Extraction and Properties of Hemagglutinin from Cell Wall Fragments of *Fusobacterium nucleatum*

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To study the hemagglutinin of *Fusobacterium nucleatum*, methods were sought to solubilize and purify this component. When cells of *F. nucleatum* were ruptured by passage through a French press, the fragments lost virtually all ability to agglutinate human erythrocytes. Extraction of the fragments with 2% Triton X-100 for 30 min at 22°C restored hemagglutinating activity (HA). Hemagglutination by these fragments could be inhibited by arginine, as can hemagglutination by intact bacteria. Treatment of active cell wall fragments with pronase and 2% Triton X-100–EDTA at 37°C or with pronase and 0.1% Triton X-100–EDTA at pH 10.0 allowed recovery of solubilized HA. The former HA was inhibited by arginine (arg+) whereas the latter was not (arg-). Fractionation of the arg+ extract by preparative isoelectric focusing showed that HA was recovered from the gel sections having a pH between 4.5 and 5.5. Hemagglutination by this preparation was still arg+. Chromatography of this hemagglutinin on DEAE-Sephadex increased the specific activity to high levels with a loss of inhibition by arginine. A fraction from the DEAE-Sephadex column containing 10,700 HA units per mg of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Solubilization at 22°C before electrophoresis revealed three Coomassie blue-staining bands which migrated with apparent molecular weights of about 21,000, 38,000 and 60,000. When the same DEAE fraction was boiled in sodium dodecyl sulfate, electrophoresis revealed only one band with an apparent molecular weight of 21,000.

*Fusobacterium nucleatum*, a gram-negative anaerobe, is capable of attachment to erythrocytes of many vertebrate species (9). The molecular interactions which permit this attachment to eucaryotic cells are poorly understood. Hemagglutination and cell attachment by other gram-negative bacteria is frequently, although not exclusively, mediated by pili on the bacterial surface (12, 27, 30). These attachments are in many cases disrupted by the presence of simple sugar molecules, implicating a lectin-like interaction between adhesive molecules and target cell receptors (2, 18, 30). For example, purified pili from *Pseudomonas aeruginosa* were shown to bind to mammalian buccal epithelial cells and to competitively inhibit the attachment of intact bacteria (32). Trypsinized human erythrocytes were agglutinated by purified type I *Escherichia coli* pili. Hemagglutination was inhibited by D-mannose, methyl α-D-manno-pyranoside, and yeast mannann (26). The binding of the fimbrial K88 antigen of *Escherichia coli* to hog intestinal membranes was shown to be inhibited by N-acetyl derivatives of galactose, mannose, and glucose (1). The role of pili as a mediator of the attachment of *Neisseria gonorrhoeae* strains to amnion cells in culture was discussed by Swan- son (31), and their function in agglutination of human erythrocytes was reported by Buchanan and Pearce (5).

Agglutination of human erythrocytes by *F. nucleatum*, in contrast to the above, does not involve filamentous surface structures. Examination of cells and cell fragments by various techniques fails to demonstrate the presence of pili or flagella (6). The hemagglutinin is therefore probably a cell surface-associated molecule.

In a previous paper we have demonstrated the inhibition of fusobacterial hemagglutination by guanido group-containing substances such as arginine (8). It was also observed that some sugars shown to interrupt hemagglutination in a similar system (9) do not affect hemagglutination by the *F. nucleatum* strain employed here. Experimental evidence was presented to suggest that the hemagglutinin is a protein or is dependent on a protein component for function.

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Hemagglutination is lost upon rupture of cells. We describe here the restoration of hemagglutinating activity (HA) to cell wall fragments and the extraction and purification of HA from detergent extracts of these fragments.

MATERIALS AND METHODS

Cultivation of *Fusobacterium nucleatum* and preparation of cell walls. *F. nucleatum* ATCC 10953 was grown and harvested as described previously (8). Bacteria were suspended to approximately 10¹⁵ cells per ml in PBS (0.15 M NaCl containing 0.01 M phosphate buffer, pH 7.5) and ruptured by two passages through a French press (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in². The lysate was diluted fivefold with PBS and centrifuged twice at 20,000 × g in a Du Pont Sorvall refrigerated centrifuge (Du Pont Instruments, Norwalk, Conn.) at 5°C. The supernatant fluid was removed, and the pellet containing the cell walls was washed twice with PBS by centrifugation and resuspension. The walls were then suspended in PBS or 0.01 M phosphate buffer (pH 7.5) to contain 1 mg of protein per ml.

Protein assay. The protein content of fractions obtained after isoelectric focusing (IEF) and DEAE chromatography was determined by the method of Bradford (4). The protein content of cell wall suspensions, detergent extracts of cell walls, and samples for electrophoresis was determined by the method of Lowry et al. (14).

Determination of HA. HA of intact bacteria, cell wall suspensions, dialyzed cell wall detergent extracts, and purified hemagglutinin preparations was assayed by serial dilution in the presence of PBS (pH 7.5). After incubation at 5°C for 2 h, tubes were scored as positive only if a mat of erythrocytes had formed at the bottom of the tube. Titters are reported as the reciprocal of the dilution of the last positive tube. The preparation of fresh type A human erythrocytes used in hemagglutination tests was described previously (8). One HA unit is the minimum amount of protein able to completely agglutinate 2.5 × 10⁹ erythrocytes. The specific activity is the number of HA units per milligram of protein. Inhibition by arginine was determined as described previously (8).

Preparation of hemagglutinating cell walls. Triton X-100 was added to cell walls suspended in PBS to a final concentration of 2% (vol/vol). The mixture was stirred at 120 rpm for 30 min at 22°C and centrifuged at 30,000 × g. The cell wall pellet was washed three times in 0.01 M phosphate buffer (pH 7.5). Cells walls so treated are designated Triton-extracted cell walls (TECW).

Extraction of TECW with various detergents and chaotrophic agents. To determine whether various agents could solubilize the hemagglutinin from the Triton-EDTA-insoluble cell wall residue, TECW were suspended in the following aqueous detergent solutions to contain 1 mg of protein per ml: 0.25% (wt/vol) sodium dodecyl sulfate (SDS), 0.5% (wt/vol) sodium deoxycholate, 0.5% (vol/vol) Brij 35, 1.0% (vol/vol) Tween 20, and 1.0% (vol/vol) Tween 80. The suspensions were rocked on a VirTis Extractomatic extractor (VirTis Research Equipment, Gardiner, N. Y.) for 2 h at 22°C and then centrifuged at 30,000 × g for 15 min, and the pellets were washed three times with 0.01 M phosphate buffer by centrifugation and resuspension. TECW were also extracted for 2 h at 22°C with 8 M urea and 6 M guanidine-hydrochloride (both pH 7.5). The mixtures were centrifuged at 100,000 × g for 15 min. The supernatant fluids from detergent extractions were dialyzed for 48 h, whereas those from urea and guanidine extractions were dialyzed for 24 h against 0.01 M phosphate buffer at 22°C and titrated. The extracted cell walls were washed by centrifugation and resuspension, suspended to their original volume in PBS, and titrated.

Protease treatment of TECW. TECW suspended in phosphate buffer (1 mg of protein per ml) were digested for 15 min at 37°C with 100 μg of K + K pronase (K + K Laboratories, Plainview, N.Y.) per ml. After incubation, the wall suspensions were cooled on ice for 5 min and then centrifuged at 30,000 × g at 5°C. Pronase-treated TECW were washed twice with phosphate buffer by centrifugation and resuspension and suspended in phosphate buffer to a protein concentration of 1 mg/ml.

Extraction of hemagglutinin. For arginine-sensitive (arg⁺) HA, pronase-treated TECW were incubated with 2% (vol/vol) Triton X-100–10 mM EDTA for 2 h at 37°C with shaking. Alternatively, for arginine-insensitive (arg⁻) HA, pronase-treated TECW prepared as detailed above were extracted with 0.1% Triton X-100 (pH 10.0) at 22°C. Each mixture was centrifuged at 100,000 × g (Beckman model L ultracentrifuge; Beckman Instruments, Palo Alto, Calif.). Each supernatant fluid was diluted 10-fold with 0.01 M phosphate buffer and dialyzed against at least 2,000 volumes of buffer for 48 h at 22°C. During dialysis, the agglutinin precipitated within the dialysis bags. The precipitate was very light and could not be concentrated by centrifugation at 100,000 × g without great loss. Therefore, the volume was reduced by ultrafiltration with constant stirring using a YM-10 membrane (Amicon Corp., Lexington, Mass.). After 10-fold volume reduction the precipitate was assayed for HA.

IEF. Preparative IEF was performed with an LBK Multiphor (LKB Instruments, Bromma, Sweden) on a glass plate (25 by 6 cm). The gel bed consisted of 50% of a 5% (wt/vol) slurry of prewashed Sephadex G75 Superfine beads and contained 2% (wt/vol) amphotolys at pH 4 9, 0.25% (wt/vol) amphotolys at pH 3 to 10, and less than 2% Triton X-100. Arg⁺ agglutinin extracts were added to the gel slurry before the bed was poured. The sample volume was usually 25 to 40 ml and contained approximately 800 μg of protein per ml.

Experiments were performed at 10°C. Power was kept at 6 W during the first 4 h of the experiment and then allowed to decrease to about 1 W by the end (20 h). The gel bed was then divided into 23 1-cm sections. The pH of alternate sections was determined. The remaining content of each section was dialyzed against 4 gel volumes of phosphate buffer (0.01 M; pH 7.5). Portions of each eluate were analyzed for protein content by the method of Bradford (4). The remainder was dialyzed for 36 h at 22°C against 2,000 volumes of distilled water and then tested for HA. Eluates of sections of the gel containing HA to be chromatographed on DEAE-Sephadex were pooled and concentrated by ultrafiltration.

DEAE-Sephadex chromatography. DEAE-Sephadex was hydrated for 18 h at 22°C in 0.05 M Tris-hydro-
chloride (pH 8.3) containing 0.1% (vol/vol) Triton X-100. The gel was loaded into a column and washed with 50 ml of equilibrating buffer. The final bed dimensions were 1.5 by 20 cm. Sample volumes were usually about 2 to 4 ml and contained up to 10 mg of protein. Dialysis of samples against equilibrating buffer before application to the column made no difference as to elution profile, quantity of protein retained by the column, or biological activity of salt-eluted materials. Therefore, this procedure was usually omitted.

After sample application, the bed was washed with 4 column volumes of equilibrating buffer and eluted with 220 ml of a linear 0 to 1.5 M NaCl gradient in equilibrating buffer. The elution rate was 15 ml/h, and the temperature was 22°C. Fractions of 5 ml were collected, and the protein content was assayed by the procedure of Bradford (4). Conductivity measurements were to determine the NaCl concentrations. Measurements were made with a Markson model 10 conductivity meter (Markson Science Inc., Del Mar, Calif.). Each fraction containing protein was dialyzed for 36 h against at least 300 volumes of distilled water at 22°C. The fractions were then tested for HA.

SDS-PAGE. SDS-polyacrylamide disc gel electrophoresis (SDS-PAGE) was performed with a Canaco Disc Gel Electrophoresis apparatus (Canaco, Rockville, Md.) at 3 mA per gel. Samples for electrophoresis were boiled for 5 min in 1% (wt/vol) SDS, 1% (vol/vol) β-mercaptoethanol, 2% (wt/vol) sucrose in 0.05 M sodium phosphate buffer (pH 7.5) unless otherwise indicated. The separating gel (5 by 50 mm) contained 15% (wt/vol) acrylamide and was overlaid with a 10-mm stacking gel containing 3% (wt/vol) acrylamide. The buffer was 0.2 M Tris-glycine (pH 8.5). Both the gel and the buffer contained 0.1% (wt/vol) SDS. The upper buffer also contained β-mercaptoethanol (1%, vol/vol) and bromophenol blue tracking dye. Electrophoresis was continued until the tracking dye had migrated to within 5 mm of the gel end. Gels were fixed in methanol-acetic acid-water (50:7:43) at 50°C for 30 min and stained with 0.1% (wt/vol) Coomassie blue in 10% acetic acid. Destaining was accomplished with ethanol-acetic acid-water (40:53:7). Myoglobin (17,000), trypsin (22,000), ovalbumin (45,000), and bovine serum albumin (68,000) were used as molecular-weight markers.

Protease treatment of purified agglutinin and intact fusobacteria. Enzymes were made from the following sources: trypsin, α-chymotrypsin, papain, subtilopeptidase A, subtilisin, and Streptomyces coelicolor protease from Sigma Chemical Co., St. Louis, Mo.; bacterial protease from K+B Laboratories; bacterial protease from Calbiochem, Los Angeles, Calif. When papain was used, the reaction mixture contained 2.5 mM EDTA and 5 mM cysteine hydrochloride. Samples of the high-specific-activity agglutinin preparation obtained after IEF and DEAE-Sephadex chromatography were diluted fivefold by the addition of 0.5 ml of 0.01 M phosphate buffer (pH 7.5) to a titer of 96. Fresh enzyme solutions were made in phosphate buffer to 1 mg/ml. To the agglutinin suspensions were added 0.1 ml of enzyme solution and 5 μl of 2% (wt/vol) sodium azide. The mixtures were incubated at 37°C for 18 h. A sample containing the diluted hemagglutinin preparation and sodium azide, but 0.1 ml of phosphate buffer instead of enzyme, was prepared as a control. The effects of these enzymes on intact fusobacteria were determined on bacterial suspensions containing 8 × 10^9 cells per ml in phosphate buffer.

Chemicals. Triton X-100, sodium deoxycholate, Brij 35, EDTA, all electrophoresis chemicals and buffer salts, and DEAE-Sephadex were obtained from Sigma. Riboflavin for acrylamide polymerization was obtained from Nutritional Biochemicals, Cleveland, Ohio, as were amino acids. All other salts were obtained from Fisher Scientific Co., Fairlawn, N.J. SDS was purchased from Matheson, Coleman and Bell, Norwood, Ohio. Sephadex G75 Superfine was from Pharmacia Fine Chemicals, Piscataway, N.J. Ampholytes of pH 4 to 9 (technical grade) were purchased from Accurate Chemical Co., Hicksville, N.Y. Ampholytes of pH 3 to 10 were obtained from LKB Instruments.

RESULTS

Restoration of cell wall HA. To reduce the number of cellular constituents to be dealt with in subsequent purification procedures, we decided to extract HA from cell wall fragments instead of intact bacteria. Upon rupture of cells in a French press it was observed that cell fragment did not agglutinate erythrocytes. HA also could not be detected in the particle-free supernatant fluid (Table 1). These results suggested that the hemagglutinin somehow was blocked or inactivated after rupture of the cells.

Inactive cell fragments were treated with 2% (vol/vol) Triton X-100 at 22°C (a method used previously to solubilize cytoplasmic membranes) (28). This procedure restored HA to the fragments and solubilized approximately 42% of the protein, while 58% of the total protein was recovered with the active particulate fraction. The specific activity of the fragments (400 HA units per mg of protein) was increased about threefold over that of intact bacteria (130 HA units per mg of protein). EDTA at 5 mM had no effect on the extraction (Table 1). Thus, under these conditions, the agglutinin is apparently part of a Triton-EDTA-resistant complex and not part of a Triton-EDTA-extractable outer membrane protein complex as found in E. coli by Schnaitman (28, 29).

Effect of various detergents and chaotropic agents on hemagglutination by TECW. Since the agglutinin was still cell wall associated after Triton X-100–EDTA extraction, we attempted to solubilize the agglutinin with other detergents and chaotropic agents. TECW were treated with detergents as described above and then assayed for HA.

Of the detergents tested, only SDS reduced the titer of the cell wall suspension from 128 to 0. HA could not be recovered from the supernatant fluid of the mixture after dialysis. The other detergents did not significantly alter the hemagglutinating ability of the cell walls, and no attempt was made to recover HA from these...
TABLE 1. Extraction of arg+ and arg− HA from F. nucleatum cell walls

<table>
<thead>
<tr>
<th>Sequential treatment of cell wall fragments</th>
<th>HA (titer) in absence/presence of arginine in:</th>
<th>% Protein solubilized</th>
<th>Sp act (HA units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell wall pellet</td>
<td>Supernatant</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>2% TX, pH 7.5</td>
<td>256/0</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>2% TX + pronase + EDTA−2% TX, pH 7.5</td>
<td>0</td>
<td>16/0</td>
<td>79</td>
</tr>
<tr>
<td>2% TX + pronase + 0.1% TX, pH 10</td>
<td>0</td>
<td>16/16</td>
<td>70−80</td>
</tr>
<tr>
<td>TX + EDTA alone</td>
<td>256/0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2% TX, 18 h</td>
<td>128/0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SDS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2% TX + 8 M urea</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2% TX + 6 M guanidine-hydrochloride</td>
<td>4/0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2% TX + sodium deoxycholate or Tween 20 or</td>
<td>256/0</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>Tween 80 (1% each)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a Samples (1 ml) of cell walls were suspended in the various extraction mixtures at a protein concentration of 1 mg/ml. The mixtures were incubated at 37°C with shaking and then centrifuged at 100,000 × g. The supernatant fluids were aspirated, diluted 10-fold with phosphate buffer, and dialyzed for 48 h against distilled water. The resultant precipitates were tested for HA by tube titration. The insoluble extraction residues were washed with 0.01 M phosphate buffer, resuspended to 1-ml volumes, and titrated.

* b Titers listed represent the reciprocal of the highest dilution of each preparation which was able to completely agglutinate 2.5 × 10^7 fresh human erythrocytes.

* c ND, not detected.

* d TX, Triton X-100.

supernatant fluids. TECW that were reextracted with Triton X-100 for up to 18 h at 22°C showed only a twofold titer reduction.

Extraction of cell wall suspensions in 8 M urea or 6 M guanidine-hydrochloride resulted in a severe drop in HA of the fragments. Urea treatment caused complete loss of cell wall HA, whereas guanidine-hydrochloride resulted in a drop in titer from 128 to 4. In neither case was HA recovered from dialyzed extraction supernatant fluids (Table 1).

**Solubilization of hemagglutinin.** Attempts were made to release the agglutinin into the suspending medium by treating TECW with pronase. This, however, left HA still cell wall associated. Reextraction of pronase-treated TECW with 2% Triton X-100–10 mM EDTA at 37°C caused the complete loss of HA from the particles. Dialysis of the supernatant fluid resulted in the appearance of a precipitate which, when titrated, demonstrated arginine-inhibitable HA. HA was associated only with this precipitate and was not detected in a soluble form after dialysis. It should be noted that all preparations of the free agglutinin were soluble only in the presence of detergent (0.1 to 2% Triton X-100).

Whenever the solubilized active preparations were dialyzed against detergent-free buffer, a precipitate which contained agglutinating activity was formed. In a typical experiment, extraction of pronase-treated TECW (1 mg of protein in 1 ml) solubilized 79% of the total residual protein. The inclusion of EDTA in the extraction mixture was necessary to effect HA solubilization. TECW extracted without prior pronase treatment did not release HA (Table 1). When concentrated by ultrafiltration to a volume of 1 ml, the dialyzed extracts had a titer of 16 with a specific activity of 86 HA units per mg of protein.

Characterization of arg− extracts (produced by treating pronase-digested particles with Triton X-100 at pH 10) was accomplished as for arg+ extracts. Typically, the precipitate from dialysis of this extract had a specific activity of 86 HA units per mg of protein (the same as that found for arg+ extracts). The inclusion of arginine in titration mixtures was without effect on titers. Incubation of pronase-treated TECW with 0.1% (vol/vol) Triton X-100 at pH 7.5, or without Triton X-100 at pH 10.0, left HA still cell wall associated and inhibitable by arginine (Table 1). The Triton-pH 10 procedure solubilized between 70 and 80% of total cell wall protein.

**Purification of HA.** The arg+ extract was subjected to preparative IEF in Sephadex G75 Superfine beads. HA was recovered in the section of gel having a pH between 4.5 and 5.5 (Fig. 1). When these fractions (9 to 14) were pooled and
dialysis, HA was recovered with a specific activity of 120 HA units per mg of protein. Specific activities of fractions from this portion of the gel did not vary significantly. They were all arg+. IEF of the arg- extract was also performed under the same conditions as above. The results were identical to those for arg+ extracts. SDS-PAGE of pooled IEF fractions containing HA revealed numerous bands (Fig. 2).

FIG. 2. SDS-PAGE of pooled HA-containing IEF fractions. After dialysis of the IEF gel eluate and assay for HA, the precipitate was solubilized in 1% SDS–1% β-mercaptoethanol at 100°C for 5 min. About 20 μg of protein was applied to the gel. After electrophoresis, the gel was stained with Coomassie blue. Numerals indicate the electrophoretic mobility of molecular weight markers (×10^3).

To effect further purification of HA, appropriate IEF gel eluates were pooled and chromatographed on DEAE-Sephadex. After removal of unadsorbed material with equilibrating buffer, the NaCl gradient was started. Protein was eluted between 0.02 and 0.40 M NaCl. HA was recovered after dialysis of the fractions only at the forefront of the NaCl-eluted peak. A plot of specific activity of each fraction is shown compared to the protein profile of the chromatogram (Fig. 3). The highest specific activity found for any of the fractions was 10,700 HA units per mg of protein for fractions 12 through 14, which eluted at about 0.03 M NaCl. However, titration of the fractions in the presence of up to 10^{-2} M arginine revealed that arginine sensitivity was lost. Codialysis of pooled unadsorbed material with pooled samples of the entire NaCl-eluted peak, or with portions of fractions containing HA, failed to restore arginine sensitivity to the hemagglutinin. Subsequent experiments showed that DEAE chromatography of the arg+ extract even without prior IEF resulted in the loss of arginine sensitivity, but still allowed recovery of HA.

Partial characterization of the hemagglutinin. After pooled, dialyzed fractions of the DEAE chromatogram containing hemagglutinin of the highest specific activity were lyophilized, samples were electrophoresed with and without prior heating (Fig. 4). The samples not heated showed the presence of three distinct bands of protein with apparent molecular weights of about 21,000, 38,000 and 60,000 (arrow). The heated material exhibited only one band with an apparent molecular weight of 21,000. These re-
RESULTS

The results suggest that, even in the presence of SDS and \( \beta \)-mercaptoethanol, at least a portion of the hemagglutinin may exist as monomer and multimer species which are apparently converted completely to the monomer form by boiling in SDS before electrophoresis.

To further confirm the protein nature of the high-specific-activity hemagglutinin preparation, hemagglutinin suspensions were digested with a variety of proteases (Table 2). Incubation of the agglutinin preparation at 37°C in the absence of enzymes (control) resulted in a fourfold drop in titer, indicating some degree of heat sensitivity. Of the proteases tested, only subtilisin caused a complete loss of HA. Subtilopeptidase A, a protease capable of inactivating HA of intact fusobacteria, was without effect. Treatment of whole bacteria with subtilisin also resulted in complete loss of HA of the cells. Thus, the HA of intact fusobacteria and purified agglutinin seem to differ in their sensitivities to subtilisin and subtilopeptidase A.

DISCUSSION

When fusobacteria are ruptured in a French press, cell-bound HA is greatly reduced. Because the whole-cell agglutinin binds to all membranes that we have tested so far, we felt that normally cryptic fusobacterial cytoplasmic membrane fragments might be bound to and thus block the agglutinin molecules. Accordingly, we extracted the fragments by methods found to solubilize cytoplasmic but not outer membrane proteins. Schnaitman (28, 29) has shown that Triton X-100 is able to completely solubilize the cytoplasmic membrane while leaving the outer membrane virtually intact. EDTA, together with Triton X-100, was required to solubilize significant amounts of outer membrane protein. Since Triton X-100 alone did free the agglutinin, it is possible that cytoplasmic membrane components did indeed block the agglutinin. Biochemical markers for fusobacterial cytoplasmic membrane, unlike those found in other organisms (11, 19, 20), have not been determined. The identification of markers specific for the fusobacterial cell membrane would help to ascertain whether the agglutinin is indeed blocked by these components.

The fact that addition of EDTA in the extraction mixture still leaves HA associated with the cell fragments indicates that it is not part of an outer membrane complex similar to that of \( E. \) coli which was solubilized by Schnaitman (29). Only after pronase treatment of cell wall fragments was a significant decrease in particle-associated HA noted upon reextraction with Triton-EDTA. Cell fragments not treated with pronase before extraction did not exhibit any significant decrease in HA. These results suggest that protein-protein interactions are responsible for the resistance of the agglutinin to detergent solubilization. The interaction of various proteins in the outer membranes of other gram-negative organisms is well documented. Cross-linking experiments and isolation of pore-forming complexes from \( E. \) coli (21, 23), \( P. \) aeruginosa (10), and \( S. \) typhimurium (13, 16, 22) demonstrate association and cooper-

### TABLE 2. Effects of various proteases on purified hemagglutinin and hemagglutinin of intact fusobacteria

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Titer after enzyme treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Purified hemagglutinin</td>
</tr>
<tr>
<td>Control (no enzyme)</td>
<td></td>
</tr>
<tr>
<td>Stored at 5°C, 96 h</td>
<td>96</td>
</tr>
<tr>
<td>Incubated at 37°C, 18 h</td>
<td>24</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>0</td>
</tr>
<tr>
<td>Subtilopeptidase A</td>
<td>24</td>
</tr>
<tr>
<td>Trypsin, papain, ( \alpha )-chymotrypsin, K+K pronase, Calbiochem protease, ( S. ) caespitosi protease</td>
<td>24</td>
</tr>
</tbody>
</table>

* HA obtained after IEF and DEAE-Sephadex chromatography of arg* Triton extracts was treated with proteases as described in the text. The effects of these enzymes on the HA while still cell wall associated on intact fusobacteria is presented for comparison. Titters listed represent the reciprocal of the highest dilution of each preparation which was able to completely agglutinate \( 2.5 \times 10^7 \) fresh human erythrocytes. 

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Fig. 4. SDS-PAGE of high-specific-activity hemagglutinin. HA-containing fractions obtained after IEF and DEAE-Sephadex chromatography were pooled and lyophilized. The residue was dissolved in a mixture of 1% SDS and 1% \( \beta \)-mercaptoethanol at 100°C (A) or 22°C (B) and electrophoresed. About 20 \( \mu \)g of protein was applied to each gel. Each was stained with Coomassie blue. Numbers indicate the electrophoretic mobility of molecular weight markers (\( \times 10^3 \)).
activity of various components. Such interactions are probably a significant feature of the fusobacterial cell wall outer membrane complex.

The extraction of arg⁺ HA from pronase-treated TECW at 37°C demonstrated that elevated temperature and a chelator seemed to destabilize the cell fragments, allowing solubilization of the agglutinin. Lower temperatures (30, 22°C) were not effective. This also suggested a temperature-dependent change in properties of the cell surface typical of a membrane structure. Elevated temperature has been shown to increase both the fluidity of isolated E. coli outer membrane (17, 25) and the efficiency of solubilization of membrane components by detergents (24).

The recovery of HA from the pH 4.5 to 5.5 portion of the isoelectric gel and its retention on DEAE-Sephadex at slightly alkaline pH show that it is an acidic protein species. When HA-containing fractions were pooled and refocused under identical conditions, the protein profile of the gel was very much like that of the first focusing. HA was also recovered from the same portion of the gel. The focusing process could be repeated up to three times with similar results. These observations suggested that the agglutinin is extracted as or becomes part of a multicomponent complex which is not completely resolved by IEF.

Subtilisin and subtilopeptidase A are proteases able to destroy HA of intact fusobacteria. HA obtained after IEF followed by DEAE chromatography, however, loses its susceptibility to inactivation by subtilopeptidase A but retains sensitivity to subtilisin. Neither the cell-bound nor the soluble agglutinin was sensitive to a variety of glycosidases (fucosidase, mannosidase, galactosidase, amylase, neuraminidase). These observations enhance the evidence presented in previous work (8) that the agglutinin is, at least in part, protein. The differences in enzyme sensitivity of cell-associated versus purified hemagglutinin preparations are not totally unexpected. Such effects undoubtedly reflect differences in accessibility of the enzymes to the agglutinin while associated with other components in situ or after undergoing purification. Similar observations have been made when the traT surface exclusion protein of E. coli outer membrane is exposed to trypsin. While still membrane associated, the protein is not digested. After partial purification, however, trypsin will cleave the molecule (15).

Attempts to reconstitute arg⁺ HA with various fractions obtained after DEAE-Sephadex chromatography were unsuccessful. Although our data show essentially quantitative recovery of protein, it is entirely possible that some other component of the extract or a small portion of the protein applied remained bound to the column. Since Triton X-100 was incorporated in all elution buffers, the role of hydrophobic forces in binding would be minimal. Efforts are currently under way to find different elution systems which allow isolation of a substance(s) which confers arginine sensitivity to the hemagglutinin.

The question of whether arg⁺ and arg⁻ agglutinin are entirely different molecules can be raised. Our evidence argues against this. IEF and SDS-PAGE revealed essentially the same migration pattern for both arg⁺ and arg⁻ agglutinins. Furthermore, when the agglutinin that was entirely arg⁺ was treated with alkali (pH 10) and Triton X-100 it was completely converted to arg⁻. Also, chromatography of an entirely arg⁻ component on DEAE resulted in elution of a completely arg⁻ agglutinin. Thus it appears that arg⁻ agglutinin is derived from the arg⁺ molecule. The best support for the single-hemagglutinin interpretation would be the successful reconstitution of arg⁺ HA by using purified arg⁻ agglutinin.

Some functions of the gram-negative outer membrane seem to depend on the integrity of multicomponent systems. This is exemplified by the in vitro reconstitution of Tula and TuB coliphage receptors from purified lipopolysaccharide and outer membrane proteins. Either component alone had no receptor activity (7). Similarly, the passive diffusion function of the lambda receptor of E. coli was reconstituted (3). However, its discriminatory function towards maltose was lost. Such a result demonstrates that radical changes in the biological activities of reconstituted systems can occur. The fusobacterial hemagglutinin may also be on a multicomponent system.

Evidence for a multimeric arrangement of the agglutinin is revealed by SDS-PAGE. Analysis of the DEAE column revealed that hemagglutinin, solubilized in SDS at 100°C, migrated as a single band with an apparent molecular weight of about 21,000. Hemagglutinin not boiled before electrophoresis revealed several bands with apparent molecular weights equal to and approximately twice and three times that of the 21,000-molecular-weight component. Apparently the multimeric association is stable at room temperature (22°C) even in the presence of SDS and β-mercaptoethanol. It is not known whether the higher-molecular-weight components are composed of homologous or heterologous subunits. End-group analysis of larger amounts of material may reveal the similarity or dissimilarity of the subunits.

LITERATURE CITED


