Purification and characterisation of haemagglutinin from *Bordetella bronchiseptica*

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Summary. A surface protein of *Bordetella bronchiseptica* was purified in one step by affinity chromatography with bovine submaxillary mucin coupled to agarose. The purified protein, with a mol. wt of 200 kDa and an iso-electric point of pI 6.5, showed haemagglutinating activity for bovine erythrocytes. This haemagglutinin (HA) inhibited the adherence of *B. bronchiseptica* to a rat lung cell line (L2) and was able to bind to *N*-acetylneuraminic acid. These findings suggest that the HA of *B. bronchiseptica* is an adhesin.

Introduction

The mechanism of adherence of bacteria to the surface of fibroblasts or epithelial cells has been examined with many species and the presence of specific adhesins on the organisms and adhesin receptors on the cells has been demonstrated.^{1–10}

Adherence to the surface of epithelial cells in the respiratory tract and subsequent colonisation and proliferation at this site are considered to be necessary for *Bordetella bronchiseptica* to cause disease.¹¹ Ishikawa *et al.*¹² suggested that the haemagglutinin (HA) of this organism might be a bacterial adhesin because a mutant strain, lacking HA activity, was incapable of adhering to the mammalian cell surface. *B. pertussis* appears to adhere to mammalian cells by a number of adhesins, namely filamentous haemagglutinin, pertussis toxin, pertactin and fimbrial agglutinogens, acting separately or together.^{13–16}

Ishikawa *et al.*¹⁷ suggested that the adhesin receptors for *B. bronchiseptica* on mammalian cells were *N*-acetylneuraminic acid, and we obtained similar results in preliminary experiments. Ravindranath *et al.*¹⁸ purified a sialic acid-specific lectin from the serum of a marine crab, *Cancer antennarius*, by affinity chromatography. Thus, since the adhesin receptors of *B. bronchiseptica* are considered to be sialic acid and the adhesins are believed to have lectin-like binding properties, we believed that affinity chromatography could be useful for simple, rapid purification of the adhesin.

The purpose of this study was to attempt to purify the adhesin of *B. bronchiseptica* by affinity chromatography and examine its properties.

Materials and methods

Bacterial strain

B. bronchiseptica strain M-3, phase I, isolated from a guinea pig,¹⁹ was used for this study.

Isolation of the crude surface proteins

B. bronchiseptica was propagated on Bordet-Gengou Agar (Difco) containing defibrinated sheep blood 7% for 48 h at 37°C, collected in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, and centrifuged twice at 10000 *g* for 10 min. Carbonate buffer (0.1 M, pH 9.8) was added to the washed cells to prepare a 10% w/v bacterial suspension.^{20, 21} The suspension was heated in a water bath for 30 min at 37°C and centrifuged at 10000 *g* for 20 min at 4°C. The supernate was dialysed overnight in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.01 M CaCl₂ (TBS) to yield a preparation of crude surface proteins.

Preparation of affinity medium

Bovine submaxillary mucin (BSM; Sigma) was coupled to agarose by the technique of Ravindranath *et al.*¹⁸ Affi-Gel 15 (BioRad Laboratories; 25 ml) was washed with cold distilled water and resuspended to 100 ml in cold 0.05 M HEPES buffer, pH 8.2, containing 100 mg of BSM. The reaction proceeded for 8 h at 4°C, after which 5 ml of 1 M ethanolamine-HCl, pH 8.0, was added to block any remaining active sites. The adsorbent was washed with TBS and stored in TBS with sodium azide 0.02% w/v at 4°C until required.

Purification of the surface protein

The crude extract of surface proteins (2.5 ml) was applied to 2 ml of TBS-equilibrated BSM-agarose in a glass column (9 × 50 mm) fitted with a water jacket. The column was washed first with 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl and 0.01 M CaCl₂ and then with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl and 0.01 M CaCl₂. The column was incubated for 10 min at 32°C and washed again with the same buffer. Elution was done at 32°C in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl and 2 mM EDTA. Fractions of 1 ml were collected into tubes, each containing 100 µl of 0.1 M CaCl₂, which were cooled rapidly in an ice bath. Each eluted fraction was concentrated to about one-tenth of its original volume for the assay of HA activity. The protein concentration was estimated by the method of Lowry *et al.*²²

Haemagglutination test

Haemagglutination was assayed according to the technique of Ishikawa *et al.*¹² Each fraction was diluted in two-fold steps up to 1 in 64 in the wells of a U-bottomed microtitration plate (Falcon), followed by the addition of an equal volume (25 µl) of bovine erythrocytes 0.5% v/v. After mixing and incubation for 2 h at 37°C, the haemagglutination titre was determined. One unit of activity was defined as the amount of protein required to give haemagglutination in one well.¹⁸

SDS-PAGE and iso-electric focusing (IEF)

SDS-PAGE was performed according to the method of Laemmli²³ with 4–12% gradient SDS-polyacrylamide gels (TEF Co., Nagano, Japan). For solubilisation, each sample was mixed in equal volumes with 20 mM Tris-HCl buffer, pH 6.8, containing SDS 2% w/v, mercaptoethanol 2% v/v and glycerol 40% v/v, and the mixture was placed in a boiling water bath for 5 min. Solubilised sample (20 µl) was added to each lane of the gel for electrophoresis at 20 mA. The mol. wt was estimated from the mobility of the marker proteins (High mol. wt range, Bethesda Research Laboratories).

The iso-electric point was determined by IEF-PAGE (TEF Co.) with a pH range of 3–10.²⁴ Each sample was mixed in equal volumes with glycerol 60% v/v. The sample solution (20 µl) was added to each lane for electrophoresis at 500 V. The iso-electric point was estimated by comparison with marker proteins (Broad pI Calibration Kit, Pharmacia).

Adherence inhibition assay

The protein (0.5 ml) purified in the BSM-agarose affinity columns was adjusted to 20, 10 or 2 µg/ml with Hanks's balanced salts solution (HBSS) and added to rat lung cells (L2 cells) obtained by monolayer culture

in eight-chamber slides (Lab-Tek[®]; Nunc, Roskilde, Denmark) and allowed to react for 30 min at 37°C. The reaction slide was washed with HBSS, to which 0.5 ml containing 10⁸ *B. bronchiseptica* cfu was added. The bacteria had been grown on Bordet-Gengou agar containing defibrinated sheep blood 7% v/v for 20 h at 37°C. The slide was incubated for 30 min at 37°C, after which it was washed with cold HBSS to eliminate non-adhering organisms and stained with May-Grünwald and Giemsa solution. The bacteria adhering to 20 L2 cells were counted by light microscopy (1000 × magnification). Data were expressed in terms of the mean number of attached bacteria/L2 cell. All assays were performed in triplicate.

Examination of the receptor specificity of the purified protein

N-Acetylneuraminic acid (NeuAc; 10, 1 or 0.1 mg) was dissolved in a solution containing the purified protein at 10 µg/0.5 ml and allowed to react for 30 min at 37°C. The solution was then added to the L2 cells obtained by monolayer culture in eight-chamber slides followed by the procedure used for the adherence inhibition assay.

Results

Purification of the surface protein

Fig. 1 shows the elution profile of the surface protein obtained by BMS-agarose affinity chromatography. Each of the fractions obtained by the various elution conditions was concentrated to one-tenth of its original volume. The presence or absence of HA activity was determined by a haemagglutination assay with bovine erythrocytes. Haemagglutinating activity was

Fig. 1. Elution profile of affinity column. BSM-agarose was equilibrated with TBS containing of 10 mM CaCl₂ at 4°C. The crude protein extract was applied and the column washed with (a) TBS containing 1 M NaCl and 10 mM CaCl₂ at 4°C. The column was transferred to a water bath at 32°C and washed with (b) TBS containing 0.3 M NaCl and 10 mM CaCl₂. (c) Elution was done at 32°C with TBS containing 0.3 M NaCl and 2 mM EDTA. Fractions of 1 ml were collected and those containing haemagglutinating activity were pooled and designated as fraction A.

Table I. Purification of *B. bronchiseptica* strain M-3 surface protein

Step	Volume (ml)	Protein (mg)	Total activity* (HA units)	Specific activity (HA units/mg)	Purification factor
Crude surface protein	2.5	1.050	800	762	1
BSM-agarose purified protein (Pool A)	0.5	0.052	320	6153	8.1

*One unit of haemagglutinin (HA) activity was defined as the amount of protein required to give one well of haemagglutination.¹⁸

Fig. 2. SDS-PAGE analysis of crude surface protein and BSM-agarose purified 200-kDa protein. The numbers indicate the sizes of the molecular mass markers. Lanes: a, molecular mass markers; b, crude protein (10 µg); c, BSM-agarose purified protein (1 µg). The gel was silver stained.

Fig. 3. Iso-electric focusing analysis of BSM-agarose purified protein. Lanes: a, iso-electric focusing markers; b, BSM-agarose purified protein. The gel was silver stained.

detected in only five fractions, numbers 128–132. These fractions, which were obtained under the same eluting conditions, were used as pooled fraction A.

The crude protein extract and purified pooled fraction A were compared with respect to the specific activity of HA (table I). The specific activity of purified pooled fraction A was 6153 units/mg, which was 8.1 times that of the crude protein (762 units/mg).

Table II. Inhibition of adherence of *B. bronchiseptica* strain M-3 to rat lung cell line (L2) by purified haemagglutinin

Haemagglutinin (µg)	Mean (SD) adherence*	Inhibition (%)
None	17.8 (0.5)	0
1	13.2 (0.4)	26
5	7.2 (0.4)	60
10	2.9 (0.3)	84

*Mean number of bacteria attached/L2 cell and SD of the mean determined from triplicate assays.

Table III. Effect of pre-treatment of haemagglutinin with *N*-acetylneuraminic acid on adherence of *B. bronchiseptica* strain M-3 to rat lung cell line (L2)

Haemagglutinin treatment	Mean (SD) adherence*
No haemagglutinin	17.8 (0.5)
Haemagglutinin 10 µg plus <i>N</i> -acetylneuraminic acid (mg)	
10	14.5 (0.8)
1	6.2 (0.2)
0.1	4.8 (0.3)
0	3.1 (0.4)

*See footnote to table II.

Electrophoretic analysis of the purified protein

The purity, mol. wt and iso-electric point of the purified protein were determined by electrophoresis. The results of SDS-PAGE analysis are shown in fig. 2. After silver staining, the crude protein extract showed > 10 bands between 200 and 29 kDa, whereas the purified protein showed only a single band at 200 kDa. The iso-electric point of the purified protein was pI 6.5 (fig. 3).

Effect of the purified protein on adherence

The effect of the purified protein on the adherence of *B. bronchiseptica* to L2 cells was examined in adherence inhibition assays. As shown in table II, pre-treatment of the L2 cells with the purified protein resulted in a marked dose-dependent decrease in adherence of the organisms.

Effect of NeuAc on binding of purified protein to L2 cells

The binding specificity of the purified protein was examined in a competitive assay. The purified protein

was treated with NeuAc and allowed to react with the L2 cells prior to examining the adherence of *B. bronchiseptica*. When the amount of NeuAc was increased, with a fixed amount of purified protein, the adherence of the organism was clearly increased (table III).

Discussion

The establishment of *B. bronchiseptica* infection requires adherence of the organism to epithelial cells in the respiratory tract for colonisation and proliferation. The receptors involved in this adherence have been found to contain sialic acid.¹⁷ However, there has been no report on the purification or properties of the adhesin of this organism. We assumed that the adhesin was a protein with lectin-like properties and attempted to purify it in one step by affinity chromatography with sialic acid as the ligand. Since sialic acid does not bind readily to agarose, bovine submaxillary mucin, which has an amino group and a high content of sialic acid, was used for binding studies. For elution in affinity chromatography, the conditions used by Ravindranath *et al.*¹⁸ to purify sialic acid-specific lectin from the serum of *Cancer antennarius* was employed. HA activity against bovine erythrocytes was observed in the fractions obtained by affinity chromatography

after heating at 32°C and with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl and 2 mM EDTA as the eluent. The specific activity of the HA was approximately eight times greater than that in the starting material. Electrophoretic analysis revealed that a protein of 200 kDa with an iso-electric point of pI 6.5 had been purified in one step by affinity chromatography. Kimura *et al.*²⁵ reported that the filamentous haemagglutinin (FHA) of *B. pertussis* was a protein with a mol. wt identical to that of the HA of *B. bronchiseptica* demonstrated in this study. Further evidence of a relationship between these proteins was provided by Domenighini *et al.*²⁶ who observed cross-reactions with antibody to the recombinant FHA of *B. pertussis*, providing evidence for the presence of an FHA in *B. bronchiseptica*.

The adhesin activity of the purified HA was also assayed. An adherence inhibition assay of *B. bronchiseptica* was performed with the L2 line of rat lung epithelial cells. Treatment of L2 cells with HA resulted in a marked decrease in the adherence of the organisms. However, bacterial adherence was not prevented when HA was allowed to react with NeuAc before treatment of the L2 cell line. These findings indicated that HA was able to bind specifically to sialic acid in the cell membrane. In conclusion, the HA of *B. bronchiseptica* acts as an adhesin and probably plays a key role in the pathogenesis of infection by this organism.

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