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Isolation and Some Properties of Exohemagglutinin from the Culture Medium of *Bacteroides gingivalis* 381

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Exohemagglutinin was found in the culture medium of *Bacteroides gingivalis* 381. Exohemagglutinin was purified *3,150*-fold from culture fluid by ultracentrifugation followed by gel filtration on Sepharose CL-4B and by affinity chromatography on arginine-agarose. Examination of the final preparation of exohemagglutinin by biochemical analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the isolated exohemagglutinin contained three major proteins but not a detectable lipopolysaccharide. Hemagglutination inhibition experiments showed that the activity of exohemagglutinin was inhibited by L-arginine and the arginine-containing peptides, although the activity was unaffected by the sugars tested. Some protein and glycoproteins that were examined also exhibited the inhibitory activity. When the bovine submaxillary mucin was chemically modified by β-elimination and bovine serum albumin was modified by guanidination, the inhibitory effects on hemagglutination were significantly enhanced. These results suggest that the hemagglutination of the isolated exohemagglutinin may be involved in arginine residues as components of ligand-binding sites on erythrocytes.

Adherence of oral microorganisms to tissue surfaces has recently gained increasing attention as an important initial event in the pathogenesis of oral diseases (18). A number of organisms that colonize the oral cavity have been often shown to possess adhesive properties that enable them to bind to soft tissue, salivary pellicle, or other bacteria (24, 28). It has been suggested that the mechanisms of oral bacterial adhesion involve various cell-cell interactions such as electrostatic (21), hydrophobic (7), and ligand-receptor (9) interactions. Recently, it has been shown that some oral bacteria possess lectinlike ligands which specifically bind to the carbohydrate receptor on the respective surfaces (2, 6, 8, 11, 17).

Relatively high proportions of *Bacteroides gingivalis* have been found in periodontal pockets (23), and it has been shown that this organism can agglutinate erythrocytes, bind to sulcular epithelium and saliva-coated hydroxyapatite, and aggregate with a number of oral gram-positive organisms (24). Thus, it may play an important role in the etiology and pathogenesis of periodontal disease. However, the molecular interactions which permit this organism to adhere to the respective surfaces are poorly understood. Hemagglutinating activity of *B. gingivalis* has been studied as one of the parameters that affect the adherence of this organism to cells of oral tissue (20). Results of several investigations have shown that the ability of *B. gingivalis* to agglutinate the erythrocytes is probably associated with fimbriae or the capsulelike outer membrane on such strains (1, 16, 19, 20, 24). However, little attention has been paid to the exohemagglutinin (exoHA) that is present in the culture broth of *B. gingivalis*.

This study deals with the isolation and characterization of the exoHA from culture medium of *B. gingivalis* 381, including the inhibitory effects of various compounds on its hemagglutination.

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MATERIALS AND METHODS

Bacterial strain and growth condition. *B. gingivalis* 381 was obtained from stock strains at Research Laboratory of Oral Biology, Sunstar Inc. The strain was maintained by weekly transfer on plates containing Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood–1 mg of yeast extract (BBL) per ml–5 μg of hemin per ml–1μg of menadione per ml. The organism was grown in 10 ml of Trypticase soy broth (BBL) supplemented with 1 mg of yeast extract per ml–5 μg of hemin per ml–1 μg of menadione per ml. The first series of cultures were transferred into 100 ml of the same broth media used for precultures. Cultures were incubated in an anaerobic system 1024 (Forma, Morietta, Ohio) in an N_2-H_2-CO_2 (85:10:5) atmosphere at 37°C.

Chemicals. Amino acids and sugars were obtained commercially from Wako Chemical Ltd., Osaka, Japan. Biologically active peptides were purchased from Peptide Institute Inc., Osaka, Japan. Bovine submaxillary mucin (BSM), α_1-acid glycoprotein, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, Mo. Isolation and purification of proline-rich glycoprotein from human parotid saliva were carried out by the method of Shibata et al. (22). The β-eliminated BSM was prepared by treating alkaline borohydride by the method of Tanaka et al. (25, 26). The guanidination of BSM with *O*-methylisourea was performed by the method of Kimmel (13).

Hemagglutination assay. The hemagglutinating activity was determined at room temperature with a microtiter plate (Linbro Division Flow Laboratories Inc., Hamden, Conn.). Because the exoHA of *B. gingivalis* was nonspecific for blood group ABO types on hemagglutinating activity in our preliminary check, human O erythrocytes were used for the hemagglutination assay in this study. The sample was diluted in twofold series in isotonic saline, and an equal volume of a 2% suspension of human erythrocytes was added. The mixtures were left to stand for 90 min, and hemagglutination

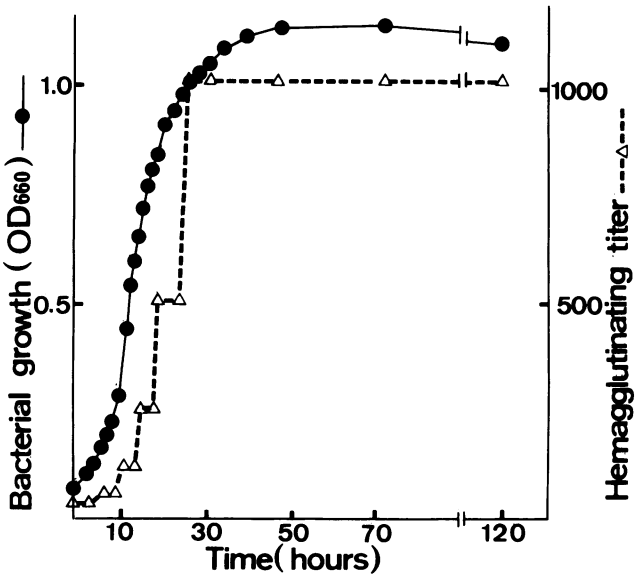

FIG. 1. Time course of hemagglutinating activity of exoHA in the culture supernatant of *B. gingivalis* 381. After centrifugation to remove bacterial cells from the culture medium, the hemagglutinating activity of exoHA was determined by a serial twofold assay with a microtiter plate. The bacterial growth was expressed as the absorbance at 660 nm. (OD, optical density).

was determined by observing the mixture with the naked eye. Titer was expressed as the reciprocal of the highest dilution showing positive agglutination; specific hemagglutinating activity was expressed as titer per microgram of protein per milliliter.

Hemagglutination inhibition test. To screen carbohydrates, amino acids, and other substances for inhibition of hemagglutination, serial dilutions of the substances were prepared; and equal volumes of exoHA (containing 4 titers of hemagglutinating activity) and of erythrocyte suspension were added to each dilution of the substrate solution. The hemagglutination was recorded after 90 min. The reaction mixture that did not contain the substrates was used as a control. The degree of inhibition was expressed as a final concentration of the maximum dilution at which the inhibitor could exhibit the inhibitory effect of hemagglutinating activity.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 10% gel (pH 8.8) was carried out by the method of Laemmli (14). Samples were dissolved in 0.05 M Tris hydrochloride buffer (pH 6.8) with 1% SDS–5% 2-mercaptoethanol–10% glycerol and immersed in a boiling water bath for 5 min. Phosphorylase *b* (molecular weight [MW], 94,000), bovine serum albumin (MW, 67,000), ovalbumin (MW, 45,000), carbonic anhydrase (MW, 30,000), and soybean trypsin inhibitor (MW, 20,000) were used for MW markers (Pharmacia, Uppsala, Sweden). Electrophoresis was done at a constant current of 2 mA per tube for 4 h at 4°C. Bromophenol blue was used as the tracking dye. Gels were stained with 0.2% Coomassie brilliant blue R-250.

Isoelectric focusing. Isoelectric focusing was carried out with a 110-ml column (LKB Instruments, Inc., Bromma, Sweden) by the method of Vesterberg and Svenson (27). The ampholite concentration was 1% with a pH range from 3.5 to 10.0 in a sucrose gradient. The run was achieved at 0 to 4°C, and the voltage was 800 V. The focusing was completed in 40 h, and 3.5-ml fractions were collected. The pH, absorbance at 280 nm, and hemagglutinating activity of each fraction were measured.

Biochemical analysis. Protein content was determined by Hartree's modification of the Lowry assay (10), with bovine serum albumin used as a standard. Total neutral carbohydrate was determined by the phenol-sulfuric acid method (5) with D-glucose used as a standard. Nucleic acid content was estimated by the ratio of UV light absorption at 280 nm compared with that at 260 nm (15). Lipopolysaccharide (LPS) was estimated colorimetrically by the method of Janda and Work (12) with LPS purified from *Escherichia coli* O111:B4 as a standard.

RESULTS

Time course of exoHA production. The relationship of exoHA production to bacterial growth was examined. The production of exoHA increased in the medium during mid-log phase and reached a plateau at stationary phase. The

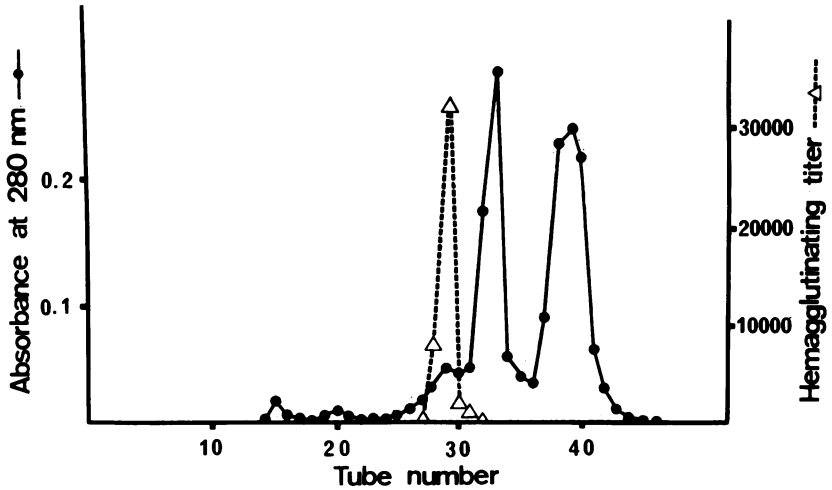

FIG. 2. Elution profile for gel filtration on Sepharose CL-4B. The sample obtained from ultracentrifugation of the culture supernatant was applied to a Sepharose CL-4B gel column and chromatographed at 10 ml/h in PBS containing 0.1% Triton X-100. Each fraction (ca. 7 ml) was assayed for the absorbance at 280 nm and hemagglutinating activity after it was dialyzed with excess distilled water.

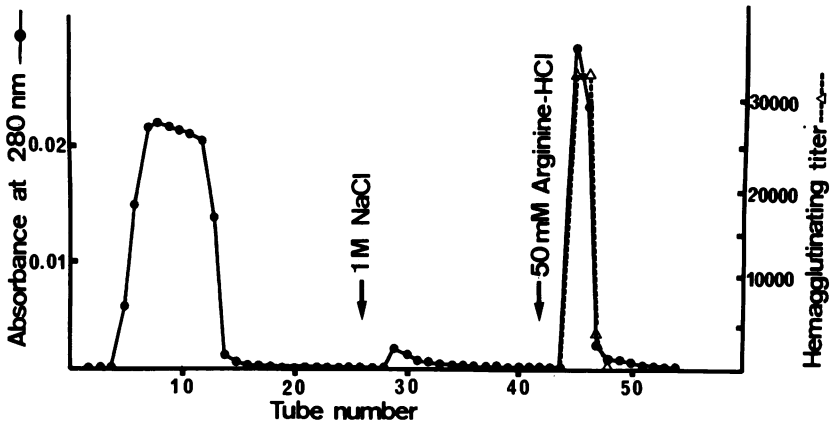

FIG. 3. Elution profile for affinity chromatography on arginine-agarose. The active fraction was applied to an arginine-agarose column. After the column was washed with 100 ml of 0.02 M PBS containing 0.1% Triton X-100, it was developed with the same buffer containing 1 M NaCl and then eluted with the same buffer containing 50 mM arginine hydrochloride.

exoHA titer did not drop during the period that it was monitored (120 h). (Fig. 1)

Isolation and partial purification of exoHA. The culture for exoHA purification was grown anaerobically in 500 ml of medium at 37°C for 30 h. The culture growth was centrifuged at 10,000 × *g* for 30 min, and the supernatant was pooled following purification. All subsequent steps in the purification were carried out at 4°C.

The supernatant was centrifuged at 200,000 × *g* for 3 h. The yellowish, gel-like, and translucent pellets obtained by ultracentrifugation were suspended in 0.02 M phosphate-buffered saline (PBS; pH 7.0) containing 1% Triton X-100.

The pellet sample was applied to a Sepharose CL-4B gel column (1.8 by 100 cm) that had been equilibrated with 0.02 M PBS containing 0.1% Triton X-100. The flow rate was 10 ml/h, and the eluate was collected into 7-ml fractions. The fractions eluted at almost twice the void volume of the column, indicating hemagglutinating activity (Fig. 2).

Because the hemagglutination of crude sample was inhibited by L-arginine in our preliminary experiments, L-arginine-agarose was used for purification of exoHA. The active fractions were pooled and applied to an affinity column (2.3 by 7 cm) packed with arginine-agarose that had been equilibrated with 0.02 M PBS containing 0.1% Triton X-100. The column was washed with 100 ml of the same buffer. Subsequently, the column was developed with the same buffer containing 1 M NaCl and then was eluted with the buffer containing 0.05 M arginine hydrochloride. The flow rate was 10 ml/h, and the eluate was collected in 7-ml fractions. The hemagglutinating activity was detected only in the fractions which eluted with the buffer containing 0.05 M arginine hydrochloride (Fig. 3). The final preparation had a specific activity of 1,260 titer/μg of protein per ml. The partial purification procedure is summarized in Table 1. Through these procedures, the exoHA was purified about 3,150-fold from the culture fluid, based on the specific hemagglutinating activity. The final preparation of exoHA was found to contain 81% protein and 8% carbohydrate, by calculation based on dry weight, and to have no detectable LPS and nucleic acid in quantities of less than 1%.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed on the final preparation of the exoHA. The partially purified exoHA possessed three major bands of protein with apparent MWs of about 24,000, 37,000, and 44,000, as well as a small number of minor bands. (Fig. 4).

Isoelectric point. The partially purified exoHA was subjected to isoelectric focusing in a pH 3.5 to 10.0 gradient. The hemagglutinating activity of exoHA was found as a single peak with an isoelectric point of pH 4.0 corresponding to a major peak that absorbed at 280 nm (Fig. 5).

Effects of pH, ionic strength, temperature, and metal ions on hemagglutinating activity. The effect of pH on hemagglutinating activity was examined in citrate-phosphate buffer (pH 5.0 and 6.0), phosphate buffer (pH 7.0), and Tris hydrochloride buffer (pH 8.0 and 9.0) containing 0.15 M NaCl. The maximum activity of hemagglutination was found at a pH range of 7.0 to 8.0, although the difference of titer by varying the pH from 5.0 to 9.0 was within fourfold of that of the hemagglutinating titer (Table 2). Some hemolysis was observed at less than pH 4.0.

In the presence of NaCl from 0.15 to 2.5 M, several changes of hemagglutinating activity were observed. The addition of NaCl to a concentration of 0.75 M caused eightfold titer increases. However, a reduction in hemagglutinating titer was found in the presense of NaCl from 0.75 to 2.5 M (Table 2).

The thermal effects on hemagglutinating activity were

TABLE 1. Purification of exoHA from *B. gingivalis* 381

Purification method	Vol (ml)	Protein (μg/ml)	Hemagglutination titer	Total titer[a]	Sp act (titer/μg per ml)	Purification (fold)	Recovery (%)
Culture medium	500	9,474	2^{12}	20,580,000	0.4	1	100
Ultracentrifugation	1.5	9,101	2^{20}	15,728,640	115.2	288	77
Gel filtration	45	65	2^{14}	7,372,800	252.1	630	36
Arginine affinity	16	26	2^{15}	6,553,600	1,260.3	3,151	32

[a] Total titer = (volume of sample [microliters]/volume of reaction mixture [100 μl]) × hemagglutination titer.

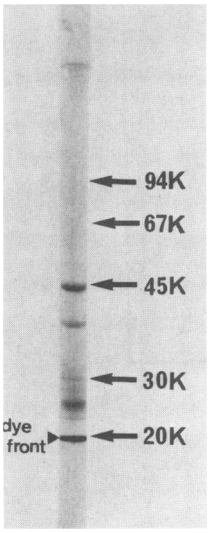

FIG. 4. SDS-polyacrylamide gel electrophoresis of exoHA partially purified from *B. gingivalis* 381. The acrylamide concentration was 10%. Gel was stained with Coomassie brilliant blue R-250. Phosphorylase *b* (MW, 94,000 [94K]), bovine serum albumin (MW, 67,000), ovalubmin (MW, 45,000), carbonic anhydrase (MW, 30,000), and soybean trypsin inhibitor (MW, 20,000) were used as MW markers.

studied at 37, 45, and 60°C. The partially purified exoHA was stable at 37°C for 40 min, but it was almost completely inactivated after the incubation at 60°C for 10 min (Fig. 6).

The effects of EDTA, Ca^{2+}, Mg^{2+}, and Mn^{2+} on the hemagglutinating activity of exoHA were examined by adding disodium EDTA (10 mM) or cation (1 mM) to the exoHA sample. It was found that EDTA, Ca^{2+}, Mg^{2+}, and Mn^{2+} had no effect on the hemagglutinating activity of exoHA.

Inhibition of hemagglutination by various compounds. The detection of specific inhibitors of exoHA was examined by a hemagglutination inhibition assay in duplicate. The results of the inhibition assay by various sugars and amino acids are summarized in Table 3. The exoHA activity was unaffected by the presence of sugars at a concentration of 0.1 M. In amino acids, L-arginine was found to have a significant inhibitory effect on exoHA. The results of exoHA inhibition by some peptides are shown in Table 4. Bradykinin and its derivatives with arginine residues exhibited an inhibitory effect, but bradykinin potentiator C, which does not contain an arginine residue, was not inhibitory at a 420 μM concentration. The most potent inhibitor tested was angiotensin I, which has an arginine residue at the second position from the amino-terminal end. The glycoproteins and proteins that were examined also inhibited hemagglutination, and the modification reaction such as β-elimination of BSM and guanidination of BSA made these compounds more potent inhibitors (Table 5).

TABLE 2. Effect of pH and ionic strength on hemagglutinating activity

Reaction mixture condition	Titer
pH[a]	
5.0	2^8
6.0	2^9
7.0	2^{10}
8.0	2^{10}
9.0	2^9
NaCl concn (M)[b]	
0.15	2^{10}
0.3	2^{12}
0.5	2^{12}
0.75	2^{13}
1.0	2^{12}
2.0	2^{10}
2.5	2^{10}

[a] 0.15 M NaCl.
[b] pH 7.0.

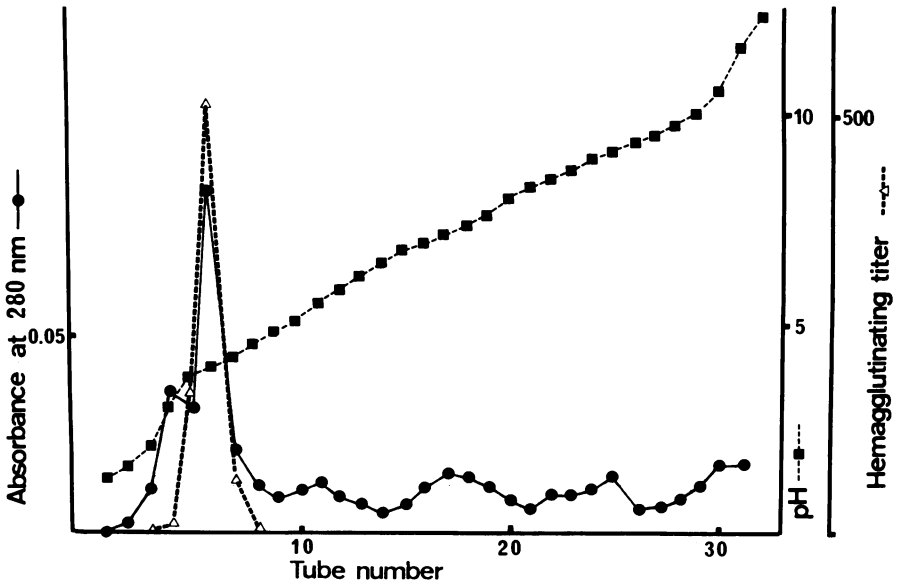

FIG. 5. Isoelectric focusing pattern of the exoHA using an ampholine column with a pH range of 3.5 to 10.0. The pH of each fraction (ca. 3.5 ml) was determined at 4°C. The fractions were assayed for the absorbance at 280 nm and for hemagglutinating activity after they were dialyzed with excess distilled water.

TABLE 3. Hemagglutination inhibition assay of exoHA with various sugars and amino acids

Sugar or amino acid	Lowest inhibitory concn (mM)
D-Fructose	>100
L-Fucose	>100
D-Mannose	>100
D-Galactose	>100
Lactose	>100
Melibiose	>100
Raffinose	>100
N-Acetyl-D-galactosamine	>100
N-Acetyl-D-glucosamine	>100
Glycine	>100
L-Alanine	>100
L-Valine	>100
L-Serine	>100
L-Arginine	1.9
L-Proline	>100
L-Lysine	>100
L-Leucine	>100

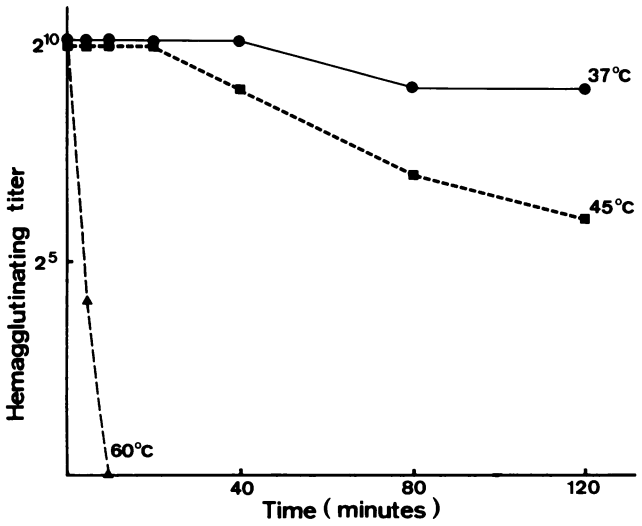

FIG. 6. Heat inactivation of the hemagglutinating activities of exoHA. exoHA was incubated in a water bath at 37, 45, and 60°C for the indicated times. After incubation, the hemagglutinating activity was determined under the conditions described in the text.

DISCUSSION

Several investigators (1, 16) have reported the presence of hemagglutinating activities in the culture medium of *B. gingivalis*. In this study, it was also found that *B. gingivalis* produced exoHA in the culture medium. Preliminary experiments revealed that better growth of the bacteria and higher total hemagglutinating titers of the exoHA were observed in the medium used here compared with several other culture media. Although the hemagglutinating activities and some properties of crude exoHA were sometimes checked throughout this experiment, no change was found in hemagglutination of exoHA during repeated passage of the stock strain. Because exoHA rapidly increased in the supernatant of mid-log-phase cultures, exoHA in the medium may be released from a membrane fragment such as the outer membrane and fimbriae, but this would not result from whole-cell lysis. Although the data are not shown, the total hemagglutinating titer of exoHA from the culture medium was two- or fourfold higher than that of the hemagglutinin isolated from bacterial cells by homogenization with a Waring blender. It is conceivable that exoHA could promote adherence of bacterial cells to oral surfaces by binding glycoproteins and proteins in saliva and serum that would interfere with adherence.

Attempts to isolate the exoHA from culture medium by the method of acidification, $MgCl_2$ precipitation, and ammonium sulfate precipitation were unsuccessful; but a simple technique of ultracentrifugation employed here was able to isolate effectively exoHA from the medium. The precipitate of exoHA obtained by ultracentrifugation was completely solubilized or disaggregated with Triton X-100. In a preliminary check, almost all hemagglutinating activity of exoHA was found in the supernatant after ultracentrifugation of exoHA in the buffer containing Triton X-100, and exoHA suspended in the buffer without Triton X-100 was eluted at the void volume on Sepharose CL-4B chromatography. exoHA was effectively purified by arginine-agarose affinity chromatography. Arginine-agarose is bound to the matrix via its α-amino group, leaving both the guanido and the α-carboxyl groups free to interact with sample substances during chromatography. exoHA was not eluted with the buffer containing 1 M NaCl, but it was specifically eluted with buffer containing 0.05 M arginine. Triton X-100 was incorporated in all elution buffer. These results suggest that the role of electrostatic or hydrophobic forces in binding would be minimal and the stereospecific effect would contribute to the resolution of exoHA from arginine-agarose.

The final sample of exoHA had a very high specific activity (as much as 1,260 titer/μg of protein per ml). Although it is difficult to compare our data on specific activity of the exoHA with those of other investigators, because of the differences in method and erythrocytes used, the activity of exoHA in our preparation was much higher than has been observed in other periodontopathic bacteria (4, 29). It was determined by SDS-polyacrylamide gel electrophoresis that the partially purified exoHA contained three major proteins with MWs of about 24,000, 37,000, and 44,000. Biochemical analyses revealed that exoHA was composed mainly of protein but did not contain either LPS or nucleic acid. The outer membrane complex isolated from *B. gingivalis* by Boyd and McBride (1) possessed both hemagglutinating and bacterial aggregating activity, and the

TABLE 4. Hemagglutination inhibition assay of exoHA with arginine derivative peptides.

Derivative	Amino acids	Lowest inhibitory concn (μM)
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	3.2
Tyrosyl-bradykinin	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1.4
Des-Arg^9-bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	57.7
Bradykinin potentiator C	Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	>420.0
Proctolin	Arg-Tyr-Leu-Pro-Thr	12.0
Angiotensin I	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu	0.1

TABLE 5. Hemagglutination inhibition assay of exoHA with proteins and glycoproteins

Protein or glycoprotein	Lowest inhibitory concn (μg/ml)
Proline-rich glycoprotein from human parotid saliva	1.9
α_1-Acid glycoprotein	0.7
Transferrin	1.0
BSA	1.9
Guanidyl BSA	0.06
BSM	0.7
β-Eliminated BSM	0.09

hemagglutinin separated from bacterial binding adhesin consisted of LPS and a small amount of protein. It has been reported that hemagglutinating activity is due to the presence of fimbriae on *B. gingivalis* (19, 20, 24). Recently fimbriae with MWs of about 43,000 have been purified from *B. gingivalis* by Yoshimura et al. (30), but the fimbriae do not show hemagglutinating activity. The MW of purified fimbriae was consistent with that of one of three major proteins seen by gel electrophoresis of exoHA. It was not possible to determine whether fimbriae or outer membrane was responsible for the activity observed here. It is conceivable that the hemagglutination of exoHA may be on the multicomponent system derived from cell surface-associated molecules.

The hemagglutinating activity of exoHA was not inhibited by any sugars examined, but was inhibited by various glycoproteins and protein present in serum or saliva. Similar results were reported by Slots and Gibbons (24) and Okuda et al. (19). In addition, because β-eliminated BSM enhanced the inhibitory effect, hemagglutination may be inhibited by a protein moiety of BSM but not by a carbohydrate chain of BSM. These findings suggest that bacterial lectin is not involved in hemagglutination by exoHA . It was found that arginine and arginine-containing peptides inhibited hemagglutination. Dehazya and Coles (3, 4) also showed the inhibition of hemagglutinating activity of *Fusobacterium nucleatum* in the presence of a low concentration of arginine and binding of dansyl-L-arginine to bacterial cells. They pointed out that arginine residues might play an important role in agglutinin-receptor binding. Interestingly, arginine-containing peptides, such as angiotensin I, showed inhibitory ability, even though the arginine residue was not located at the amino-terminal residue of the peptide chain. Furthermore, the guanidination of BSA made the compound a more potent inhibitor. These results suggest that the inhibitory effect of arginine on hemagglutination can be attributed to the guanido group of arginine. The guanido group gives arginine a uniquely cationic character at physiological pH. Because the exoHA was an acidic protein, the electrostatic effect might influence the interaction between exoHA and arginine residues. However, based on our data concerning affinity chromatography on arginine-agarose and the specifically inhibitory effects of arginine and arginine-containing peptides, arginine-specific binding would be involved in this interaction. It is conceivable that arginine may function as a contact residue between exoHA and erythrocytes during agglutination.

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