaemolysin from S. canis.

3. MATERIAL AND METHODS

3.1. Purification of the cohaemolysin

For purification of the cohaemolysin the group G streptococcal culture of S. canis 2643 was used; originally obtained from R. Weiss (Institut für Hygiene und Infektionskrankheiten der Tiere, Giessen, F.R.G.) and characterized biochemically and serologically [12]. The organism was cultivated in Todd-Hewitt-Broth (THB, Gibco, Europe, Karlsruhe, F.R.G.) for 24 h at 37°C, the culture supernate was filtered (Pellicon filter, 0.45 μm, Millipore Eschborn, F.R.G.) and finally precipitated with 70% ammonium sulphate (472 g/l) for 72 h at 4°C. After dialysis of the precipitate against PBS (0.05 mol/l phosphate, 0.15 mol/l NaCl, pH 7.3) the precipitated proteins were further concentrated by ultrafiltration (Ultrafiltration membrane, Millipore) to a final volume of 5 ml. This preparation was applied to a Sephadex G 75 superfine column (2.5 cm × 100 cm, Pharmacia-LKB, Freiburg, F.R.G.) previously washed with PBS. The proteins were eluted with the same buffer at a flow rate of 8 ml/h. The fractions with synergistic haemolytic activities were pooled and applied to a 110 ml isoelectric focusing column with pH 3.5 - 10 carrier ampholytes (Pharmacia-LKB) and electrophoresing was done for 24 h [13]. The synergistic haemolytic activities of the isolated preparations were determined according to Huser et al. [14]. For this, sheep erythrocytes were washed three times in PBS (0.05 mol/l phosphate, 0.9% NaCl, pH 7.4) and finally diluted 1:10 in the same buffer. A 20 μl volume of this suspension was added to 1.4 ml TBS (0.01 mol/l Tris, 0.01 mol/l MgCl₂ and 0.15 mol/l NaCl, pH 7.4) and preincubated for 10 min at 37°C with 50 μl sphingomyelinase (S. aureus sphingomyelinase, 1.25 U/ml, Sigma, Deisenhofen, F.R.G.). After the addition of 50 μl of the cohaemolysin preparations, and 5 min incubation at 37°C, the undamaged erythrocytes were removed by centrifugation (1000 × g, for 3 min). Lysis of the cells was followed spectrophotometrically at the A₅₄₆nm of the supernate; one unit of cohaemolysin was defined as the amount of protein that caused a change of 0.1 in A₅₄₆nm. The protein content of the cohaemolysin preparation was determined according to Peterson [15] with bovine serum albumin (Behring-Werke, Marburg, F.R.G.) as standard.

3.2. SDS-PAGE and Western blotting of the cohaemolysin

The proteins were further analysed by SDS-PAGE electrophoresis and Western blotting [16,17]. For this the proteins were subjected to SDS-PAGE in 11% polyacrylamide with 25 mM Tris-192 mM Glycine-0.1% SDS as running buffer and subsequently transferred onto nitrocellulose for 16 h at 30 V/cm at 7°C. The non-specific binding sites of the nitrocellulose were blocked by treatment with skimmed milk [18].

3.3. Preparation of rabbit anti-cohaemolysin serum

To produce specific antibodies 200 μg of the purified cohaemolysin was mixed with 1 ml Freund's complete adjuvant (Difco) and injected subcutaneously into rabbits at multiple sites. A booster injection was given at week 3. In parallel experiments serum of non-immunized rabbits and purified human immunoglobulin G (Sigma) was used. The Western blots were developed with peroxidase-labelled specific immunoglobulins as described [18].

4. RESULTS

The cohaemolysin from S. canis 2643 was concentrated by ammonium sulphate precipitation and ultrafiltration. Purification of the protein was achieved by gel filtration and subsequent preparative isoelectric-focussing. The major synergistic haemolytic activity was detected at pH 5.1 (Fig. 1, Fig. 2). The purification steps are summarized in Table 1.

SDS-PAGE of the purified protein revealed a single protein band. After transfer of the cohaemolysin onto nitrocellulose membranes a reaction
with specific antiserum could be observed (Fig. 3). The protein did not react with sera from non-immunized rabbits or with human IgG. The molecular mass of the purified protein was 18600. The cohaemolysin from *S. canis* appeared to be relatively heat-stable. When the protein was boiled for 5 min the cohaemolytic activity remained largely unaffected, 92% of the activity remained. Even after boiling for 30 min 25% of the activity remained. The cohaemolytic activity was greatly reduced by trypsin treatment (0.4 mg/ml) of the preparation; 11% of the activity remained.

5. DISCUSSION

The synergistic haemolytic reaction of the CAMP-protein of group B streptococci in the diffusion zone of the β-lysine of *Staphylococcus aureus* has already proved to be a useful diagnostic tool for presumptive identification of group B streptococci. In addition, this CAMP-protein plays a role as a virulence factor in group B streptococcal infections [6,19].

More recently, a comparable synergistic haemolysin was demonstrated with canine pathogenic group G streptococci, previously designated as *Streptococcus canis* [12]. In the present study, this cohaemolysin of *S. canis* was purified by ammonium sulphate precipitation and ultrafiltration.
Table 1
Purification of the cohaemolysin from *Streptococcus canis* 2643

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Cohaemolysin activity (coHU/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (coHU/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernate</td>
<td>72</td>
<td>0.33</td>
<td>218.18</td>
<td>0</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>3360</td>
<td>2.45</td>
<td>1371.43</td>
<td>6.28</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>20480</td>
<td>17.42</td>
<td>1175.66</td>
<td>5.39</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>2160</td>
<td>0.214</td>
<td>10093.46</td>
<td>46.26</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>3280</td>
<td>0.186</td>
<td>17634.41</td>
<td>80.82</td>
</tr>
</tbody>
</table>

Purification followed by gel filtration and preparative isoelectric-focusing. The isolated cohaemolysin appeared as a single protein band in SDS–PAGE, with a molecular mass of 18 600. The isolated group B streptococcal CAMP-factor had a molecular mass of 23 500–25 000 [3,4] respectively. In contrast to the B streptococcal CAMP-factor, the *S. canis* cohaemolysin had an isoelectric point at pH 5.1 and did not show any non-specific binding of IgG. The latter proved to be a specific property of the CAMP-factor (protein B) of group B streptococci [6]. Similar to group B streptococcal CAMP-factor, the cohaemolysin of *S. canis* appeared to be heat-stable and sensitive to trypsin treatment. However, the structural relationship of both of the haemolytically active streptococcal substances remains to be elucidated. The complete amino acid sequence of the CAMP-factor (protein B) of group B streptococci has been determined recently [20].

REFERENCES


