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CHARACTERIZATION OF PORPHYROMONAS (BACTEROIDES) GINGIVALIS HEMAGGLUTININ AS A PROTEASE

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A hemagglutinin (HA) was purified to homogeneity from the membrane fraction of the oral bacterium Porphyromonas gingivalis. The HA possessed protease activity hydrolyzing proteins and arginine-containing synthetic substrates. The protease activity was inhibited by thiol-blocking reagents, and hence the HA can be characterized as a cystein protease. The HA functions as an attachment factor and its substrate-binding site is responsible for the attachment to an erythrocyte.

Porphyromonas gingivalis, a putative periodontal pathogen (1), has unique hemagglutinin (HA) which, unlike lectins, is not inhibited by sugars but is inhibited by arginine and arginine-containing peptides (2,3). Our previous investigation (4) demonstrated that hemagglutination by the P. gingivalis membrane fraction is inhibited by protease inhibitors such as leupeptin, antipain and TLCK, and that the binding of the bacterial cells to erythrocytes is inhibited by leupeptin. We therefore speculated that P. gingivalis HA has both HA and protease activities and that the same site of the HA molecule participates in erythrocyte binding and substrate binding. In this study, we have attempted to obtain direct evidence that P. gingivalis HA is a protease by using a purified HA preparation.

Abbreviations: HA, hemagglutinin; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TLCK, Tos-Lys chloromethyl ketone; ALCK; Ac-Lys chloromethyl ketone; Bz, benzoyl; Z, benzyloxycarbonyl; Boc, t-butyloxycarbonyl; Tos, tosyl; Ac, acetyl; pNA, p-nitroanilide; MCA, 4-methyl-coumaryl-7-amide.

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METHODS

Assay of HA: Serially twofold-diluted samples in 0.5 ml of SSC (15 mM trisodium citrate and 0.15 M NaCl, pH 7.5) containing 4 mM 2-ME were mixed with an equal volume of 0.5% (v/v) chicken erythrocyte suspension in SSC using a titer plate with U-bottom wells. The mixture was left standing for 90 min at room temperature. The titer was expressed as the reciprocal of the highest dilution showing hemagglutination.

Assay of Protease: Unless otherwise stated, protease activity was assayed with Bz-Arg-pNA as the substrate. A sample was mixed with 1 ml of 50 mM Tris-HCl (pH 8.5) containing 50 mM 2-ME and 0.2 mM Bz-Arg-pNA. After an appropriate incubation time at 25°C, 0.2 ml of 50% acetic acid was added and the increase in absorbance at 410 nm was measured.

Purification of HA: The membrane fraction (wet) of P. gingivalis 381 prepared as described previously (5) was homogenized with a Teflon-pestle homogenizer in 50 volumes of 20 mM phosphate buffer (pH 7.3) containing 1% CHAPS, 25 mM 2-ME and 5 mM methylguanidine. The homogenate was incubated for 2 h at 40°C to effect solubilization and centrifuged at 110,000 x g for 1h. After the supernatant had been incubated for 30 h at 40°C to dissociate HA aggregates, HA was purified from this supernatant (10 ml) by sequential steps of chromatography consisting of Bio-Gel A-0.5m (Bio-Rad) gel filtration (Fig. 1A), DE-52 (Whatman) ion-exchange chromatography (Fig. 1B) and arginine-cellulose affinity chromatography (Fig. 1C). The affinity adsorbent was prepared by coupling of arginine methyl ester with CM-52 (Whatman) using a water-soluble carbodiimide followed by trypsin treatment for the removal of the methoxy group.

RESULTS

Purity of HA: SDS-PAGE of the HA preparation obtained from an arginine-cellulose affinity column (Fig. 1C) gave a single band with an apparent molecular mass of 44 kDa under reduced and non-reduced (not shown) conditions (Fig. 2).

Attempt to separate HA and protease activities: As the chromatogram of Fig. 1C shows, the HA activity was accompanied by the protease activity hydrolyzing Bz-Arg-pNA. To learn whether the protease activity was due to a trace of contaminating protease(s) in the purified HA preparation, the HA preparation was analyzed by PAGE in the presence of CHAPS (Fig. 3). The HA and the protease activities could not be separated from each other. Gel filtration on Bio-Gel A-0.5m in the presence of CHAPS also failed to separate the two activitie (not shown). Next, we tried to separate the two activities under dissociative conditions. The purified HA preparation was treated with the radioactive active site-directed irreversible protease inhibitor [Ac-^{3}H]ALCK (the synthesis and properties of this compound will be published elsewhere) and

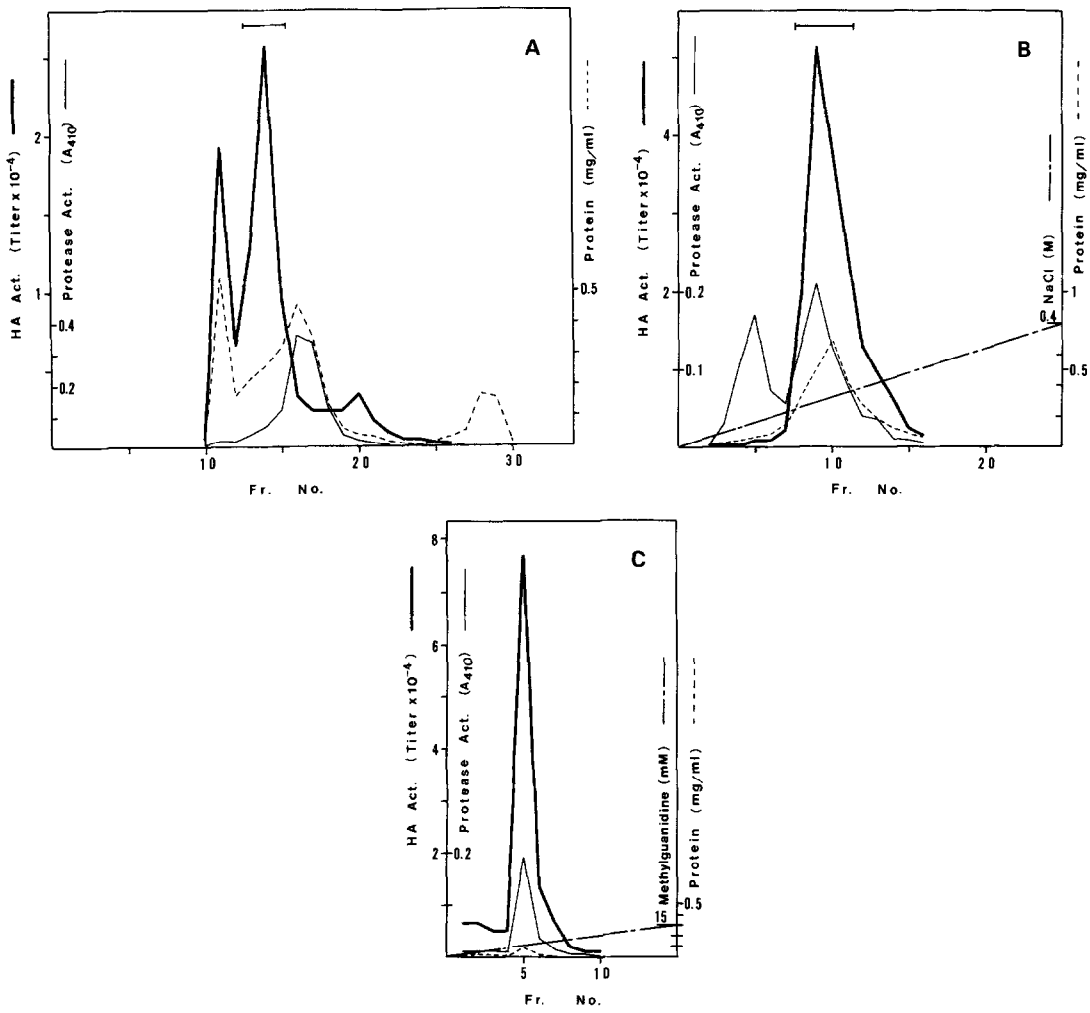

Fig. 1. Purification of P. gingivalis HA. (A) Gel filtration on a Bio-Gel A-0.5m column (3.1 x 74 cm). (B) Ion-exchange chromatography on a DE-52 column (1.0 x 6.4 cm). (C) Affinity chromatography on an arginine-cellulose column (1.0 x 6.4 cm). Elution buffer: (A) 20 mM phosphate buffer (pH 7.3)/ 0.1% CHAPS/25 mM 2-ME/1 mM methylguanidine; (B and C) the same as in A except that methylguanidine was excluded. Fraction volume: (A) 20 ml; (B and C) 4 ml. All chromatography steps were done at 4°C. Fractions were assayed for HA activity, protease activity (10-µl aliquot, 10-min incubation) and protein content (estimated from fluorescence of tryptophan). Fractions pooled are indicated by the bar. The first HA peak in A seemed to contain aggregates and therefore was not fractionated further.

subjected to SDS-PAGE. As Fig. 4 shows, radioactivity and a Coomassie blue-stained band were detected at the same position. Direct radioactivity measurement of excised fractions of the stained gel suspended in Econofluor containing 3% Protosol (DuPont-NEN) confirmed that the radioactivity was incorporated exclusively into the stained band (not shown). These results demonstrate that the protease activity is not due to contamination, i.e., the HA and the protease activities are exhibited by the same molecule.

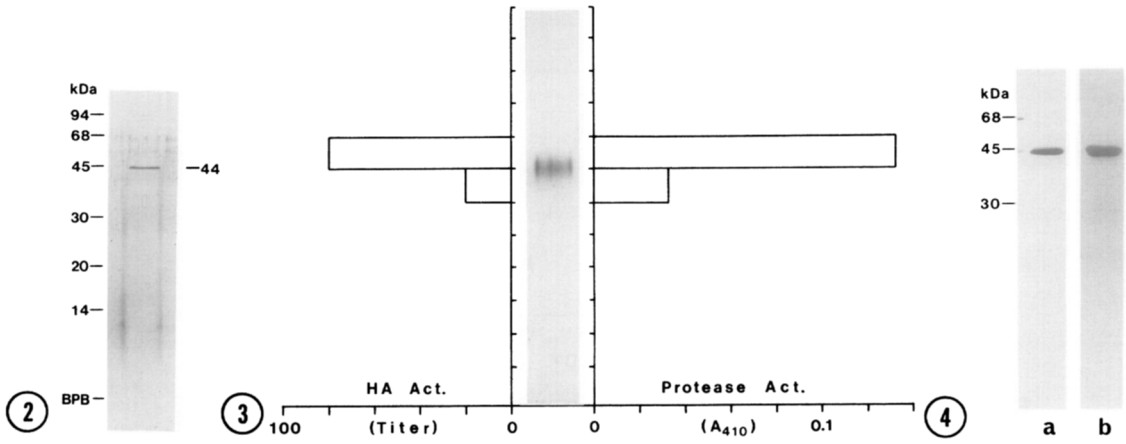

Fig. 2. SDS-PAGE of purified HA. The electrophoresis was performed using the Laemmli buffer system (10) in 12.5% polyacrylamide slab gel. The sample was reduced with 5% 2-ME. The gel was silver-stained.

Fig. 3. PAGE of purified HA. The electrophoresis was performed in duplicate using the Davis buffer system (11) in 5% polyacrylamide slab gel in the presence of 0.6% CHAPS. One lane was stained with Coomassie brilliant blue R-250. The other was excised (5 mm) and each gel fraction was pulverized with a spatula in 0.5 ml of 50 mM Tris-HCl (pH 8.5) containing 50 mM 2-ME. After incubation of the suspension at 4°C for 18 h, the supernatant (100 µl) was assayed for HA activity and the whole of the remainder was assayed for protease activity (30-min incubation).

Fig. 4. SDS-PAGE of [Ac-^{3}H]ALCK-treated HA. The purified HA, after removal of methylguanidine by dialysis, was incubated with 50 µM [Ac-^{3}H]ALCK at 25°C for 30 min in 20 mM phosphate buffer (pH 7.3) containing 0.5% CHAPS and 1 mM 2-ME. The incubation inactivated protease activity by 94%. The reaction mixture was dialyzed against dilute HCl and lyophilyzed. SDS-PAGE was performed in duplicate as in Fig. 2. One lane was stained with Coomassie blue (a) and the other was subjected to fluorography (b).

Effects of inhibitors: Various compounds inhibited both the HA and protease activities. Of the compounds examined, leupeptin (6) was the strongest inhibitor: it inhibited the HA activity by 98% and the protease activity by 99% (measured with 0.04 mM Bz-Arg-pNA after 10-min preincubation at 25°C) at 0.1 µM. Salmine sulfate, an arginine-rich polypeptide, inhibited the HA activity by 81% and the protease activity by 69% at 2 µg/ml (roughly 0.2 - 0.4 µM). Benzamidine, methylguanidine, agmatin and arginine also inhibited both activities, though much more weakly. The active site-directed irreversible protease inhibitors TLCK (7) and ALCK, and the thiol-blocking reagents N-ethylmaleimide and iodoacetamide inactivated both activities. The serine enzyme inhibitor diisopropylfluorophosphate was not inhibitory. Sugars and amino acids other than arginine did not inhibit either activity. Inactivation of the two activities by TLCK was investigated with various TLCK concentra-

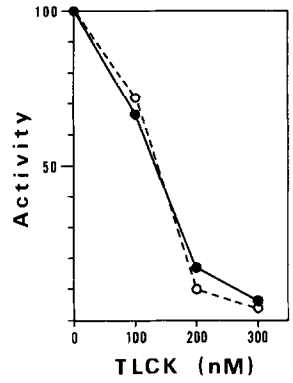

Fig. 5. Inactivation of HA (●) and protease (○) activities by TLCK. The purified HA, after removal of methylguanidine by dialysis, was incubated with TLCK at 25°C for 1 h in 20 mM phosphate buffer (pH 7.3) containing 0.5% CHAPS and 5 mM 2-ME. Longer incubation did not cause further inactivation. Probably, TLCK reacted with 2-ME as well as with the HA.

tions. As Fig. 5 clearly shows, the two activities decreased in parallel with increasing TLCK concentrations. These results indicate that the HA can be characterized as a cystein protease and that its catalytic activity, or at least the substrate-binding site, is essential for hemagglutination.

Role of HA in hemagglutination: No protease activity could be detected in the supernatant of the wells showing hemagglutination. Addition of leupeptin to these wells (final concentration of 100 μM) and resuspension of the agglutinated erythrocytes resulted in the collapse of hemagglutination (8-fold decrease in titer). As described above, hemagglutination was strongly inhibited in the presence of 0.1 μM leupeptin. From the wells that showed negative hemagglutination, the well with the highest HA concentration was chosen and the protease activity in the supernatant was measured (6-day incubation at 37°C with 0.5 mM Bz-Arg-pNA). About 80% of the total protease activity was found to be unattached to erythrocytes. These findings demonstrate that the HA functions as an attachment factor and that its substrate-binding site is the sole site for erythrocyte attachment.

Hydrolysis of substrates by HA: The catalytic properties of the HA were investigated with small synthetic substrates and proteins. As shown in Table 1, the HA hydrolyzed only those synthetic substrates containing arginine. The ester substrate (Bz-Arg-OC_2H_5) was hydrolyzed much more slowly than the amide

Table 1. Hydrolysis of synthetic substrates by purified HA

Substrate	Hydrolysis rate
Bz-Arg-pNA	100
Z-Arg-pNA	45
Bz-Arg-MCA	111
Tos-Gly-Pro-Arg-pNA	155
Bz-Arg-OC_2H_5	1.6

Hydrolysis not observed: glutaryl-Phe-pNA, Bz-Tyr-pNA, succinyl-$(Ala)_3$-pNA, Z-Gly-Pro-pNA, Bz-DL-Lys-pNA (0.08 mM), Tos-Gly-Pro-Lys-pNA, Boc-Val-Leu-Lys-MCA

Substrates (0.04 mM) were incubated with the purified HA at 25°C in 50 mM Tris-HCl (pH 8.5) containing 50 mM 2-ME. Hydrolysis was followed by the continuous monitoring of the change of absorbance at 410 nm (p-nitroanilide), 370 nm (4-methylcoumaryl-7-amide) and 253 nm (Bz-Arg-OC_2H_5). The rate of Bz-Arg-pNA hydrolysis was assumed to be 100.

substrates. Proteins such as bovine serum albumin, chicken ovalbumin and bovine fibrinogen were also hydrolyzed as demonstrated by SDS-PAGE of the hydrolysates (not shown).

DISCUSSION

In this work we purified an HA from the membrane fraction of P. gingivalis 381 and found it to be a protease able to hydrolyze proteins and arginine-containing synthetic substrates, which confirmed our previous speculation (4). Vibrio cholerae also produces an HA with protease activity. This HA, a metalloprotease, is assumed to change surface characteristics of erythrocytes, thereby allowing them to autoaggregate (8). Our HA, in contrast, is a cystein protease and serves as an attachment factor to erythrocytes via its substrate-binding site. Whether the HA is di- or multimeric, or has more than one substrate-binding site per molecule remains to be determined. The residue on an erythrocyte that specifically interacts with the substrate-binding site seems to be arginine, because (i) the HA binds to arginine-cellulose, (ii) the HA is inhibited by arginine and related compounds, (iii) the HA hydrolyzes arginine-containing substrates, and (iv) treatment of erythrocytes with arginine deiminase results in an appreciable loss of agglutinability (unpublished). Inoshita et al. (2) also suggested the importance of arginine as a contact residue between erythrocytes and their HA from the culture supernatant of P. gingivalis 381. It is not known whether

our HA hydrolyzes erythrocyte proteins or peptides as a prerequisite action for hemagglutination. This should be verified by chemical modification of the HA with reagents that inactivate protease activity without altering erythrocyte-binding ability.

Recently, Li et al. (9) demonstrated partial inhibition of the adherence of P. gingivalis to Actinomyces viscosus by protease inhibitors such as TLCK. It is possible that the HA obtained in the present work and other protease activity-possessing HAs in P. gingivalis, if any, participate, at least in part, in the cohesion of the two bacteria.

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REFERENCES

1. Slots, J., Bragd, L., Wikström, M., and Dahlén, G. (1986) J. Clin. Periodontol. 13, 570-577.
2. Inoshita, E., Amano, A., Hanioka, T., Tamagawa, H., Shizukuishi, S., and Tsunemitsu, A. (1986) Infect. Immun. 52, 421-427.
3. Okuda, K., Yamamoto, A., Naito, Y., Takazoe, I., Slots, J., and Genco, R.J. (1986) Infect. Immun. 54, 659-665.
4. Nishikata, M., Yoshimura, F., and Nodasaka, Y. (1989) Microbiol. Immunol. 33, 75-78.
5. Yoshimura, F., Nishikata, M., Suzuki, T., Hoover, C.I., and Newbrun, E. (1984) Archs Oral Biol. 29, 559-564.
6. Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T., and Umezawa, H. (1969) J. Antibiotics 22, 558-568.
7. Shaw, E., Mares-Guia, M., and Cohen, W. (1965) Biochemistry 4, 2219-2224.
8. Booth, B.A., Sciortino, C.V., and Finkelstein, R.A. (1986) In Microbial Lectins and Agglutinins (D. Mirelman, Ed.), pp.169-182. John Wiley & Sons, Inc., New York.
9. Li, J., Ellen, R.P., Hoover, C.I., and Felton, J.R. (1991) J. Dent. Res. 70, 82-86.
10. Laemmli, V.K. (1970) Nature 227, 680-685.
11. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427.