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Fimbriae from the Oral Anaerobe *Bacteroides gingivalis*: Physical, Chemical, and Immunological Properties

FUMINOBU YOSHIMURA,[1]* TOSHIHIDE TAKASAWA,[2]† MICHIO YONEYAMA,[3] TAIHEI YAMAGUCHI,[1] HIROYUKI SHIOKAWA,[2] AND TAKESHI SUZUKI[1]

Department of Microbiology, School of Dentistry,[1] Section of Biochemistry, Institute of Immunological Sciences,[2] and Section of Biopolymer Science, Department of Polymer Science, Faculty of Science,[3] Hokkaido University, Sapporo 060, Japan

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Circular dichroism spectra indicated the predominance of β-sheet structure in *Bacteroides gingivalis* fimbriae regardless of the presence of sodium dodecyl sulfate. By using a computer program, the α-helix, β-sheet, and β-turn contents and the remainder were estimated to be 0, 55, 18, and 27%, respectively, judging from the circular dichroism spectra of the fimbriae. Heating for 5 min at 100°C in sodium dodecyl sulfate was necessary to denature the fimbriae into their constituent protein (fimbrilin) monomers with a reduced content of β-sheet structure. The amino-terminal amino acid sequence of the fimbrilin was different from partial or complete amino acid sequences of fimbrilins so far determined from *Bacteroides nodosus*, which falls into the same nonfermentative species of the genus *Bacteroides* as *B. gingivalis*, and from various other bacteria. Fimbrilin monomers had an isoelectric point of 6.0. Examination of antibodies against fimbriae and sodium dodecyl sulfate-denatured fimbrilin by enzyme-linked immunosorbent assay reinforced a previous notion (F. Yoshimura, K. Takahashi, Y. Nodasaka, and T. Suzuki, J. Bacteriol. 160:949–957, 1984) that different sets of antigenic determinants seemed to be exposed on their surfaces.

Bacteroides gingivalis, a gram-negative oral anaerobe, is a suspected periodontal pathogen and is believed to have several characteristics of an overt pathogenic bacterium (12, 26, 27). *B. gingivalis* cells can colonize periodontal pockets and can sometimes be prominent members of the pocket microflora at the advanced stage of adult periodontal diseases (27). Their prevalence in the dental pockets is probably fostered by their affinity for sulcular epithelium, saliva-coated hydroxyapatite, or a number of oral gram-positive organisms (28).

Fimbriae, filamentous appendages, seem to be one of the ligands for several adhering functions in this organism. Recently, *B. gingivalis* fimbriae have been purified, and their chemical, morphological, and immmunological properties have been characterized (35). The purified fimbriae have a number of unique characteristics; for example, they are curly filaments composed of a single strand ca. 5 nm in width, and the apparent molecular weight (43,000) of their constituent protein (fimbrilin) is considerably higher than the molecular weights previously reported for fimbriae from other bacteria (22).

Research in our laboratory is aimed at the elucidation of the precise function of fimbriae as an adherent factor, as well as at determining the molecular basis for their unique structure, i.e., stable single-stranded filaments without a hollow structure. In this paper, we show that the *B. gingivalis* fimbriae have a high content of β-sheet structure and an amino-terminal acid sequence different from the sequences of fimbrilins so far reported, and we present data on further chemical and immunological properties of this material.

* Corresponding author.

† Present address: Laboratory of Chemistry, Liberal Arts Division, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain used was *B. gingivalis* 381 (originally isolated from subgingival plaque by S. S. Socransky, Forsyth Dental Center, Boston, Mass.). Mass cultures were grown anaerobically in General Anaerobic medium (Nissui, Tokyo, Japan) at 37°C as described previously (34).

Purification of fimbriae from the bacterial wash fluid. Freshly harvested cells (18 g [wet weight] from 3 liters of culture) were suspended in 300 ml of 20 mM Tris-hydrochloride (pH 7.4)–0.15 M NaCl–10 mM $MgCl_2$ by repeated pipetting. The suspension was stirred magnetically for 30 min. The bacterial wash was obtained as the supernatant after centrifugation at 8,000 × *g* for 20 min. The above procedure was carried out at room temperature to avoid cell lysis. The following procedures of purification were carried out as described in our previous report (35), except that the final preparation was dialyzed against a different buffer, such as 5 mM Tris-hydrochloride (pH 8.0)–0.1 M NaCl. The purity, judged by the scanning of the stained sodium dodecyl sulfate (SDS)-polyacrylamide gel, was at least 98%.

Preparation of fimbrilin monomers, LPS, and antisera. Fimbrilin monomers were prepared by heating the purified fimbrial preparation in 1% (wt/vol) SDS at 100°C for 10 min, followed by precipitation by addition of 2 volumes of cold acetone to remove SDS. The precipitated denatured fimbrilin monomers were collected by centrifugation (8,000 × *g* for 30 min) and washed twice with 70% cold acetone, with each wash followed by centrifugation. The amount of SDS bound to the protein was reduced to a level as low as several micrograms of SDS per milligram of protein (9) by these acetone treatments. The final precipitate was dried under an N_2 stream and then suspended in a small volume of 50 mM bicarbonate buffer (pH 9.6). Fimbrilin preparation for carbohydrate analysis was carried out by using preparative SDS-polyacrylamide gel electrophoresis (PAGE), followed

by electroelution and dialysis (4). Lipopolysaccharide (LPS) of *B. gingivalis* ATCC 33277, isolated by phenol-water extraction, and anti-LPS serum were generously donated by E. Isogai, Higashi-Nihon College, Tobetsu, Japan. Antisera against native fimbrial polymers and denatured fimbrilin monomers were the same preparations used previously (35).

CD spectra. A Jasco J-20 automatic recording spectropolarimeter was used for measuring circular dichroism (CD) spectra. The spectra were measured in quartz cells at a protein concentration of 0.22 mg/ml with or without 0.2% SDS. The data were expressed in terms of mean residue ellipticity [θ], in units of degrees · square centimeters per decimolephenylthiohydrantoin. The mean residue weight of protein was assumed to be 107.5 (apparent fimbrilin molecular weight of 43,000 per total residues of 400) (35). The secondary structure of the protein was analyzed according to the method of Provencher and Glöckner (23). The computer program was originally provided by S. W. Provencher.

Amino-terminal sequencing. A Beckman model 890C sequencer was used for the automatic amino-terminal amino acid sequencing. Polybrene was used as a nonprotein carrier to retain the protein in the spinning cup as described by Tarr et al. (31). Fractions from the sequencer were evaporated under N_2 gas and treated with methanol at 55°C for 5 min (13), and the solvent was evaporated to dryness. The thiazolinone derivatives were converted to (PTH)-amino acids in 1 N HCl–methanol at 55° C for 10 min by the method of Tarr (29, 30). Identification of PTH-amino acids was carried out with an Hitachi liquid chromatography apparatus, model 635A (Unisil 5C18 column, 4 by 250 mm; ca. 17,000 plates per column; Gasukuro Kogyo Co., Ltd., Tokyo, Japan).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed essentially as described by Engvall and Perlmann (7), with the conjugate and substrate system described by Ruitenberg et al. (24). Antigens were dispersed in 50 mM bicarbonate buffer (pH 9.6) to a concentration of 15 μg/ml. Disposable polystyrene microtiter trays (100-81; Sanko Junyaku Co., Tokyo, Japan) were filled with antigen suspension (1.5 μg per well) and incubated for 3 h at 37°C. Nonattached antigen was removed by two washings with tap water, followed by one washing with buffer A (0.05% Tween 20–0.1% bovine serum albumin–10 mM phosphate buffer [pH 7.2]–0.15 M NaCl). Dilution series of the rabbit antisera were made with buffer A. Wells were filled with 100 μl of diluted serum. Trays were incubated for 2 h at 37°C. After the three washes described above, 100 μl of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Cappel Laboratories, Cochranville, Pa.) diluted 1,500 times with buffer A was added to each well, followed by 2 h of incubation at 37°C. Trays were washed three times with tap water, and 200 μl of peroxidase substrate solution was applied to each well. Peroxidase substrate was composed of 100 ml of 0.1 M phosphate buffer (pH 6.0), 100 mg of *o*-phenylenediamine, and 34 μl of H_2O_2 (30% [wt/wt]). The reaction was developed for 30 min at room temperature and terminated by the addition of 4 N HCl (50 μl). The absorbance at 490 nm was measured by an ELISA reader (MicroELISA Minireader MR590; Dynatech Laboratories, Inc., Alexandria, Va.). The endpoint titer was determined as the highest serum dilution giving an absorbance 0.3 U higher than that of negative controls.

Analytical methods. Sugars were determined by the phenol-sulfuric acid method (11), with D-glucose as the standard. Total phosphorus was assayed by using ashed samples (2). The method of Lowry et al. (16) was routinely used for protein determination with bovine serum albumin as the standard. SDS-PAGE was performed in a 1.0-mm-thick slab gel as described by Lugtenberg et al. (17). Preparative electrophoresis was carried out in the same system by the method of Deal and Kaplan (4). Gels were stained with Coomassie brilliant blue or silver. LPS was stained by a modified version of the method of Tsai and Frash (32). By this staining method, as little as 0.03 μg of protein of *B. gingivalis* fimbriae could also be stained.

Isoelectric point. The isoelectric point of the proteins was determined by running two-dimensional gel electrophoresis, which was done according to the method of O'Farrell (21) with the SDS solubilization procedure of Ames and Nikaido (3). A blank tube was subjected to isoelectric focusing, and then the gel was extruded from the tube. This gel was sliced into 1-cm segments, and each piece was put into 2 ml of water. After 2 to 3 h, the pH of each segment was measured with a pH meter. The pH varied linearly along the length of the gel from 4.0 to ca. 7.5. The isoelectric point of the protein was estimated by measuring how far it had migrated in the isoelectric focusing direction.

RESULTS

Secondary structure in the fimbrial preparations. We have shown that *B. gingivalis* fimbriae are very stable, unique filaments even in the presence of SDS (35). Heat stability of this material in SDS at 70°C prompted us to study secondary structure in the fimbriae preparations by measuring the CD spectra in 5 mM Tris-hydrochloride (pH 8.0)–0.1 M NaCl between 200 and 250 nm. Only one negative peak, at 216 nm, was seen with the preparations, regardless of the presence of SDS (0.2%) (Fig. 1, curve 1), a result indicating the presence of large amounts of β-sheet structure (data not shown in the absence of SDS) (1). There was no indication of the presence of significant amounts of α-helices; i.e., there was no negative peak or shoulder at 208 or 222 nm (1). For the purpose of determining the α-helix, β-sheet, and β-turn contents and the remainder more precisely, computer analyses were performed with the help of Fumio Arisaka and Shin-ich Ishii, Hokkaido University. The α-helix, β-sheet, and β-turn contents and the remainder were estimated to be 0, 55, 18, and 27%, respectively, from the CD spectrum of the fimbriae between 200 and 240 nm (Fig. 1, curve 1). We studied the effect of heating on the physical structure, both by measuring the CD spectra and by applying SDS-PAGE because a conformational change was expected upon heating. The fimbrial preparation (0.22 mg of protein per ml) was first heated at 80°C for 40 min in the same buffer. In the previous experiments, dissociation of fimbriae started under the same conditions. There were no gross changes in either the CD spectrum (Fig. 1, curve 2) or the pattern on SDS-PAGE (data not shown); i.e., there was no fimbrilin band (the 43,000-molecular-weight protein) seen on the SDS-PAGE gel. *B. gingivalis* fimbriae seemed to be more stable in the presence of salt. Therefore, we carefully measured all CD spectra in the preparations containing 0.1 M NaCl. Upon heating at 100°C for 1 min, the profile of the CD spectrum changed significantly. The negative peak became deep and broad (Fig. 1, curve 3). Heating at 100°C for 5 min caused the disappearance of the peak at 216 nm and the appearance of a large negative peak at 207 nm with a shoulder at 220 nm, a profile resembling those of α-helical polypeptides (1). Simultaneously, some fimbriae migrated as fimbrilin monomers on SDS-PAGE when heated at 100°C for 1 min, whereas all the fimbriae migrated when heated at 100°C for 5 min (data not shown). From the CD spectrum of SDS-denatured fimbrilin

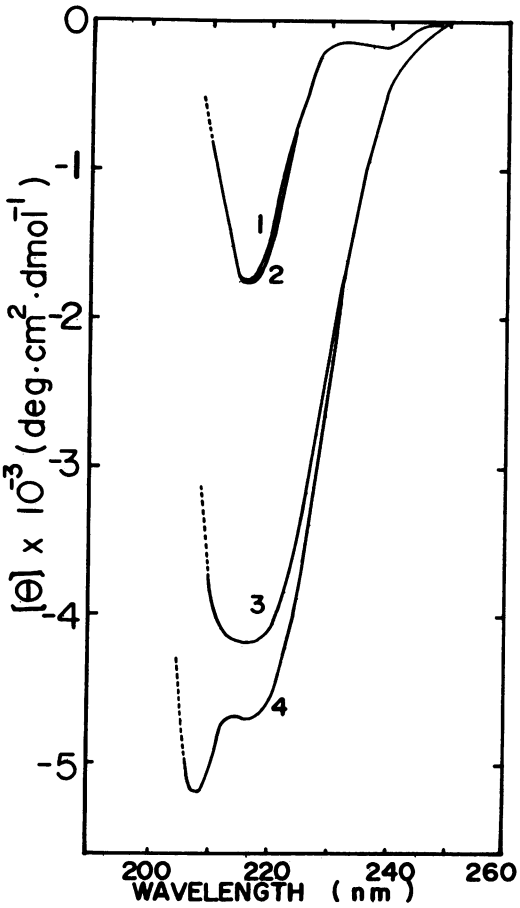

FIG. 1. CD spectra of purified fimbriae dissolved in SDS solution at various temperatures. Fimbriae (0.22 mg/ml) were treated in 0.2% SDS solution containing 0.1 M NaCl at room temperature for 60 min (curve 1), 80°C for 40 min (curve 2), 100°C for 1 min (curve 3), or 100°C for 5 min (curve 4). Dotted lines indicate regions close to the upper limit of the photomultiplier voltage of the spectropolarimeter.

monomers produced by heating at 100°C for 5 min, the α-helix, β-sheet, and β-turn contents and the remainder, however, were estimated to be 5, 28, 38, and 29%, respectively, although the smaller α-helix content was unexpected.

Amino-terminal amino acid sequence. A recent report dealing with *B. nodosus* has shown that fimbrilins from four taxonomically distinct bacteria have highly homologous amino-terminal amino acid sequences with a common unusual amino-terminal amino acid, *N*-methylphenylalanine. On the other hand, partial (5, 6, 10) or complete amino acid sequences (14, 15) of fimbrilins derived from several strains of *Escherichia coli* have been reported to be heterogeneous and different from these homologous N-terminal amino acid sequences.

It is of interest to know whether fimbrilin from *B. gingivalis*, falling into the same genus and the same nonfermentative species of the genus as *Bacteroides nodosus*, belongs to the homologous or heterogeneous group. With a liquid-phase sequencer, fimbrilin preparations (three different lots, 5 to 20 nmol, with the assumed molecular weight of 43,000), were sequenced six times up to 36 residues. The consecutive terminal sequence up to 24 residues is shown in Fig. 2 with those of fimbrilins, reported previously (8, 10, 14, 15, 25). The figure clearly shows that the sequence of *B. gingivalis* fimbrilin belongs to the heterogeneous group with unique N-terminal amino acid sequences. Furthermore, there was no significant homology with any fimbrilin in the heterogeneous group.

Carbohydrate and phosphorus analyses. With the fimbrial preparation (100 to 430 μg of protein used, 98% purity, judged by scanning stained electrophoresis gels), the total carbohydrate analysis determination gave a value of 7.6 ± 2.0 μg of hexose (as glucose) per mg of protein (1.8 ± 0.47 mol of hexose per mol of fimbrilin). When fimbrilin was further purified by a preparative SDS-PAGE from the purified fimbrial preparation, an average of 1.4 ± 0.35 mol of hexose (as glucose) per mol of fimbrilin was detected by using 34 to 240 μg of fimbrilin protein.

Total phosphorous analysis determination gave a value of less than 0.05 mol of phosphorus per mol of fimbrilin with the fimbrial preparation (100 to 430 μg of protein used).

Immunological reactions. Our previous report showed that different sets of antigenic determinants seemed to be exposed on the surfaces of fimbriae and SDS-denatured fimbrilin; anti-fimbriae serum produced no precipitation with SDS-denatured fimbrilin, and anti-fimbrilin serum showed no interaction with native fimbriae in conventional immunological assays. We applied a more sensitive method, ELISA, in a quantitative way to this problem. Antisera against native fimbrial polymers or denatured fimbrilin monomers showed high antibody titers against their specific antigens in ELISA (Table 1). In other words, fimbriae and fimbrilin showed a very low cross-reactivity (about 1/10 that of specific anti-

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
B. gingivalis	Ala	Phe	Gly	Val	Gly	Asp	Asp	Glu	Ser	Lys	Val	Ala	Lys	Leu	Thr	Val	Met	Val	Tyr	Asn	Gly	Glu	Gln	Gln	Heterogeneous group
E. coli, Type 1	Ala	Ala	Thr	Thr	Val	Asn	Gly	Gly	Thr	Val	His	Phe	Lys	Gly	Glu	Val	Val	Asn	Ala	Ala	X	Ala	Val	Asp	
E. coli, K88	Trp	Met	Thr	Gly	Asp	Phe	Asn	Gly	Ser	Val	Asp	Ile	Gly	Gly	Ser	Ile	Thr	Ala	Asx	Gly	–	–	–	–	
E. coli, CFA/1	Val	Glu	Lys	Asn	Ile	Thr	Val	Thr	Ala	Ser	Val	Asp	Pro	Val	Ile	Asp	Leu	Leu	Gln	Ala	Asp	Gly	Asn	Ala	
B. nodosus	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Val	Ala	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Phe	Ala	Ile	Pro	Ala	Tyr	Homologous group
N. gonorrhoeae	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala	Ile	Val	Gly	Ile	Leu	Ala	Ala	Val	Ala	Leu	Pro	Ala	Tyr	
M. nonliquefaciens	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Ile	Ala	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Ile	Ala	Leu	Pro	Ala	Tyr	
P. aeruginosa	MePhe	Thr	Leu	Ile	Glu	Leu	Met	Ile	Val	Val	Ala	Ile	Ile	Gly	Ile	Leu	Ala	Ala	Ile	Ala	Ile	Pro	Gln	Tyr	

FIG. 2. Amino-terminal amino acid sequences of fimbrilins from *B. gingivalis* and other bacteria. Determination of the amino-terminal amino acid sequence of *B. gingivalis* fimbrilin was carried out with a liquid-phase sequencer (Beckman model 890C) as described in the text. The sequence was compared with those of fimbrilins thus far reported from various bacteria, including *B. nodosus*. Boxed residues are identical among four homologous fimbrilins. MePhe, *N*-methylphenylalanine.

gens) with anti-fimbrilin and anti-fimbriae sera, respectively. In addition, LPS had no significant reactivity with the anti-fimbriae and anti-fimbrilin sera. Although fimbrilin monomers showed detectable reactivity with anti-LPS serum, the reactivity of anti-LPS serum with fimbriae was insignificant, and the LPS preparation (as much as 3 μg) had no detectable fimbrilin band on the SDS-PAGE gel stained with silver (data not shown). There were no LPS bands in the fimbrial preparations on the gel either.

Isoelectric point of fimbrilin. The purity of this preparation was confirmed by two-dimensional gel electrophoresis (Fig. 3), where a single spot with only slight contamination could be seen. *B. gingivalis* fimbrilin was found to be acidic, with an isoelectric point of 6.0.

DISCUSSION

In the present study, CD spectra indicated the predominance of β-sheet structure in fimbriae from *B. gingivalis* for the first time. There have been few reports on the secondary structure of bacterial fimbriae, except that native fimbriae and fimbrilin-octyl glucoside from *Pseudomonas aeruginosa* were calculated to have 40% α-helix and 38% β-structure (33). Based on their CD spectra and computer analyses, *B. gingivalis* fimbriae are rich in β-structure and much less rich in α-helix, if it is present at all. *B. gingivalis* fimbriae with rich β-structure polypeptides were stable at 80°C for at least 40 min in SDS (Fig. 1, curve 2). The great stability of this conformation would have evolutionary advantages, considering that fimbriae are always exposed to the environment. In respect to stability, we have noticed that *E. coli* fimbriae (type 1) seem to be more stable than *B. gingivalis* fimbriae (18). It is of interest to know whether the type 1 fimbriae are rich in β-structure polypeptides, since some outer membrane proteins such as porin in *E. coli* and other gram-negative bacteria, which are also exposed to the outside, are known to be unusually rich in β-structure and to have a stable oligomeric structure in SDS solution (20). The CD spectra of *B. gingivalis* fimbriae are similar to those of outer membrane proteins with an unusually high content of β-structure (19).

We have shown that *B. gingivalis* fimbriae are a novel type in several respects (35). The N-terminal amino acid sequence of the fimbrilin was shown to be completely different from those of fimbrilins reported thus far. Although a search for sequence homology for the N-terminal sequence of *B. gingivalis* fimbrilin was carried out by using amino acid sequence data from the Peptide Institute, Inc., (Osaka, Japan), there was no significant homologous sequence found.

TABLE 1. Quantitative determination of antibody titers by ELISA[a]

Antiserum elicited against:	Reciprocal antibody titer against (as antigen):		
	Fimbriae	Fimbrilin	LPS
Fimbriae	102,400	3,200	<100
Fimbrilin	12,800	25,600	<100
LPS	<100	800	3,200

[a] Antisera against antigens were elicited by subcutaneous administration to rabbits. The ELISA was carried out on microtiter trays coated with suspensions of different antigens at 15 μg/ml in 50 mM bicarbonate buffer (pH 9.6). Sera were diluted with phosphate saline buffer (pH 7.2) containing 0.1% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20. The second incubation was performed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G diluted 1,500-fold. *o*-Phenylenediamine was used as peroxidase substrate. Titers are given as the reciprocal of the highest serum dilution showing an absorbance 0.3 U higher than that of negative controls at 490 nm after 30 min at room temperature. Fimbrilin, SDS-denatured constituent protein monomer of fimbriae.

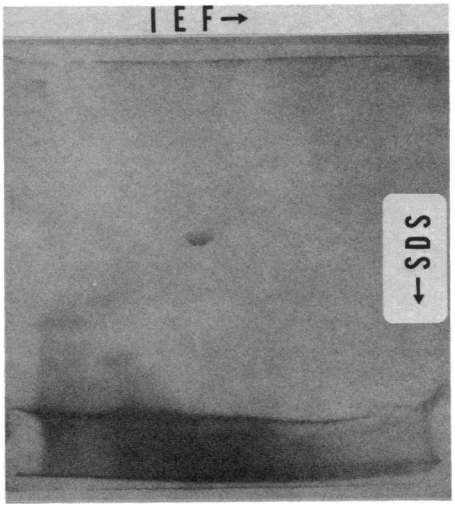

FIG. 3. Two-dimensional gel electrophoresis of *B. gingivalis* fimbriae. According to the procedure described in the text, *B. gingivalis* fimbriae (24 μg in 12 μl) was added to 3 μl of 10% SDS solution, and the mixture was heated at 100°C for 5 min. The heated sample was cooled to room temperature and was added to 30 μl of Ames lysis buffer (2). This preparation was applied to an isoelectric focusing tube gel for the first dimension. For the second dimension, the acrylamide gel was extruded from the tube and layered on top of a standard 12% SDS-polyacrylamide gel. The gel was immersed with a melted equilibration buffer containing 1% agarose and 2-mercaptoethanol. The arrows show the direction of isoelectric focusing (IEF) and SDS-PAGE (SDS).

We have also examined sugar content in fimbrial preparations, as well as pure fimbrilin preparations obtained through a preparative SDS-PAGE from the purified fimbrial preparations. Although it is difficult to draw a definite conclusion because of the very low sugar content, *B. gingivalis* fimbriae seem to have no more than one hexose residue, if any, per peptide.

The present results by ELISA confirmed our previous observation that native fimbriae and denatured fimbrilin differ greatly in their immunological properties. Native fimbriae reacted strongly with anti-fimbriae serum but showed much less cross-reactivity with anti-fimbrilin serum. In a similar manner, fimbrilin monomers were observed to react strongly with a specific antiserum, anti-fimbrilin, and to have a lower cross-reactivity with the anti-fimbriae serum (Table 1).

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