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Isolation and Characterization of Fimbriae from a Sparsely Fimbriated Strain of *Porphyromonas gingivalis*

HAKIMUDDIN T. SOJAR,[1]* NOBUSHIRO HAMADA,[2] AND ROBERT J. GENCO[1,3]

Department of Oral Biology, School of Dental Medicine,[1] and Department of Microbiology, School of Medicine and Biomedical Sciences,[3] State University of New York at Buffalo, Buffalo, New York 14214, and Department of Microbiology, Kanagawa Dental College, Yokosuka 238, Japan[2]

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***Porphyromonas gingivalis* W50 (ATCC 53978) possesses the gene for fimbriae; however, the surface-expressed fimbriae are sparse and have not been previously isolated and characterized. We purified fimbriae from strain W50 to homogeneity by ammonium sulfate precipitation and reverse-phase high-performance liquid chromatography [H. T. Sojar, N. Hamada, and R. J. Genco, Protein Expr. Purif. 9(1):49–52, 1997]. Negative staining of purified fimbriae viewed by electron microscopy revealed that the fimbriae were identical in diameter to fimbriae of other *P. gingivalis* strains, such as 2561, but were shorter in length. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the apparent molecular weight of isolated fimbrillin from strain W50 was found to be identical to that of the fimbrillin molecule of strain 2561. Unlike 2561 fimbriae, W50 fimbriae, under reducing conditions, exhibited a monomeric structure on SDS-PAGE at room temperature. However, under nonreduced conditions, even at 100°C, no monomer was observed. In immunoblot analysis as well as immunogold labeling of isolated fimbriae, polyclonal antibodies against 2561 fimbriae, as well as antibodies against peptide I (V-V-M-A-N-T-G-A-M-E-V-G-K-T-L-A-E-V-K-Cys) and peptide J (A-L-T-T-E-L-T-A-E-N-Q-E-A-A-G-L-I-M-T-A-E-P-Cys), reacted. However, antifimbrial antibodies against strain 2561 reacted very weakly compared to anti-peptide I and anti-peptide J. Negative staining of whole W50 cells, as well as immunogold electron microscopy with anti-peptide I and anti-peptide J, showed fimbriae shorter in length and very few in number compared to those of strain 2561. Purified fimbriae showed no hemagglutinating activity. Amino acid composition was very similar to that of previously reported fimbriae of the 2561 strain.**

Porphyromonas gingivalis has been shown to comprise a large proportion of the cultivable flora in periodontal lesions (6, 23, 24, 30). *P. gingivalis* fimbriae are believed to play an important role in the pathogenesis of periodontal diseases (1, 2). Inactivation of the *fimA* gene diminishes the periodontal bone loss in gnotobiotic rats (16). Colonization of the sublingual area by *P. gingivalis* is likely mediated by fimbriae through binding affinity to crevicular cells (7), human buccal epithelial cells (11), salivary proteins (14), and other existing microflora in the vicinity (5). Fimbriae are also found to stimulate both humoral and cellular immune responses of the host. Patients with periodontal diseases have high titers of antibody to *P. gingivalis* fimbriae. Moreover, induction of various proinflammatory cytokines such as interleukin 1, interleukin 6, and interleukin 8 by *P. gingivalis* fimbriae has also been reported (8, 9, 18–21).

Earlier studies (10, 18, 22, 32) have shown that the amount of fimbriae produced by *P. gingivalis* varies from strain to strain. In some strains, such as W50 and W83, fimbriae were expressed in negligible amounts; hence, they are not detected by immunological methods. However, these strains are believed to be fimbriated because a few fimbriae can be detected by electron microscopy. In *P. gingivalis* D40C-28 and D83T-3, fimbriae were not detected under the electron microscope (27). Therefore, among *P. gingivalis* strains, some seem highly fimbriated, some seem sparsely fimbriated, and some strains are afimbriated. Extensive heterogeneity among *P. gingivalis* strains has been shown elsewhere (28). Van Steenbergen et al. (31) used a mouse model to demonstrate that strains W50 and W83 are very virulent, causing a severe phlegmonous abscess or secondary lesions and septicemia. These investigators also recognized that these strains are invasive. In order to confirm whether all *P. gingivalis* strains have the *fimA* gene, Takahashi et al. (29) used Southern blot analysis of various strains with cloned DNA fragments carrying the *fimA* gene as a probe. All *P. gingivalis* strains tested had a single-copy *fimA* gene on their chromosomal DNAs. The *fimA* gene of *P. gingivalis* was present on a 2.5-kb *Sac*I fragment, except in strains W50 and W83. The fimbrillin gene of these two strains was within a 5-kb *Sac*I fragment.

Pure preparation of proteins is a prerequisite for in vitro study, as well as in antibody production for subsequent study of the antigenic diversity in the major fimbrial proteins. Various laboratories (15, 26, 33) have purified *P. gingivalis* fimbriae from highly fimbriated strains. However, difficulties have been found in purifying fimbrial proteins from sparsely fimbriated strains because of a negligible amount of fimbriae. The present study describes an isolation method for fimbrial protein from strains such as W50 and W83 as well as partial characterization of the same.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* ATCC 53978 (W50) was grown in brain heart infusion broth (Difco Chemical Co.), supplemented with 5 mg of yeast extract, 5 μg of hemin, and 0.2 μg of menadione per ml (pH 7.4) at 37°C for 2 days in a Forma anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2).

Isolation of crude fimbriae from the W50 strain. Since the W50 strain has a negligible amount of fimbriae on the surface, in order to achieve maximum fimbria solubilization, the method of Morris et al. (17) for *Escherichia coli* fimbria preparation was used with slight modifications. *P. gingivalis* W50 cells were harvested from a 2-liter culture by centrifugation and washed once with 50 mM Tris-Cl buffer (pH 8.0), containing 1 mM the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The cell pellet was suspended in the same buffer and heated at 60°C for 15 min to enhance detachment of fimbriae from cells. The

* Corresponding author. Mailing address: Department of Oral Biology, School of Dental Medicine, State University of New York at Buffalo, 213 Foster Hall, 3435 Main St., Buffalo, NY 14214. Phone: (716) 829-2551. Fax: (716) 829-3942. E-mail: Hakim_Sojar@sdm.buffalo.edu.

cell suspension was then cooled by placement on ice. The cell suspension was sonicated with a 3-mm microtip at a 20-W output with a pulse setting and a 50% duty cycle for 10 min in an ice bath by using a Vibra Cell model VC250 sonicator (Sonic and Materials, Inc.). After this treatment, whole cells were centrifuged at 20,000 × *g* for 30 min in a Sorvall RC 5C centrifuge. The supernatant which contained fimbriae, along with other cell surface components, was made 60% saturated by the stepwise addition of solid ammonium sulfate and stirred at 4°C overnight. The precipitated proteins were collected by centrifugation at 20,000 × *g* for 30 min at 4°C and suspended in a minimum volume of high-performance liquid chromatography (HPLC)-grade water with 1 mM PMSF. Suspended fimbriae were dialyzed against HPLC-grade water with two changes. After extensive dialysis, the clarified crude fimbria preparation was collected by centrifugation at 20,000 × *g* for 5 min.

HPLC separation of fimbriae. The crude fimbria preparation was subjected to reverse-phase HPLC separation on Rainin's rabbit model (Rainin Instrument Co. Inc., Emeryville, Calif.) HPLC system with semipreparative, wide-pore C_8, reversed-phase 10-mm-inside diameter Dynamax modules (catalog no. 83-313-C5) on a 5-μm-by-25-cm-long column. About 1 ml (1 mg/ml) of the crude fimbriae was loaded and eluted with a gradient of solvent B (0.1% trifluoroacetic acid in acetonitrile) in solvent A (0.1% trifluoroacetic acid in HPLC-grade water) for 70 min with a flow rate of 2 ml/min at room temperature. The initial gradient was set from 0 to 40% of solvent B for 10 min, and a gradient of 40 to 70% of solvent B was set for another 60 min. About 25 peaks were recorded, and peak 25 at 38.2 min was collected repetitively. Only 5% of the HPLC-recorded peak area of the chromatogram was found to be a fimbria peak. Hence, in each run only 50 μg of fimbriae was collected. Certainly, the yield could be improved by connecting a higher-volume sample loop and by injecting a higher volume of crude fimbria preparation onto the preparative column. The collected peak was dialyzed against 10 mM phosphate buffer containing 1 mM PMSF and 10 mM $MgCl_2$ with Spectra/Por 3500 cutoff dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) for 24 h with three to four changes.

Analytical procedures. Protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's directions with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (12). Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes in 25 mM Tris-glycine buffer (pH 8.3), for Western immunoblot analysis as described elsewhere (13).

Electron microscopy. The purified fimbriae were examined with a transmission electron microscope. Ten microliters of fimbriae (1 mg/ml) was applied on a copper grid coated with a thin Formvar film and air dried. The samples were then negatively stained with 2% (wt/vol) uranyl acetate for 1 min, air dried, and examined and photographed with a Hitachi H-600 electron microscope operating at 75 kV.

Immunogold labeling. Purified fimbriae were applied to nickel grids coated with Formvar film, air dried as described above, and incubated with 10 μl of rabbit polyclonal antibodies raised against peptide I (1:500 in phosphate-buffered saline [PBS] containing 1% bovine serum albumin) at 37°C for 1 h. Controls consisted of incubation of the sample either (i) in the absence of the rabbit serum or (ii) in the presence of normal rabbit serum. After being washed four to five times with PBS, grids were incubated for 30 min with sheep anti-rabbit immunoglobulin G conjugated with 5-nm gold particles (1:20; AuroProbe EM; Amersham) at 37°C for 30 min. The samples were rinsed twice with PBS and negatively stained with 2% (wt/vol) uranyl acetate for 1 min, air dried, and examined and photographed with a Hitachi H-600 electron microscope operating at 75 kV.

RESULTS AND DISCUSSION

In this study, we have shown biochemical purification of fimbriae from sparsely fimbriated strains of *P. gingivalis*, such as W50. Morphological studies of *P. gingivalis* have revealed that fimbriae distributed peritrichously on the surface of *P. gingivalis* strains are thin, curly, and variable in length (27). Suzuki et al. (28) screened 65 isolates, representing a wide geographic distribution, for the presence of fimbriae on the cell surface under the electron microscope by negative staining. Forty-nine strains were found to be clearly fimbriated, and only two strains were nonfimbriated. The presence of fimbriae on the remaining 14 strains is yet to be determined. Since in many cases bacterial fimbriae or fimbria-associated adhesins are responsible for bacterial binding, it is essential to evaluate how the fimbriae contribute in particular strains of bacteria. It is also of interest to observe structural and immunological heterogeneity among fimbrial proteins of *P. gingivalis* in order to determine the number of serologically moving targets at which a fimbrial vaccine might aim. To obtain valuable information

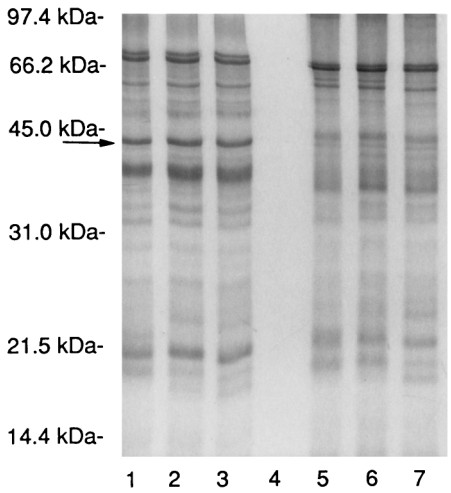

FIG. 1. SDS-PAGE analysis of isolated fimbriae. Samples were electrophoresed on reduced SDS–15% PAGE and stained with Coomassie blue R-250. Lane 1, crude fimbriae were mixed with an equal volume of 2× reduced sample buffer (0.25 M Tris base [pH 6.8], 4% SDS, 20% glycerol, 0.002% bromophenol blue, 10% β-mercaptoethanol) and incubated for 10 min at room temperature. Lane 2, crude fimbriae were mixed with an equal amount of 2× reduced sample buffer and incubated at 80°C for 10 min. Lane 3, crude fimbriae were mixed with an equal amount of 2× reduced sample buffer and boiled for 10 min. Lane 4, blank with nonreduced sample buffer only. Lane 5, crude fimbriae were mixed with an equal volume of 2× nonreduced sample buffer (0.25 M Tris base [pH 6.8], 4% SDS, 20% glycerol, 0.002% bromophenol blue) and incubated for 10 min at room temperature. Lane 6, crude fimbriae were mixed with an equal amount of 2× nonreduced sample buffer and incubated at 80°C for 10 min. Lane 7, crude fimbriae were mixed with an equal amount of 2× nonreduced sample buffer and boiled for 10 min.

on whether the fimbrial protein of the W50 strain is appropriate to target for prevention of *P. gingivalis* binding to oral surfaces or matrix components, purified fimbriae will be important.

In earlier reports of purification of *P. gingivalis* fimbriae from fimbriated strains such as 2561 (15, 25), a considerable amount of fimbriae was detached by sonication. However, recovery of fimbriae from strain W50 by sonication alone was negligible; hence, the method has been modified. Prior to sonication, cells were heated at 60°C for 15 min to release and solubilize maximum fimbriae. When cells were sonicated without preheating and crude fimbriae were recovered by ammonium sulfate precipitation, this crude fimbrial preparation demonstrated no significant fimbrial peak on HPLC. When the same preparation was sonicated after heating of the cells, a significant peak was observed at 38 min on HPLC. Hence, to purify a sufficient quantity of fimbriae from a sparsely fimbriated strain such as W50, preheating of cells is an essential step, as in K99 antigen recovery from *E. coli* B41 (17). Precipitation of detached fimbriae was optimized by using various ammonium sulfate concentrations from 20 to 80%. Maximum fimbriae was precipitated at 60% ammonium sulfate saturation. Crude fimbriae prior to placement on HPLC were found to be in a highly polymerized form when run under nonreducing conditions without β-mercaptoethanol. Even after heating at 100°C for 5 min, fimbriae did not appear on the gel. As soon as β-mercaptoethanol is added to the sample buffer, even without heating, crude fimbriae showed a prominent band at about 43 kDa. Unlike other strains, the sulfhydryl group of W50 fimbriae seems important in polymerization (Fig. 1).

The crude fimbria preparation obtained by ammonium sulfate precipitation was resolved on a C_8 semipreparative col-

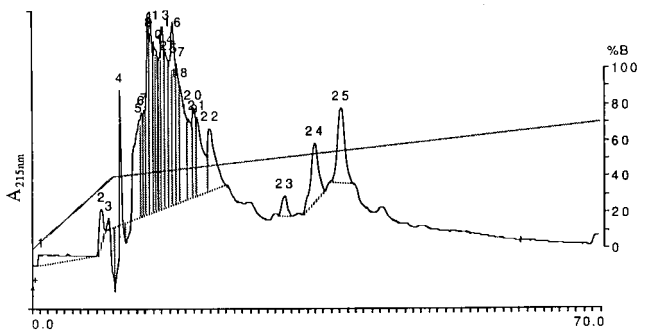

FIG. 2. The HPLC chromatogram of crude *P. gingivalis* fimbriae on a semipreparative, wide-pore C_8, reverse-phase column. The initial gradient was set up with 0 to 40% solvent B (0.1% trifluoroacetic acid in acetonitrile) for 10 min, and then fimbriae were eluted with a gradient of 40 to 70% solvent B for another 60 min. %B is percent acetonitrile; time is from 0 to 70 min.

umn. The peak around 38 min (peak 25) was found to be the fimbrial peak (Fig. 2). Even though only 5% of protein peak was recovered as fimbrial peak from HPLC, this method has an advantage over conventional methods (15, 25) in that the method is very rapid and fimbriae can be recovered from the crude preparation in 1 h in both analytical and preparative scale (26).

Purified fimbriae were found to be in a highly polymerized form when run under nonreducing conditions without β-mercaptoethanol (data not shown). Even after heating at 100°C for 5 min, fimbriae did not appear on the gel. In the presence of 2-β-mercaptoethanol and with heating at 100°C for 10 min, the fimbriae showed a prominent band at about 43 kDa when stained with Coomassie blue. However, the same gel, when stained with silver, showed an additional, very minor band at 50 kDa (Fig. 3). Fimbriae prepared from the W50 strain did not appear on the gel in SDS-PAGE, even after heating at 100°C for 10 min. However, when a reducing agent such as β-mercaptoethanol or dithiothreitol is added to the purified fimbrial preparation, even without heating the preparation

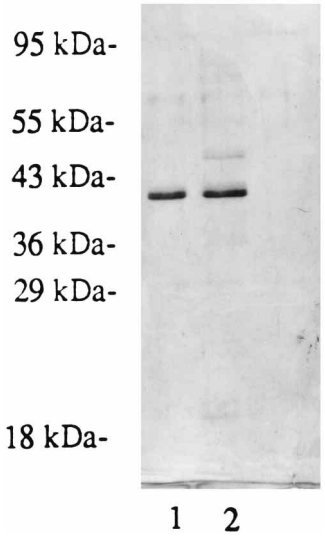

FIG. 3. SDS-PAGE analysis of purified *P. gingivalis* W50 fimbriae. Lane 1, *P. gingivalis* 2561 purified fimbriae boiled with an equal volume of 2× reduced sample buffer for 10 min. Lane 2, *P. gingivalis* W50 purified fimbriae boiled with an equal volume of 2× reduced sample buffer for 10 min. The gel was stained with silver.

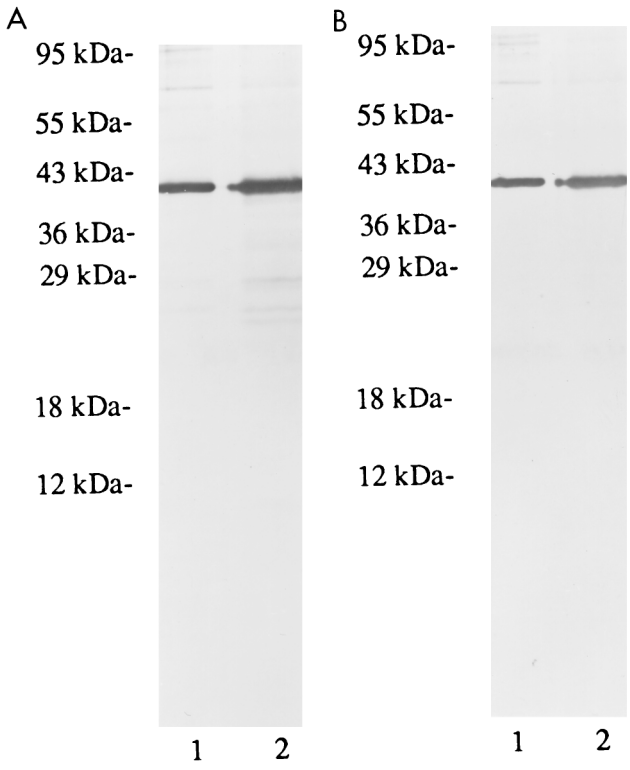

FIG. 4. Immunoblot analysis with rabbit polyclonal antibodies against *P. gingivalis* fimbriae. Lanes 1, *P. gingivalis* 2561 purified fimbriae boiled with an equal volume of 2× reduced sample buffer for 10 min. Lanes 2, *P. gingivalis* W50 purified fimbriae boiled with an equal volume of 2× reduced sample buffer for 10 min. (A) Peptide I polyclonal antibodies. (B) Peptide J polyclonal antibodies.

shows a single prominent band at about 43 kDa, suggesting that a sulfhydryl group of the 43-kDa subunit may play an essential role in the tertiary structure of fimbriae. On Coomassie blue staining, a fimbria preparation showed a prominent band at about 43 kDa, but when the same gel was stained with silver, an additional minor band appeared at 50 kDa and a very faint band appeared at 18 kDa. At present, it is difficult to say whether this 50-kDa band is an aggregate of 43 kDa and the 18-kDa band is a proteolytic product or whether both are fimbria-associated proteins (34) copurifying with fimbriae. The fimbria purified with an HPLC column is a major band of about 43 kDa on SDS-PAGE.

Immunoblotting of the fimbrial protein was carried out with polyclonal antibodies raised against peptide I and peptide J of the 2561 fimbrillin molecule (Fig. 4). The protein band of the W50 fimbriae was shown to react with the 43-kDa band under reduced conditions only. No reaction was seen under nonreduced conditions with or without heating. However, no reaction was observed when preimmunized sera were used as control (data not shown). These results indicated that under nonreduced conditions the fimbria preparation was in oligomeric form and did not appear on the gel. It seems that the presence of the sulfhydryl group plays a major role in the oligomeric structure of W50 fimbriae. Fimbriae from W50 shared regions of antigenicity identical to those of previously reported fimbriae from strain 2561 (25).

Antisera against 2561 fimbriae, peptide I, and peptide J (13) reacted with W50 fimbriae. These results were not in agreement with our previously reported results (13). Perhaps in

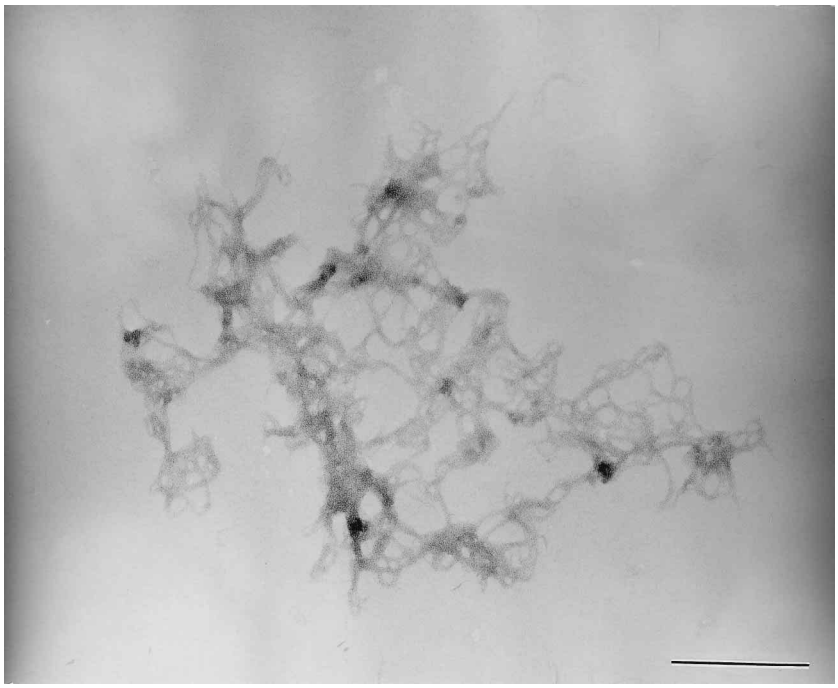

FIG. 5. Electron micrograph of purified fimbriae from *P. gingivalis* W50. The purified fimbria preparation was negatively stained with 2% uranyl acetate. Bar, 0.2 μm.

earlier studies the crude fimbrial preparation of W50 had no fimbriae or the concentration of fimbriae was below detectable levels. When a sufficient amount of purified fimbriae was run on SDS-PAGE, very prominent reactivity was observed with antisera against 2561 fimbriae. Our results are identical to the results of Fujiwara et al. (3, 4), who also observed on immunoblot analysis marginal reactivity with anti-381 fimbria antibody.

Moreover, the nucleotide sequence of the *fimA* gene of strain W50 was found to be 98% similar to that of strain

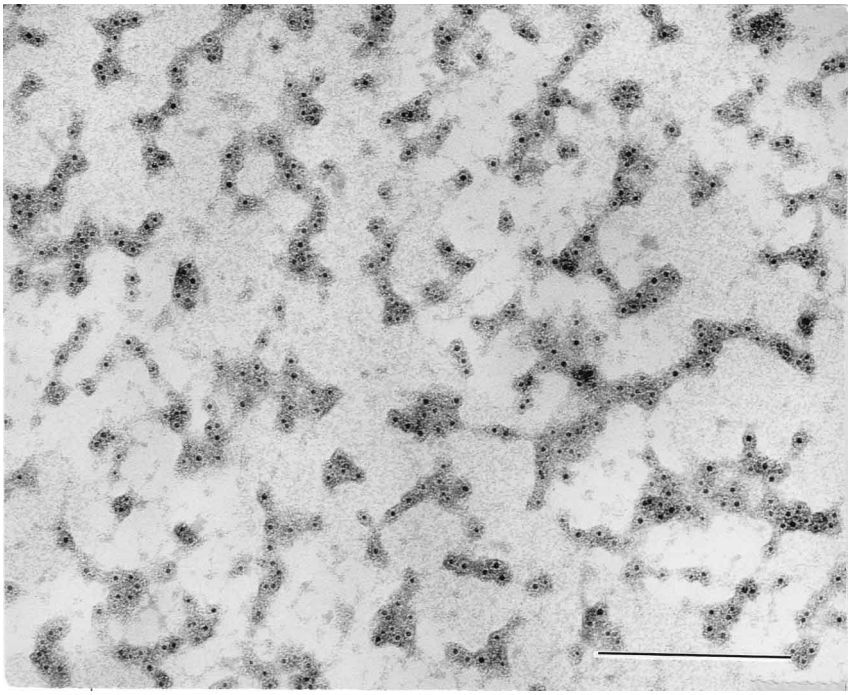

FIG. 6. Immunogold labeling of purified fimbriae from *P. gingivalis* W50. Purified fimbriae were incubated with anti-peptide I polyclonal antibodies followed by 5-nm colloidal gold-labeled goat antirabbit serum. Samples were prepared by negative staining with 2% uranyl acetate. Bar, 0.2 μm.

HG564 (3, 4). Antibody against peptide I and J 20-mers which recognized fimbriae on immunoblot analysis of most *P. gingivalis* strains (13) also recognized W50 purified fimbriae. Peptide I of the 2561 strain has 25% sequence identity in the same region and peptide J of the 2561 strain has 50% sequence identity according to the sequence reported by Fujiwara et al. (3), which may be a common epitope recognized in both strains.

Under the electron microscope, examination of fimbriae on *P. gingivalis* W50 whole cells revealed a distinct short (5 nm in width and 0.1 to 0.5 μm in length) type of fimbriae, fewer in number, compared to those of 2561 or other fimbriated strains (data not shown). Purified fimbriae from W50, when treated with uranyl acetate for negative staining, show thin hair-like structures under the electron microscope (Fig. 5). Further, purified W50 fimbriae were immunogold labeled with peptide I polyclonal antibodies (Fig. 6). Nonspecific labeling was assessed by evaluation of controls which consisted of normal rabbit sera (data not shown). Handley and Tipler (10) examined strains of *P. gingivalis* by negative staining, and the electron microscope revealed that strain W50 possessed fimbriae (4.6-nm width and 1.3-μm length), and the number of fimbriae as well as the number of fimbriated cells was much lower than in other strains. Our isolated fimbriae were different in appearance compared to fimbriae isolated from strain 2561 (25). Fimbriae purified from the W50 strain appeared as a mesh, perhaps due to the nature or size of the fimbriae.

Taken together, we have shown that a significant amount of fimbriae can be purified biochemically from the W50 strain. To understand the role of various kinds of fimbriae in pathogenicity, the purified fimbriae from fimbriated strains and sparsely fimbriated strains will be helpful. Since the fimbrial protein retains its native antigenicity, this method can be used to purify fimbrial proteins from various strains of *P. gingivalis* which are sparsely fimbriated to produce antisera for studying antigenic heterogeneity, as well as humoral response to various fimbriae. Further, the study of physicochemical characterization of fimbrial protein from strains such as W50, which has been hampered by difficulties in purifying the proteins, can be further developed.

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