Purification of a Galactosyl-α1–4-galactose-binding Adhesin from the Gram-positive Meningitis-associated Bacterium Streptococcus suis*

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Streptococcus suis causes meningitis, sepsis, and other serious infections in newborn and young pigs and in adult humans. The Galα1–4Gal-binding adhesin of S. suis was purified to homogeneity by ultrasonic treatment, fractional ammonium sulfate precipitation, and preparative polyacrylamide gel electrophoresis. Pigeon ovomucoid, a glycoprotein with Galα1–4Gal terminals, was used to detect the adhesin by blotting. The purified adhesin appeared as single band of an apparent size of 18 kDa and of a pI of 6.4; no disulfide bridges were present. The amount of adhesin as revealed by pigeon ovomucoid binding correlated with the hemagglutination activity of different S. suis strains. The purified adhesin bound to latex particles induced hemagglutination which was specifically inhibited with the same inhibitors as hemagglutination by the intact bacteria, thus demonstrating that the purified protein was the Galα1–4Gal-recognition adhesin of S. suis. Two adhesin variants (P₁ and P₂) with differing Galα1–4Gal binding specificity had the same electrophoretic mobilities and the same N-terminal peptide sequences, indicating that they were closely related. This represents the first isolation of an adhesin with well-defined cell surface carbohydrate binding activity from Gram-positive bacteria associated with meningitis.

The first event in the establishment of infectious diseases is the adhesion of bacteria to the surface of host cells (1). Adhesins mediating this interaction are thus essential factors in bacterial pathogenesis and important virulence factors (2, 3). Many adhesins act as lectins, recognizing specific carbohydrate moieties on host cell surface glycoconjugates. Most of the knowledge on bacterial carbohydrate-binding adhesins is derived from studies on Gram-negative bacteria (reviewed in Ref. 4), whereas only little information exists on the molecular identity and detailed binding properties of such adhesins in Gram-positive bacteria.

Streptococcus suis is an important Gram-positive pathogen which causes meningitis, sepsis, and other serious infections in pigs (5–7) and meningitis in humans who have been in contact with pigs (8, 9). The identification and characterization of the molecules responsible for the interaction of the bacteria with host cells would give valuable information for the understanding of the pathogenesis of the infection and is a key factor in the development of new antibacterial agents and vaccines.

In previous studies, the binding of S. suis bacteria to host cells was found to be mediated by an adhesin activity which recognizes the disaccharide galactosyl-α1–4-galactose (10, 11). In the present study, the adhesin was identified, purified to homogeneity, and characterized and was found to retain the hemagglutination activity and specificity of the intact bacteria.

EXPERIMENTAL PROCEDURES

Materials—Pigeon ovomucoid was purified as described previously (12). Hen ovomucoid, N-acetylgalactosamine, and N-acetylgalactosamine were obtained from Sigma, Galactose and mannose were obtained from Fluka, Buchs, Switzerland. Sialidase (Vibrio cholerae) was obtained from Behringwerke AG, Marburg, Germany. Phenylmethylsulfonyl fluoride was obtained from Boehringer Mannheim, Germany. Bovine serum albumin was obtained from Sigma, ammonium sulfate from Riedel-de Haën, Seelze, Germany, and Na125I from Amersham, UK. Acrylamide, bisacrylamide, and N,N,N’,N’-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad, sodium dodecyl sulfate from Fluka. Low molecular weight standards, isoelectric focusing standards (IEF standard 3–10), and Phast Gel 3–9 were obtained from Pharmacia, Sweden. Bacto-latex 0.81 standardized suspension of latex particles was purchased from Difco.

Todd-Hewitt broth and a Gas-Pak anaerobic system were purchased from Becton Dickinson and Co., Cockeysville, MD. Microwell Microtiter Plates were from Dako, Roskilde, Denmark, and polystyrene flat bottom Microtrips from Labsystems, Helsinki, Finland. IODOBEADS were purchased from Pierce. Bio-Gel P-6DG Desalting Gel was from Bio-Rad. Nitrocellulose sheets were from Schleicher & Schuell, Dassel, Germany, and PVDF-P membrane from Millipore.

Bacterial Strains—The S. suis strains studied have been described before (13). Strain 628 was obtained from Dr H. C. Zanen, Academic Medical Centre, Amsterdam, the Netherlands; strains TEW/2 and R75/L1 were obtained from Dr F. Clifton-Hadley, Clinical Veterinary School, University of Cambridge, UK, and strains 836, 825, 752, 3031, 598, 3027, and 1045 from Dr J. Hommez, Regional Veterinary Investigation Laboratory, Torhout, Belgium. The bacteria were maintained in Todd-Hewitt broth at −20 °C and were grown on sheep blood agar plates overnight at 37 °C under anaerobic conditions (Gas-Pak system). The bacteria were harvested from the plates, washed twice, and suspended in phosphate buffer A (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) and adjusted to a concentration that gave an A₆₀₀ nm of 0.5 at 1:100 dilution.

Hemagglutination and Hemagglutination Inhibition Assays—Erythrocytes obtained from healthy adults were washed and treated with sialidase as described before (14) and used at 2% concentration. The microtiter and slide hemagglutination assays were done as described before (14) on microtiter plates using 25 μl of bacterial suspension prepared as described above.

Protein Determination—Protein determination was carried by an adaptation of the method described by Bradford (15) on polystyrene Microtrips using Bio-Rad Protein Assay Dye Reagent Concentrate. The absorbance at A₅₉₅ nm was measured by SLT Labinstruments Easy Reader SF plus ELISA reader.

Radio-labeling of Pigeon Ovomucoid—Pigeon ovomucoid was labeled with 125I using the IODOBEAD method according to the instructions of

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the manufacturer. A total amount of 0.3 mCi of Na251 was added to 250 μg of ovomucoid in 250 μl of buffer A, and a bead was added. The reaction mixture was incubated for 30 min at room temperature with occasional shaking. The reaction mixture was removed from the bead, and 0.5 mM NaI was added to a concentration of 10 mM. Nonincorporated iodine was separated by chromatography on a desalting column (Bio Gel P-6DG, 1 × 3 inch) equilibrated wads on a slide and incubated. Water. Bovine serum albumin was added to the labeled pigeon ovomucoid to a concentration of 1 mg/ml, and the preparation was stored at −80 °C and used within 2 weeks.

Dot Assay and Blot Binding of Radiolabeled Pigeon Ovomucoid—Adhesin activity was identified from whole bacterial cells and electrophoretically separated proteins by dot and blot binding, respectively, of radiolabeled pigeon ovomucoid. In dot assays, 1–μl dots of bacterial suspensions diluted 1:1, 1:10, and 1:50 in phosphate buffer A were pipetted onto a grided nitrocellulose paper. For blot binding analyses, the polyacrylamide gel electrophoresis (16) was used without sodium dodecyl sulfate in order to retain the pigeon ovomucoid binding activity of the adhesin. Proteins from the bacterial sonicates were separated in a 6% polyacrylamide gel electrophoresis in a Bio-Rad Mini Protein II device. The bacterial extracts in a volume of 75 μl were mixed with 23 μl of sample solution containing 0.65 mM sucrose and 4 mM EDTA in 87 mM Tris-HCl, pH 8.0, and 2 μl of 0.1% bromphenol blue, and 20 μl of the mixtures were layered into the sample wells. After electrophoresis, the proteins were transferred electrophoretically to a PVDF-P membrane (5, 10, or 25 ml) by the method of Towbin et al. (17) by using 10 mM CAPS buffer (pH 11.2) methanol (9:1, v/v).

Nonhybridizing sites were saturated by incubation of the membranes for 1.5 h in phosphate buffer C (0.1 mM sodium phosphate buffer, 0.5% Tween 20, 150 mM NaCl, pH 5.3). The membranes were incubated with the 125I-labeled pigeon ovomucoid (6 × 106 cpm/25 μl), specific activity about 2.5 × 108 cpm/μg, in phosphate buffer C for 1 h at +8 °C. The membranes were washed three times for 10 min in phosphate buffer C, dried between filter papers, and exposed to an X-ray film with an intensifying screen at −80 °C for 5–48 h.

Extraction of Adhesin—Adhesin protein was extracted from the bacteria by sonicating the bacterial suspension with an ultrasonic probe in phosphate buffer A five times for 15 s on ice with a chilling interval of 1–2 min between the sonications. After sonication, phenylmethylsulfonyl fluoride was added to 2 mM final concentration, and insoluble material was removed by centrifugation at 15,800 × g for 20 min at 8 °C.

Ammonium Sulfate Precipitation—The sonication supernatant (16 ml) was made 60% with respect to ammonium sulfate saturation by the addition of cold saturated ammonium sulfate (24 ml) in an ice bath. After 1 h, the resulting pellet was removed by centrifugation at 15,800 × g for 20 min, and the supernatant was made 70% with respect to ammonium sulfate saturation by the addition of cold saturated ammonium sulfate (13.3 ml). The pellet was collected after 1 h by centrifugation, and resuspended in 16 ml of phosphate buffer B (10 mM sodium phosphate, 0.05 mM NaCl, pH 7.4), dialyzed overnight at +8 °C against water, lyophilized, and dissolved in 4 ml of phosphate buffer A.

Preparative Gel Electrophoresis—Preparative gel electrophoresis was performed in the absence of SDS in a Bio-Rad 491 Prep Cell device. The heights of the cylindrical stacking and separating polyacrylamide gels (6%) were 2 and 15 cm, respectively. Dialyzed and lyophilized 70% ammonium sulfate pellet (3 ml) was mixed with 920 μl of sample solution (920 μl) and bromphenol blue (80 μl) and pipetted onto the gel which was run at constant current of 40 mA. The elution buffer was 25 mM Tris-HCl, pH 8.3. Fractions of 4 ml were collected and analyzed by analytical electrophoresis in 6% polyacrylamide gels that were stained with Serva Blue G. Fractions containing the adhesin protein were combined, dialyzed overnight at +8 °C against phosphate buffer B, lyophilized, and stored at −20 °C.

SDS-Gel Electrophoresis and Isoelectric Focusing—For gel electrophoresis in the presence of SDS (16), the purified adhesin was run in 15% polyacrylamide gels under reducing and nonreducing conditions. Low Molecular Weight Standards (Pharmacia, Sweden) were run parallel for calibration. The focusing was performed using the Phast Gel electrophoresis device with the Phast isoelectric system (Pharmacia). The Phast Gel 3–9 and the isoelectric focusing standard 3–10 were used. The gel was stained by using Silver IEF-Method 6 of the Phast system (18).

Amino Acid Analysis—For the amino acid analysis, 7 nmol of purified adhesin was dissolved in 100 μl of 6 M HCl. 60 nmol of norleucine was added as an internal standard. The solution was hydrolyzed at 110 °C for 24 h, lyophilized, and analyzed with an LKB 4151 Alpha Plus Amino Acid Analyzer according to the instructions of the manufacturer.

Amino Acid Sequencing—The N-terminus amino acid sequence of the adhesin was determined by Applied Biosystems 477A Purified Liquid Protein/Peptide Sequencer with 120A Amino Acid Analyzer according to the instructions of the manufacturer. Purified adhesin or sonicates were subjected to electrophoresis in 6% polyacrylamide gels and transferred to PVDF-P membranes as described above. The membranes were stained with Serva Blue for 10 min, and, after destaining of the extra stain, the adhesin band was cut off for peptide sequencing. The position of the adhesin was identified by pigeon ovomucoid blotting of adjacent lanes.

Latex-induced Hemagglutination—A suspension of 5 × 106 latex particles in a volume of 100 μl was centrifuged at 8000 × g for 2.5 min and washed twice with phosphate buffer A. The particles were suspended into 90 μl of phosphate buffer A, and 0.4 μg of either purified adhesin or bovine serum albumin was added in a volume of 10 μl and the suspensions were incubated for 2 h at room temperature on a tilting table. The suspensions were centrifuged and washed twice as above. Phosphate buffer A (45 μl) and bovine serum albumin (5 μl, 5 mg/ml) were added, and the mixtures were incubated on a tilting table at room temperature for 1 h. The suspensions were centrifuged and washed twice as above, and the latex pellets were suspended into 50 μl of phosphate buffer A.

Latex Hemagglutination and hemagglutination inhibition assays were performed as described before (14). Adhesin-covered latex particles (5, 10, or 25 μl) were mixed with a 4% suspension of sialidase-treated human erythrocytes (5, 10, or 25 μl, respectively) on a ceramic slide and incubated on ice for 10 min. In agglutination inhibition assays, 10 μl of adhesin-covered latex particles were mixed with 10 μl of the inhibitory compounds on a slide and incubated on ice. After 15 min, 20 μl of sialidase-treated 4% human erythrocytes were added, and the mixture was incubated for 15 min on ice. Similar agglutination reactions were obtained in volumes of 5 to 25 μl of latex with 5 to 25 μl of sialidase-treated erythrocytes, respectively. Bovine serum albumin-coated latex particles (0–0.1 mg/25 × 104 particles) used as controls gave no hemagglutination reactions.

RESULTS

Detection of S. suis Adhesin Activity with Pigeon Ovomucoid—Pigeon ovomucoid contains blood group P1 (Galα1–4Galβ1–4GlCNacβ1–1) terminals on its glycans (19). In hemagglutination inhibition studies, pigeon ovomucoid was shown to be highly active as an inhibitor of the hemagglutination induced by Galα1–4Gal-binding S. suis (10). Pigeon ovomucoid was therefore tested as a ligand for the detection of the adhesin activity in S. suis cells in a dot binding assay. Radiolabeled pigeon ovomucoid was found to bind efficiently to S. suis cells with hemagglutination activity, whereas the nonhemagglutinating control strain did not bind pigeon ovomucoid (Fig. 1). Furthermore, the binding correlated with the hemagglutination activity of the strains. Pigeon ovomucoid binding was subsequently used for the detection of the adhesin activity during purification of the adhesin from the bacteria.
Isolation of Galα1-4Gal Adhesin of S. suis

Figure 2. Purification of the adhesin protein by fractional ammonium sulfate precipitation. The sonication supernatant of S. suis strain 628 was subjected to fractional ammonium sulfate precipitation, and the precipitates of the 60, 70, and 80% ammonium sulfate saturation precipitates and the starting sonication supernatant (S) were subjected to electrophoresis in 6% polyacrylamide gels in the absence of SDS. The gel was stained for protein (A), or the proteins were transferred to PVDF-P membrane which was probed for adhesin activity with 125I-labeled pigeon ovomucoid (B).

Purification of the Adhesin—Several methods used previously for the extraction of adhesins from bacteria were tried for the extraction of the S. suis adhesin. These included heat treatments with or without mechanical homogenization (20, 21) or extraction with lithium 3,5-diiodosalicylate (22) or alkali (23). These methods were, however, not suitable for the isolation of the S. suis adhesin. Among the methods tried, sonication with high energy was the only successful method for detaching the adhesin from S. suis cells.

In fractional ammonium sulfate precipitation of the sonicate (50 mg of protein/16 ml of sonicate), the adhesin activity was precipitated with ammonium sulfate with a saturation degree of 70% (Fig. 2). Ammonium sulfate having a saturation degree of 60% precipitated the contaminating proteins running near the adhesin, as analyzed by polyacrylamide gel electrophoresis in the absence of SDS and Western blotting with radiolabeled pigeon ovomucoid.

The adhesin was finally purified to homogeneity from the 70% ammonium sulfate precipitate (1 mg of protein/16 ml of sonicate) by preparative gel electrophoresis. Fractions from the preparative gel were collected and analyzed by gel electrophoresis. The adhesin was eluted as a single band (Fig. 3).

Characteristic of the Purified Adhesin—The purified adhesin appeared in SDS-polyacrylamide gel electrophoresis as a single homogeneous band of an apparent size of 18 kDa (Fig. 4A). The mobility was not changed by the use of a reducing agent, which suggested the absence of disulfide bridges. This was also confirmed by amino acid analysis which revealed the absence of cysteine residues (Table I). The amino acid composition indicated that the adhesin is rich in glutamic acid and glycine and also contains high concentrations of alanine and lysine. The adhesin contained somewhat more acidic (21.7%) than basic (16.0%) residues and a relatively high proportion of hydrophobic amino acids (40.3%). The isoelectric point of the purified adhesin was 6.4 (Fig. 4B).

Table I. Amino acid composition of the S. suis adhesin

| Amino acid                        | Mol % |
|-----------------------------------|-------|
| Aspartic acid or asparagine       | 7.2   |
| Threonine                         | 4.5   |
| Serine                            | 3.2   |
| Glutamic acid or glutamine        | 14.5  |
| Glycine                           | 12.2  |
| Alanine                           | 9.3   |
| Valine                            | 5.7   |
| Cysteine                          | 0.0   |
| Methionine                        | 0.1   |
| Isoleucine                        | 6.3   |
| Leucine                           | 6.8   |
| Tyrosine                          | 2.1   |
| Phenylalanine                     | 3.3   |
| Lysine                            | 8.7   |
| Histidine                         | 3.6   |
| Arginine                          | 3.7   |
| Proline                           | 6.8   |

Gala1-4Gal binding specificity (11). The N termini of adhesins determined from electrophoretic blots of four other S. suis strains, two of type P_N (TEW/2, R75/L1) and two of type P_O (752, 825), were identical with the purified adhesin.

Correlation of Amount of Adhesin with Hemagglutinating Activity—In order to investigate whether the varying hemagglutination activities of different S. suis strains could be ex-
the presence of the Galadhesin, erythrocytes on a glass slide (Table II). As compared to the free strong hemagglutination reaction was achieved with sialidase-adhesin polyvalent, it was adsorbed onto latex particles. A presumably due to its monovalent nature. In order to make the (Fig. 5, strain 628).

The intensity of the band correlated with the hemagglutinating titers of the corresponding strain. Like in many other bacteria (24), the P hemagglutination of S. suis undergoes spontaneous phase variation. Also in bacteria extracted in the highly agglutinating or low agglutinating phases, the intensity of the adhesin bands correlated with the agglutinating titer (Fig. 5, strain 628).

Agglutination Activity of the Purified Adhesin—The purified adhesin expressed only weak hemagglutinating activity, presumably due to its monovalent nature. In order to make the adhesin polyvalent, it was adsorbed onto latex particles. A strong hemagglutination reaction was achieved with sialidase-erythrocytes on a glass slide (Table I). As compared to the free adhesin, 0.2 μg of which agglutinated sialidase-treated human erythrocytes on a microtiter plate weakly with a titer of 1:1, 0.2 μg of the adhesin adsorbed to latex particles hemagglutinated the erythrocytes with a titer of 1:16. No agglutination was achieved with latex particles coated with bovine serum albumin. On the other hand, the hemagglutination induced by the adhesin-bound latex particles was inhibited specifically with the same inhibitory compounds, galactose, N-acetylgalactosamine, and pigeon ovomucoid as hemagglutination induced by whole bacteria (10, 11).

DISCUSSION

In previous studies, we found that pigeon ovomucoid, due to the presence of the Galα1-4Gal-containing blood group P1 active glycans, is an effective inhibitor of the P adhesins of S. suis (10, 11). This glycoprotein was therefore used as an indicator for the presence of the adhesin in S. suis cells by dot binding assay and for the identification of the adhesin protein during its purification. Different lines of observations suggest that the protein isolated was indeed the adhesin responsible for the hemagglutinating activity of the bacteria. The hemagglutination activity correlated with pigeon ovomucoid binding both in whole cells and in the sonication extracts of the cells. In the latter, only one pigeon ovomucoid-binding component was present and corresponded in mobility to the purified protein. Furthermore, the purified protein adsorbed to latex particles inhib}

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**TABLE II**

| Assay condition | Hemagglutination |
|-----------------|------------------|
| Adhesin-latex   | +++              |
| Control latex   | -                |
| Adhesin-latex with |                |
| Galactose (100 μM) | -              |
| Mannose (100 μM) | +++              |
| Glucose (100 μM) | +++              |
| N-Acetylglactosamine (25 μM) | - |
| N-Acetylgalactosamine (25 μM) | +++ |
| Pigeon ovomucoid (25 μg/ml) | - |
| Hen ovomucoid (25 μg/ml) | +++ |

*Latex particles coated with purified adhesin or bovine serum albumin (control) were mixed with sialidase-treated human erythrocytes. The hemagglutination activities were rated from absent (−) to strong (+++).*
able to serve as binding ligands for \textit{S. suis} containing P adhesin to frozen sections of pig pharyngeal tissue (10). However, the in vivo roles of the different adhesion specificities remain so far unresolved. Identification and purification of the P adhesin of \textit{S. suis} will now make possible the cloning of the corresponding gene and thus facilitate studies designed to elucidate the molecular mechanism of the binding interaction, the basis of the differential binding of the variant adhesins, and the pathogenic role of the adhesin.

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