

Purification and Properties of a 75-Kilodalton Major Protein, an Immunodominant Surface Antigen, from the Oral Anaerobe *Bacteroides gingivalis*

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Received 26 April 1989/Accepted 8 August 1989

A 75-kilodalton major protein (75K protein) was purified to homogeneity from the cell lysate fraction and the envelope of *Bacteroides gingivalis* 381. The 75K protein was originally present in the outer membrane or the outermost part of this organism as a large, stable complex with an apparent molecular weight of about 2,000,000. Heating at 80°C and at higher temperatures in the presence of sodium dodecyl sulfate was needed to completely dissociate it to monomers. Amino acid analysis revealed that the 75K protein had about 50% nonpolar amino acids. Various strains of *B. gingivalis* but not other bacteria, including oral *Bacteroides* species tested, contained serologically related 75K proteins when tested in Western blotting (immunoblotting) analysis. The abundance and localization of the 75K protein in this organism suggest that it has the potential to participate in the host-parasite interaction in infection. The 75K protein was, indeed, strongly recognized in patients with adult periodontal diseases. Immunoblotting with sera from patients and with rabbit antisera generated by intravenous inoculations of whole *B. gingivalis* cells revealed that the 75K protein was an immunodominant antigen on the surface of *B. gingivalis*.

Bacteroides gingivalis, a gram-negative oral anaerobe, is thought to be one of the most prominent periodontopathogens (19, 24, 25), and it is believed to have several characteristics of an overt pathogenic bacterium (8); for instance, the organism has fimbriae and hemagglutinin, considered to be attachment factors, and proteases, regarded as destructive factors.

The envelope of a gram-negative bacterium usually has peptidoglycan, an outer membrane, capsular polysaccharides, and protein surface layers as major components. The outer part of the envelope is involved in most of the specific recognition processes in the host-bacterial interaction because it is the most exposed region of the bacterial cell. Our major interest is to elucidate the structures and the functions of the outermost part of the *B. gingivalis* cell. Major cell surface proteins recognized by the human immune system in natural infection are of especially great interest because they might be involved in the progress of adult periodontitis. They also have the advantage of being easily studied because they are abundant in the bacterial cells. Although the outer membrane, its constituent proteins (12, 16, 17, 31), and lipopolysaccharides (4, 11, 18) in oral *Bacteroides* species have been studied so far, none of the surface proteins except for fimbriae (3, 27) has been purified or thoroughly characterized in *B. gingivalis*.

During the course of studies on fimbriae of *B. gingivalis*, we found that a major envelope protein of *B. gingivalis* with an apparent molecular weight of 75,000 (75K protein) was present in a large complex form even in sodium dodecyl sulfate (SDS) (34) and that this protein was strongly recognized by the host (33).

In this study, we describe methods for the complete purification of the 75K protein from the cell lysate and the

envelope fractions and present data on some chemical and immunological properties of this material.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. gingivalis* strains used were 381, 1112, 14018, ATCC 33277, W83, JKG-10, D40C-28, D67D-9, and Shirai. Other bacterial strains were *B. asaccharolyticus* ATCC 25260; *B. endodontalis* ATCC 35406; *B. intermedius* ATCC 25611 and ATCC 33563; *B. corporis* VPI 9342; *B. melaninogenicus* ATCC 25845; *B. denticola* ATCC 33185; *B. loeschei* ATCC 15930; *B. fragilis* IID 1638; *B. macacae* ATCC 33141; *B. levii* B-151; *Actinobacillus (Haemophilus) actinomycetemcomitans* ATCC 29523, Y4, ATCC 29522, and SUNYaB 67; *Fusobacterium nucleatum* FN-2; *Actinomyces naeslundii* ATCC 12104; *A. viscosus* PK 455; *Veillonella criceti* ATCC 17747; *Streptococcus mutans* ATCC 27351; and *S. sanguis* ATCC 10557. *Bacteroides* species and other anaerobes were grown anaerobically at 37°C as described previously (32).

Purification of the 75K protein from a cell lysate fraction (method A). Cells (ca. 60 g [wet weight]) from 10 liters of a 24- to 48-h culture were washed twice with 20 mM Tris hydrochloride (pH 7.4)–0.15 M NaCl–10 mM MgCl₂ to strip off the fimbriae. The washed cells, still retaining some fimbriae, were suspended in 20 mM Tris hydrochloride (pH 8.0)–10% sucrose–0.1 mM dithiothreitol containing about 1 mg each of pancreatic DNase and RNase, and the suspension was shaken in a flask together with 30 g of glass beads with a Braun cell homogenizer as described previously (32). After the large debris was removed by centrifugation at 1,000 × g for 10 min, the cell lysate fraction was obtained as a supernatant after centrifugation at 143,000 × g for 60 min. Ammonium sulfate was added to the supernatant fraction to 40% saturation, and the salted-out proteins were collected by centrifugation and then suspended in a small volume of 20

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mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-LiOH (pH 8.0)-0.5 M LiCl-1% lithium dodecyl sulfate (buffer A). After incubation for 60 min at 50°C, undissolved debris was removed by centrifugation at 15,000 $\times g$ for 30 min at 20°C and the supernatant (ca. 15 ml) was applied to a column of Sephacryl S-200 (superfine, 1.6 by 90 cm) and eluted with 20 mM HEPES-LiOH (pH 8.0)-0.1% lithium dodecyl sulfate-0.1 M LiCl at room temperature. Fractions eluted at the void volume (fractions 42 to 54, total of 160 ml) were collected, and cold acetone (2 volumes) was added. The acetone precipitate was suspended in ca. 7 ml of buffer A, and the solution was left overnight at room temperature. The aggregates that emerged were removed by centrifugation at 10,000 $\times g$ for 30 min. At this step, fimbriae identified as a 43K protein were largely fractionated out as a pellet. Although about 70% of the 75K protein remained in solution, the solution was still contaminated with fimbriae and other proteins, presumably the membrane proteins. The 75K protein-rich fraction was again applied to the same column of Sephacryl S-200, the 75K protein fractions eluted were pooled, and the sample was subjected to preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the 75K protein band from the others.

Purification of the 75K protein from the bacterial envelope fraction as a complex form (method B). A bacterial crude envelope fraction was obtained as a pellet after ultracentrifugation at 143,000 $\times g$ for 60 min as described in method A. The envelope fraction was extensively extracted four times with 50 ml of 20 mM Tris hydrochloride (pH 7.4)-1% Triton X-100 at 0°C, each time followed by centrifugation at 143,000 $\times g$ for 60 min at 4°C. These extraction steps removed most of the cytoplasmic membrane proteins and some of the outer membrane proteins, presumably leaving behind most outer membrane proteins. The pellet was suspended in 30 ml of 20 mM Tris hydrochloride (pH 7.4), and then a 20% SDS solution was added to the suspension to make a final concentration of 1% SDS, and the final suspension was incubated at room temperature for about 10 min. Centrifugation (143,000 $\times g$ for 40 min at 20°C) produced a supernatant containing most of the 75K protein from the suspension. Part (about 3 ml) of this supernatant was applied to a column of Sephacryl S-400 (superfine, 1.5 by 96 cm) and eluted with buffer B (10 mM Tris hydrochloride [pH 8.0], 1% SDS, 0.4 M NaCl, 5 mM EDTA) at room temperature. The 75K protein was eluted as a nearly symmetrical peak at the K_{av} (partition coefficient) value of about 0.13, followed by the other proteins and nucleic acids (see Fig. 1 and 2). The purity, judged by scanning of the stained SDS-PAGE gel, was at least 95% (see Fig. 2).

Preparation of antisera. An antiserum was raised in rabbits against the purified 75K protein from the envelope fraction. The 75K protein (0.1 mg) was mixed with Freund complete adjuvant, and the mixture was injected into rabbits subcutaneously three times at 2-week intervals. Two weeks after the third injection, the 75K protein (0.2 mg) was injected intravenously as a booster. Rabbit antisera against whole *B. gingivalis* cells were generated by intravenous inoculations (10 times) of whole cells (2 ml) suspended in phosphate-buffered saline every 3 days. The concentrations of cell suspensions inoculated were increasingly varied from an optical density at 600 nm of 0.1 (first inoculation) to 0.8 (fourth and succeeding inoculations).

A monoclonal antibody against the 75K protein was obtained during the course of studies on the monoclonal anti-fimbriae antibodies. The procedure was described earlier (9).

Liposome swelling assay. This was carried out essentially as described earlier (14) but usually by using a mixture of acetone-extracted egg phosphatidylcholine and dicetylphosphate as the phospholipid (20, 36).

Amino acid analysis. The solutions were dialyzed against 5 mM sodium bicarbonate (pH 8.0) and dried in vacuo. The samples were hydrolyzed in evacuated, sealed tubes either with 6 N HCl at 110°C for 22 or 24 h or with 4 N methanesulfonic acid containing 3-(2-aminoethyl)indole (23) at 115°C for 22 h to determine tryptophan content. The amino acid contents were determined with a Hitachi 835-10 amino acid analyzer.

SDS-PAGE and Western blotting analysis. SDS-PAGE was performed in a 1.0-mm-thick slab gel as described by Lugtenberg et al. (15). A 0.5% agarose-2% acrylamide composite gel was made essentially as described previously (21) but with a modification for proteins (A. Hattori, manuscript in preparation). The samples were heated with SDS at 100°C for 5 min unless otherwise specified. Preparative electrophoresis was done in the same system (2). Whole cell extracts of various strains were prepared by sonication, followed by centrifugation at 1,000 $\times g$ for 10 min. Western blotting (immunoblotting) analysis was carried out essentially as described earlier (28) but usually by using a Trans-Blot cell with a cooling coil (Bio-Rad Laboratories) at 6 V/cm overnight. Human sera and horseradish peroxidase-conjugated antibodies were diluted 2,000- and 3,000-fold, respectively, with the buffer before use as described previously (33). Both sera were used at rather high dilution to minimize nonspecific reactions.

Analytical methods. The amount of protein was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard. The purity of protein was judged by the scanning of stained SDS-PAGE gels with a Shimadzu CS-930 dual-wavelength scanner.

RESULTS

Purification of the 75K protein. During the purification of *B. gingivalis* fimbriae from the cell lysate fraction, the 75K protein was always detected as the second major component in the main peak of gel filtration. The 75K protein was coeluted with fimbriae at the void volume from a column of Sephacryl S-200, suggesting that it existed as aggregates either with itself or with fimbriae even in SDS (see reference 34, Fig. 2, lanes B and C). In either case, the aggregates or the complex seemed to have a molecular weight of more than 250,000, on the basis of the elution position in gel filtration. Unless preparative SDS-PAGE was carried out, the preparations from these procedures (method A) always contained contaminating proteins, including fimbriae detected as the 43K protein in SDS-PAGE. One of the reasons is that fimbriae were not completely removed by differential centrifugation on the basis of their solubility.

A second method of purification was sought to elucidate how the 75K protein exists, whether it associates to fimbriae or forms a homologous complex, and where it is located in the cells. As described earlier (34), the 75K protein was also present in abundance in a crude envelope fraction in which the 43K protein was not detected, and it has been partially purified from the envelope by gel filtration. It has also been noted that the 75K protein was enriched in the envelope fraction extracted with a buffer containing Triton X-100. In this study, we extensively extracted the crude envelope fraction with the buffer to remove other proteins first. The resulting sediment was extracted with a buffer containing 1%

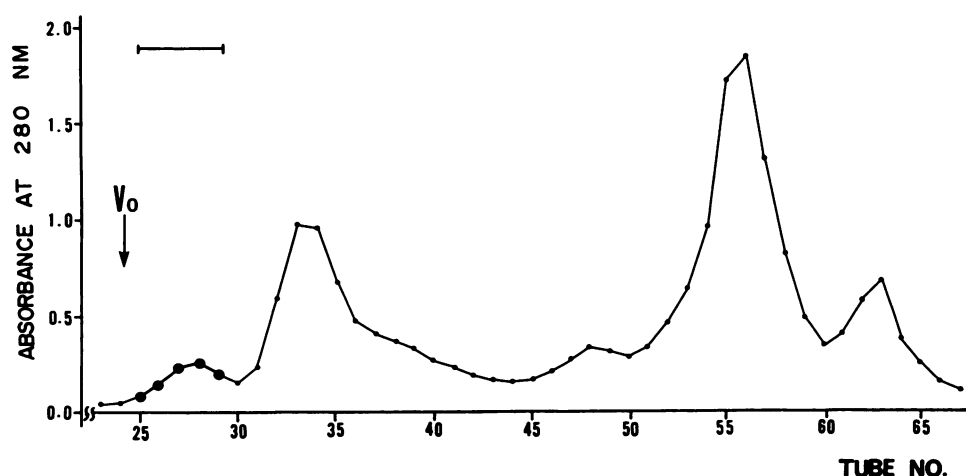


FIG. 1. Gel filtration of the *B. gingivalis* protein complex from the envelope fraction by SDS-Sepharcl S-400 column chromatography. •, A_{280} ; —, ●, fractions collected for the next step; V_0 , elution peak of Blue Dextran 2,000.

SDS without salt. This extraction solubilized almost all proteins in the sediment. None of the proteins tightly associated to peptidoglycan sheets could be detected; they were usually left behind in the last pellet. Finally, the 75K protein complex was simply purified by gel filtration on a Sepharcl S-400 column that could separate proteins in the molecular weight range of 20,000 to 8,000,000. A typical elution pattern and SDS-PAGE patterns of some fractions are shown in Fig. 1 and 2, respectively. The final preparation resulting from these procedures (method B) produced a single band of the 75K protein on a SDS-PAGE gel (Fig. 2, lane d).

Amino acid composition. The preparation obtained by method A was applied to a preparative SDS-PAGE gel to separate the 75K protein from other minor proteins. Amino acid compositions of this preparation as well as the prepa-

ration obtained from the envelope fraction (method B) were determined (Table 1). The two preparations purified from different starting materials had essentially the same amino acid composition, reinforcing the previous supposition that they are identical (34). The 75K protein had nearly 50% nonpolar amino acids. Waugh's nonpolarity index (30), i.e., the occurrence frequency of nonpolar side chains, which include valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and tyrosine, was 0.34. Cysteine was not detected, even with methanesulfonic acid hydrolysis and by the method of air oxidation (6).

Immunological reactions. By using the antiserum prepared earlier (34) and an antiserum raised against the purified 75K protein complex in this study, Western blotting analysis confirmed that the two preparations of the 75K protein from

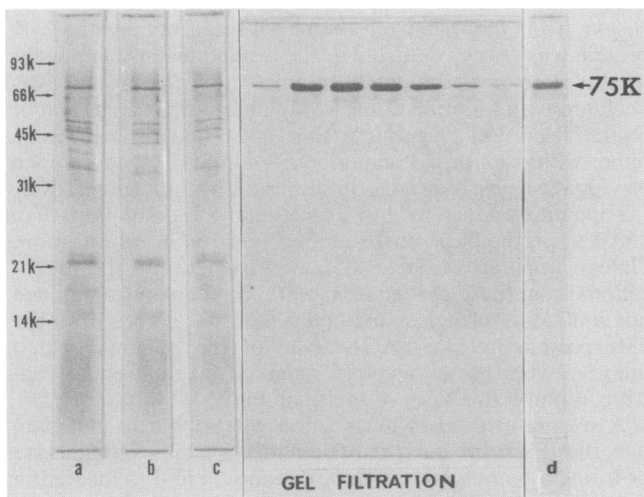


FIG. 2. Purification of the *B. gingivalis* 75K protein by method B. SDS-PAGE patterns of some fractions are shown. The envelope fraction (lane a) from strain 381 was washed four times with the buffer containing 1% Triton X-100, and the pellet (lane b) was extracted with the buffer containing 1% SDS. The extract (lane c) was applied onto a Sepharcl S-400 column. The electrophoretogram of the proteins of fractions indicated by tubes 23 through 29 (Fig. 1) is shown from right to left in the gel filtration panel. The final preparation after dialysis is shown in lane d.

TABLE 1. Amino acid composition of *B. gingivalis* 75K protein

Amino acid	Residues/molecule ^a	
	Sample A	Sample B
Asx	89.1 (89)	88.5 (89)
Thr	52.9 (53)	53.2 (53)
Ser	40.3 (40)	40.3 (40)
Glx	64.1 (64)	63.4 (63)
Pro	55.9 (56)	51.6 (52)
Gly	58.1 (58)	57.0 (57)
Ala	70.5 (71)	70.8 (71)
Cys	ND ^b	0.2 (0)
Val	58.9 (59)	59.6 (60)
Met	10.7 (11)	10.1 (10)
Ile	30.1 (30)	32.2 (32)
Leu	36.9 (37)	36.9 (37)
Tyr	28.4 (28)	28.4 (28)
Phe	17.1 (17)	18.2 (18)
Lys	46.9 (47)	48.0 (48)
His	11.2 (11)	11.3 (11)
Arg	14.6 (15)	14.5 (15)
Trp	ND	11.0 (11)

^a Analytical values listed are expressed as residues per molecule, based on a molecular weight of 75,000. Values in parentheses are nearest integers. Samples A and B were derived from method A (cell lysate) and method B (envelope), respectively. Samples were hydrolyzed either with 6 N HCl (A) at 110°C for 24 h or with 6 N HCl (B) at 110°C for 22 h.

^b ND, Not determined.

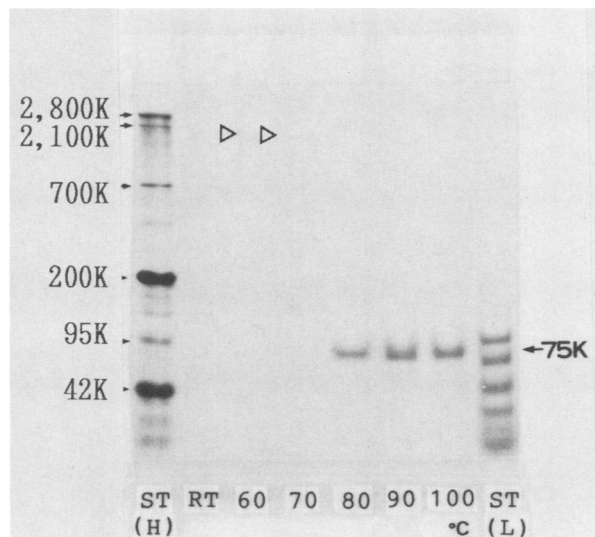


FIG. 3. Electrophoretogram of the 75K protein and its complex in 0.5% agarose-2% acrylamide composite gel electrophoresis. The samples (2 μ g of protein) were heated at various temperatures for 20 min before electrophoresis. Numbers indicated at the bottoms of lanes show temperatures (in degrees Celsius) at which the samples were heated (RT, room temperature). \triangleright , Position of the 75K protein complex (indicated because of faint bands). ST(H) and ST(L), Standard proteins for higher- and lower-molecular-weight proteins, respectively. From the top to the bottom in lane ST(L), 93K, 66K, 45K, 31K, 21K, and 14K proteins are shown. 93K, 93,000; 75K, the 75K protein. In lane ST(H), 2800K, α -connectin; 2100K, β -connectin; 700K, neblin; 200K, myosin (heavy chain); 95K, α -actinin; and 42K, actin (all identified by using rabbit myofibrillar proteins).

different starting materials were immunologically identical (data not shown).

Molecular weight. As mentioned earlier, the 75K protein appeared to be present as a large stable complex in the presence of SDS, since the complex did not dissociate during gel filtration in the presence of SDS and SDS-PAGE at room temperature. Even heating at 70°C in the presence of SDS for 20 min did not cause dissociation. To determine the molecular weight of the 75K protein complex, we subjected it to agarose-acrylamide composite gel electrophoresis, developed originally for ribonucleic acids, that was modified for molecular weight estimation and separation of muscle proteins (A. Hattori and K. Takahashi, personal communication). The 75K protein had an apparent molecular weight of about 2,000,000, on the basis of the positions of α -connectin, β -connectin, and neblin, which had apparent molecular weights of 2,800,000, 2,100,000, and 700,000, respectively. The estimation of the molecular weight of the 75K protein complex by electrophoresis was supported by the results from gel filtration: the 75K protein was sharply eluted at the K_{av} value of 0.13 from the Sephacryl S-400 column, as mentioned above (Fig. 1 and 2). From the selectivity curves in gel filtration with Sephacryl S-400 (provided by Pharmacia Fine Chemicals), the K_{av} value of 0.4 corresponds to a molecular weight of about 1,000,000 for globular proteins, indicating that K_{av} values of less than 0.4 represent molecular weights of more than 1,000,000. The degree of band intensity produced by staining a given amount of the 75K protein complex in composite gels was always much less than what was produced by staining the same amount of 75K protein monomers (Fig. 3). This could

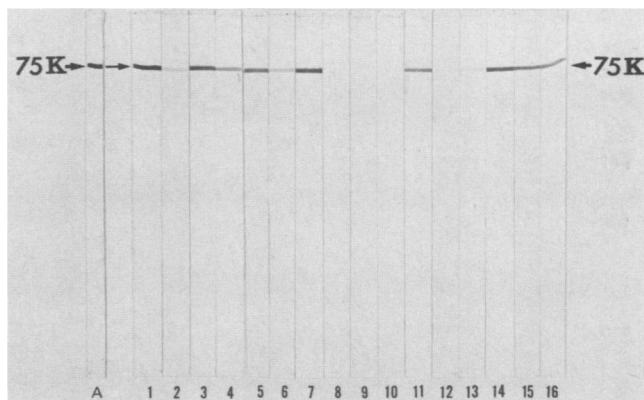


FIG. 4. Detection of specific antibodies against the 75K protein in patients with adult periodontitis. Purified *B. gingivalis* 75K protein (usually ca. 20 μ g of protein) was applied onto SDS-PAGE with a single lane of a preparative type. After electrophoresis, proteins in the gel were electrophoretically transferred to a nitrocellulose sheet. After being treated with bovine serum albumin for blocking, the sheet was cut into strips, and each strip was used for the detection of specific immunoglobulin G antibodies. The strips were incubated separately with different sera (2,000-fold dilution, 2.5 μ l into 5 ml of the buffer) in screw-capped tubes overnight. Strips 1 through 16 correspond to each serum sample from patients with adult periodontitis. Ten patients showed clear positive reactions to the protein. Strip A was treated with anti-75K protein serum from a rabbit as a control. 75K, 75K protein.

be due to the 75K protein complex having limited ability to bind dye because of its native conformation. Since the 75K protein existed in a large, stable complex, it is of great interest from a structural point of view to examine the morphology of the complex. An attempt to observe it by electron microscopy after negative staining remained unsuccessful because the 75K protein complex caused much larger aggregates after dialysis for removing SDS, and the aggregates tended to lose the ability to adsorb to a collodion membrane on a grid but not to the polystyrene microdilution plate for an enzyme-linked immunosorbent assay.

Immunoresponses to the 75K protein in patients with periodontal diseases. In a previous work (33), the 75K protein, fimbriae, and some proteins in the envelope were strongly recognized by the humoral immunological system in patients with periodontal diseases. Although an immunoblotting technique resulted in clear, positive reactions to what was blotted to membranes, surface components except for fimbriae were used as whole cell extracts because of the unavailability of these materials as pure forms. We, therefore, intended to confirm the results by using a homogeneous 75K protein preparation. The presence of immunoglobulin G antibodies against the 75K protein in patient sera is shown in Fig. 4. Of the 35 patients with adult periodontitis, who had to fulfill the earlier-described criteria (33), 27 had specific antibodies with high titers against the 75K protein. Moreover, when we considered the high dilution of the sera (2,000-fold) used in immunoreactions, some sera seemed to have very high titers of the antibodies on the basis of the degree of intensity in positive 75K protein bands (Fig. 4, lanes 1, 3, 5, 7, 14, and 15). When we used *B. gingivalis* whole cell extracts as antigens and the human sera at a high dilution as antibodies in the Western blotting assay, we often found that the 75K protein was the only band or a clear, major band detected, suggesting that the 75K protein is an immunodominant protein. Healthy subjects (16 people) did

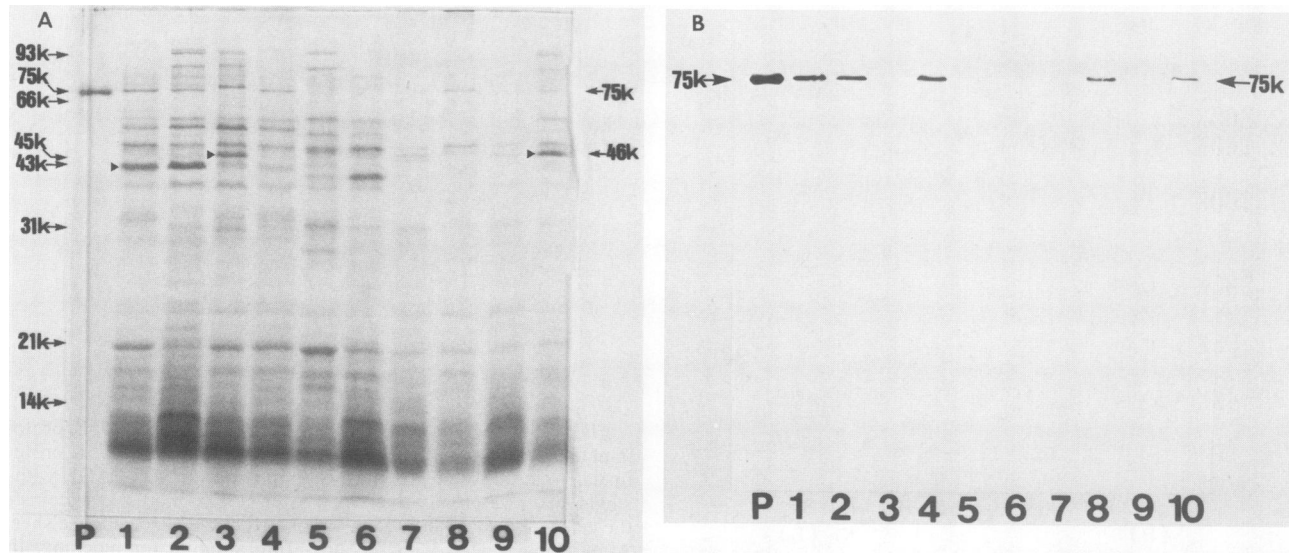


FIG. 5. Species specificity of the 75K protein in *B. gingivalis*. (A) The electrophoretogram of the protein pattern of the whole cell lysates from *B. gingivalis* strains. Lanes: P, purified 75K protein (2 μ g); 1, 381; 2, 1112; 3, 14018; 4, ATCC 33277; 5, W83; 6, JKG-10; 7, D40C-28; 8, D43B-4; 9, D67D-9; 10, Shirai. Each 40 μ g of protein was subjected to electrophoresis. 75K, 75K protein; 46K, fimbriin of type 14018; 43K, fimbriin of type 381. \blacktriangleright , Fimbrilins identified (27). (B) Identification of the 75K protein in the electrophoretogram by Western blotting analysis. The letters, the numbers, and the amounts of protein applied are the same as those identified for panel A. A monoclonal antibody against the 75K protein was used in this experiment (1,000-fold dilution of ascites, 30 μ l into 30 ml of the buffer, overnight).

not have sera that showed any positive reactions to the protein (data not shown).

Species specificity and prevalence of the 75K protein in *B. gingivalis*. The *B. gingivalis* 75K protein was shown to be species specific when SDS-PAGE analysis and the above-described immunoassay were done by using whole cell lysates from related *Bacteroides* species, as well as some other bacteria listed in Materials and Methods and a polyclonal antibody. None of the species except for *B. gingivalis* strains had any immunoreactive 75K protein detected on membranes (data not shown). In 5 (381, 1112, ATCC 33277, D43B-4, and Shirai) of 10 *B. gingivalis* strains tested, the 75K protein was detected in SDS-PAGE gels as well as on the immunoblotting membranes by using a monoclonal antibody (Fig. 5A and B). In some fimbriate strains such as JKG-10, the 75K protein was not detected, suggesting that the presence of the 75K protein is not essential to fimbriation. Western blots with the rabbit antisera generated by intravenous inoculations of whole *B. gingivalis* cells also showed that the 75K protein was strongly antigenic regardless of the route of immunization and the degree of purity (data not shown). Again, we found that in numerous proteins blotted to the membrane, the 75K protein was a clear, major band detected with the anti-whole cell antisera.

Channel-forming activities. Since gram-negative bacteria have channel-forming proteins, porins, as major proteins in the outer membrane even in the whole cell lysate in most cases, the channel-forming activities of various preparations were determined to identify porin in this organism by reconstitution into liposomes at the early stage of this research. Major proteins such as the 43K and the 75K proteins in *B. gingivalis* were initially thought to be porins because they had some characteristics of porin proteins; for example, they formed oligomeric structures even in the presence of SDS. We used the 43K and the 75K protein-rich fractions during the purification to measure channel-forming activities. The degree of permeability (toward L-arabinose) produced by the

incorporation of a given amount of the 75K protein-rich fraction (ca. 60% purity) was about 50% of what was produced by the incorporation of an equivalent amount of *Pseudomonas aeruginosa* porin (98% purity), whose channels have been shown to have much less permeability than *Escherichia coli* porin (OmpF) channels do (36). The 43K protein fraction (ca. 80% 43K protein and some 75K protein) resulted in an even lesser degree of permeability than the 75K protein-rich fraction did, showing only a trace of channel-forming activity. When more purified preparations of the 75K protein were used, a lesser degree of permeability was observed by the same liposome assay. Since the 75K protein preparation used had some other membrane proteins such as 48K and 50K proteins, and since the whole envelope of *B. gingivalis* showed a degree of permeability comparable to that of *P. aeruginosa*, neither the 75K nor the 43K protein was considered to be a porin protein. The 43K protein has recently been established as a constituent protein of fimbriae, fimbriin (27, 34).

DISCUSSION

In this study, we have shown that the 75K protein could be prepared from an envelope fraction or a cell lysate from *B. gingivalis* 381. The 75K protein had several interesting characteristics. (i) It was present in a stable, large complex with an apparent molecular weight of about 2,000,000 in the presence of SDS. (ii) It existed as a major component in the outer membrane or the outermost part of the bacterial cell. (iii) It was strongly recognized by patient humoral immune system, suggesting that it was largely exposed to the outward portion of the cell surface; that is, it might not be buried at the bottom of "dense" lipopolysaccharide with "long" O-antigenic chains (7, 29). More detailed information on *B. gingivalis* lipopolysaccharide is necessary to discuss this problem further. It is also possible that the 75K protein could easily be released as outer membrane blebs or vesicles

from the envelope, as described previously (5, 31) and that they could play a role as strong antigens. Fresh *B. gingivalis* cells brought preferentially strong antibodies against the 75K protein, detected in Western blotting analysis, as well as those against fimbriae in rabbits when they were injected intravenously every 3 days (data not shown). The strong reactivity of the rabbit antisera and the patient sera with the 75K protein indicates that the 75K protein is an immunodominant surface antigen of this organism.

An amino-terminal sequence of the first 27 residues of the 75K protein (F. Yoshimura, T. Takasawa, K. Watanabe, and H. Kato, unpublished data) indicates that the 75K protein has a primary structure different from that of *B. gingivalis* fimbriae (3, 35).

The purification procedure (method B) was simple and efficient enough to allow us to obtain a large amount of the 75K protein, a major surface protein in this organism, suggesting that it could be possible to use this 75K protein as a material for clinical application and to study the process of infection, by using it as a model system by following immunological responses against it. Experiments along this line are in progress in our laboratories.

Results obtained in this study suggest that the 75K protein complex is present in or on the outer membrane, that it is, at least in part, exposed outward, and that the amount brought into cell lysate varies depending on how *B. gingivalis* cells are broken. In this context, if the 75K protein is on the outer surface of the outer membrane, it might compose crystalline bacterial surface layers (S layers). There is, however, no way to simply distinguish whether it is an outer membrane protein or a protein composing the S layers because some surface proteins are tightly associated to or partially embedded in the outer membrane (26). Although S layers in oral *Bacteroides* species have been reported, bacteria causing human infections have rarely been shown to contain an S layer (10, 22). Even an attempt to separate the inner and outer membranes of *B. gingivalis* in a preparative way, by following the method applicable to *E. coli* and *P. aeruginosa*, remains unsuccessful (F. Yoshimura, unpublished data). More work on the surface structures of this interesting oral anaerobe, *B. gingivalis* is needed.

Although some *B. gingivalis* strains did not contain serologically related 75K proteins, these strains may have proteins that are similar to but immunologically different from the 75K protein because some surface proteins, as well as fimbriae, are known to be antigenically diverse as the result of selective pressure exerted by the host immunodefense system through a long host-bacterial interaction (1). Like fimbriin, the strains such as 14018 had a corresponding protein with a slightly larger apparent molecular weight than the 75K protein in SDS-PAGE (27) (Fig. 5A, lane 3).

ACKNOWLEDGMENTS

We thank M. Nishikata for critical review, Y. Takahashi, T. Miyashita, A. Uchida, K. Sagisaka, and K. Takahashi for carrying out some studies and T. Watanabe, A. Hattori, K. Takahashi, and T. Suzuki for their valuable suggestions. We also thank K. Barrymore for helpful advice and discussion of the manuscript.

This work was supported by a grant from the Akiyama Foundation and by Grants-in-Aid for Scientific Research and Co-operative Research from the Ministry of Education, Science and Culture of Japan.

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