Collagenolytic Activity of Bacteria

FRANCIS A. WALDVOGEL AND MORTON N. SWARTZ

Department of Medicine, Harvard Medical School, and Medical Service (Infectious Disease Unit), Massachusetts General Hospital, Boston, Massachusetts 02114

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Actively growing aerobic and anaerobic bacteria were screened by a plate assay, with reconstituted guinea pig collagen as a substrate, for their ability to produce a collagenolytic factor. Collagenolytic activity was not demonstrated among the aerobic organisms tested, with the exception of one strain of Staphylococcus aureus (only when grown under anaerobic conditions). Collagenolytic activity, however, was detected in cultures of Clostridium tetani and Bacteroides species other than B. melaninogenicus. Collagenolytic activity of these organisms could be confirmed by measuring the amount of hydroxyproline liberated from the collagen gel during growth. Although collagenase production by Pseudomonas aeruginosa has been suggested in previous reports, our results were negative. An extracellular fraction of P. aeruginosa was able to hydrolyze a synthetic hexapeptide Cbz-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine, but was without detectable effect on reconstituted collagen.

Breakdown of tissue proteins has been observed in many bacterial infections and has stimulated interest in the study of proteolytic enzymes of a variety of microorganisms. Although many of these bacterial enzymes readily attack randomly coiled polypeptide chains, as in gelatin, they are generally inactive against the undenatured collagen molecule. Among bacteria, true collagenolytic activity has been conclusively demonstrated only with Clostridium perfringens (16), C. histolyticum (10), and Bacteroides melaninogenicus (4) in studies employing native or purified reconstituted collagen as a substrate. Recently, it has been suggested that Pseudomonas aeruginosa produces a collagenolytic factor, since this organism can cause severe damage to collagenous structures such as the cornea (3) and is capable of hydrolyzing a synthetic hexapeptide which possesses an amino acid sequence susceptible to the C. histolyticum collagenase (17).

The present study was undertaken to screen for collagenase-producing microorganisms by means of a simple, reliable test with reconstituted collagen as a substrate. In addition to C. perfringens and C. histolyticum, other bacteria that have been found to exhibit collagenolytic activity include strains of C. tetani, various strains of Bacteroides (in addition to B. melaninogenicus), and one strain of Staphylococcus aureus which produced lysis of collagen under anaerobic conditions only.

MATERIALS AND METHODS

Bacterial strains and cultural methods. B. ruminicola (ATCC 59189), B. melaninogenicus (ATCC 15032), and B. succinogenes (ATCC 19169) were obtained from the American Type Culture Collection. The strains of Clostridium were obtained from the Department of Bacteriology of Harvard Medical School. One of the strains of C. tetani, used in the production of tetanus toxoid, was kindly provided by the Division of Biologic Laboratories, Massachusetts Department of Health. All other organisms were isolated from clinical specimens and identified according to Bergey's Manual of Determinative Bacteriology. All isolated Bacteroides strains failed to grow on horse blood-agar unless cultured under strict anaerobic conditions. These organisms were unevenly staining, gram-negative, pleomorphic bacilli with blunt ends. Bacteroides strains were classified as B. melaninogenicus if they fulfilled the following criteria: production of a black pigment on horse blood-agar after less than 6 days of incubation; absence of growth in a synthetic thioglycolate medium; rapid growth in thioglycolate medium supplemented by heme or menadione, or both. Bacteroides strains not fulfilling these requirements were grouped as "Bacteroides species." There was total correlation among these three tests which have already been shown by Gibbons and McDonald (4) to be of great value for characterizing B. melaninogenicus.

Horse blood-agar plates were used for growth of bacteria in the screening tests for collagenolytic activity. For other experiments, Nutrient Broth (Difco) was employed. When anaerobic organisms were screened for collagenolytic activity, they were cultured on horse blood-agar plates and incubated in
Brewer jars under 95% N₂ and 5% CO₂. For other experiments, freshly prepared thioglycollate medium was used.

Growth curves for broth cultures were established by measuring turbidity in a Klett colorimeter.

Substrates and enzymes. Reconstituted collagen was extracted and purified according to the method described by Glimcher et al. (3); however, 0.1 M acetic acid was used as the sole extraction solution and the heat gelation step was omitted. Finally, the preparation was clarified by centrifugation at 48,000 × g for 1 hr, lyophilized, and stored at -20 C until used. For each experiment, small amounts of collagen were prepared by dissolving lyophilized material in cold sterile phosphate buffer (1/2 = 0.4 M, pH 7.6) in a proportion of 2:1 (w/v). The viscous solution was shaken overnight at 4 C, dialyzed against 0.4 M NaCl-0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.6, for 12 hr, and centrifuged at 105,000 × g for 1 hr. The final solution gelled very rapidly when incubated at 37 C. Hydroxyproline determinations showed that 85 to 90% of the protein content of the gel was collagen.

The synthetic polypeptide (Cbz-glycyl-l-prolyl-glycyl-L-prolyl-L-alanine) was obtained from Mann Research Laboratories, New York, N.Y. Radioactive (14C-glycine) collagen was kindly provided by M. Dresden. The preparation had a specific activity of 2.6 × 10⁸ counts per min per mg. Trypsin and C. perfringens collagenase were obtained from Worthington Biochemical Corp., Freehold, N.J.

Chemical and other methods. Hydrolysis of the synthetic hexapeptide was measured by the ninhydrin method described by Grassmann and Nordwig (6). Hydroxyproline determinations were performed by a modification of the method of Bergman and Loxley (1). Protein concentrations were determined by the Lowry procedure (13).

Disruption of bacteria was carried out with a Branson Sonifier (model S75) at 110 v and 6 amp. Measurement of radioactivity. Samples of 0.1 ml of 14C-glycine-labeled collagen were gelled overnight at 37 C. A 0.2-ml amount of a buffer solution containing 0.05 M Tris (pH 7.6) and 0.005 M CaCl₂ was added to each tube and incubated with 0.3 ml of the undiluted enzyme preparation or cell extract. After centrifugation for 10 min at 100,000 × g, radioactivity from degraded collagen (0.1-ml samples of supernatant solution) was measured in a Nuclear-Chicago liquid scintillation counter.

RESULTS

Screening for collagenolytic activity in aerobic organisms. The method of Gross (7) for detecting collagenolytic activity was modified for use with bacteria. Samples of 1.0 ml of a 0.2% collagen solution were gelled in small petri dishes at 37 C overnight, forming a gray opaque film. Circles of agar (8 to 10 mm) covered by actively growing overnight cultures were punched out, inverted, and applied steriley to the collagen gels. These preparations were incubated in a moist atmosphere for another 4 or 5 days at 37 C. All experiments were run in duplicate. Collagen lysis, when it occurred, appeared as a black circular area around the culture on a gray opalescent plate. Preliminary experiments showed that as little as 0.03 µg of purified C. perfringens collagenase produced clear-cut lysis after 3 hr. Controls consisting of 25 µg of trypsin and sterile blood-agar discs were run with each batch of experiments and were uniformly negative after 5 days of incubation.

The aerobic organisms screened for collagenolytic activity were as follows: Diplococcus pneumoniae, 3 strains; S. aureus, 8 strains; S. epidermidis, 3 strains; nonhemolytic streptococci, 4 strains; α-hemolytic streptococci, 5 strains; β-hemolytic streptococci, 7 strains (4 that were group A by the bacitracin test and 3 that were not); Proteus mirabilis, 6 strains; P. reitgeri, 2 strains; P. morganii, 2 strains; Pseudomonas aeruginosa, 30 strains; E. coli, 6 strains, Klebsiella, 5 strains; Aerobacter, 2 strains; Citrobacter, 1 strain; Serratia, 3 strains; Salmonella, 1 strain; Shigella, 1 strain; and Herella, 1 strain. None of the strains tested showed detectable collagenolytic activity (Fig. 1A).

Collagenolytic Activity of P. aeruginosa. Of the 30 strains of P. aeruginosa tested by the method previously described, all except one yielded negative results; one strain (9419) showed questionable collagen lysis on initial cultivation but none in any subsequent test. Supernatant fractions and centrifuged cells from overnight cultures of six Pseudomonas strains grown in Nutrient Broth and in enriched medium (14) were assayed with the same technique and failed to produce lysis. Sonic extracts of 18-hr cultures (Nutrient Broth) of two strains (9419 and 9150) also showed no collagenolytic activity. The pellet and the supernatant fraction obtained by mitomycin-induced lysis of strain 9419 also failed to show collagenolytic activity.

C. histolyticum collagenase, in addition to cleaving native collagen (10), hydrolyzes the synthetic polypeptide Cbz-Gly-Pro-Gly-Pro-Ala. This hexapeptide has been shown to be split by an extracellular fraction of P. aeruginosa (18). To ascertain the relation of this peptidase activity to true collagenase activity, culture filtrate fractions were assayed simultaneously for both. Pseudomonas strain 9419 was grown in Nutrient Broth on a Brunswick rotary shaker with vigorous aeration for 24 hr at 37 C. The cells were then removed by centrifugation at 3,000 × g for 30 min. Ammonium sulfate fractionation of the culture supernatant fluid was carried out, and 0 to 30%, 30 to 50%, and 50 to 70% fractions were obtained as described by Schoellman and Fisher (18). The precipitates were redis-
solved in cold 0.05 M Tris (pH 7.4) and dialyzed against the same buffer for 4 hr. Samples of 200 μg of protein were assayed by the collagen gel plate method with negative results. The 0 to 30% ammonium sulfate fraction was then chromatographed on a diethylaminoethyl (DEAE) Sephadex A-50 column (1.9 × 60 cm; flow rate, 0.2 ml/min). Elution was effected by the procedure of Schoellman and Fisher (18) with a linear gradient of 0 to 2.0 M NaCl in 0.05 M Tris (pH 7.4). Three major protein-containing peaks were obtained. After dialysis against 0.01 M calcium acetate (pH 6.3) for 4 hr at 4 C, 0.5 ml (100 to 180 μg) of each of three fractions (A, B, C) was incubated with the synthetic polypeptide according to the method of Grassman and Nordwig (6). Release of ninhydrin-positive material was produced only by fraction B after 6 hr of incubation. Neither fraction B nor the other DEAE-Sephadex fractions, when assayed on a collagen gel, produced any visible lysis.

To increase the sensitivity of the collagenase assay, 300-, 400-, and 500-μg portions of the three DEAE-Sephadex fractions (A, B, C) were incubated at 37 C for 6 hr with 100 μg of pregelled 14C-glycine-labeled collagen (2.6 × 10^4 counts per min per mg). No radioactivity (<5%) was released by fraction B into the supernatant fluid after centrifugation of the gel. Fractions A and C showed the release of 6 and 8%, respectively. This is not considered significant, since trypsin treatment may produce the same results (7). Incubation of each of the three fractions with collagen of higher specific activity (4.57 × 10^4 counts per min per mg) for 84 hr confirmed the absence of collagenolytic activity.

Collagenolytic activity of anaerobic bacteria.

When the same plate assay was used under anaerobic conditions, three strains of C. tetani and two strains of Bacteroides species could be shown to produce lysis of collagen (Table 1; Fig. 1B). Since collagenolytic activity could be demonstrated under anaerobic conditions only, five strains of S. aureus and four strains of S. epidermidis were incubated and assayed for collagenolytic activity under aerobic and anaerobic conditions. None of the strains lysed collagen except for S. aureus (S8440), which showed this activity only under anaerobic conditions.

To confirm the presence of collagenolytic activity in bacteria showing lysis on the plate assay, release of hydroxyproline from reconstituted collagen into liquid culture was measured. A 5-ml amount of freshly prepared thiglycolate broth was added to 1.0 ml of pregelled, sterile collagen solution; the tubes were then inoculated with 0.2 ml of an overnight culture and incubated for 5 days at 37 C. Cultures were then centrifuged at 10,000 × g for 30 min, and the pellets, containing bacteria and residual collagen, were analyzed for hydroxyproline. Collagenolysis was determined by comparing the amount of hydroxyproline released from culture residues with that in uninoculated controls. Significant hydroxyproline release was demonstrated for all strains which showed lysis with the screening method (Table 2). The pH of the culture medium did not change throughout the experiments. Autoclaving of the bacterial cultures prior to adding them to the collagen gels eliminated all collagenolytic activity. Release of hydroxyproline from uninoculated controls never exceeded 2% after 5 days of incubation.
In addition, to investigate the possible production of collagenase by *P. aeruginosa* when grown anaerobically, three strains (including strain 9419) were grown and incubated with collagen under strict anaerobic conditions (Brewer jar) in liquid medium containing nitrate as a terminal electron acceptor (12). Release of hydroxyproline after 5 days of incubation did not exceed that found in uninoculated controls.

Comparison of the growth curves of *Bacteroides* 5891, *C. tetani* HMS, and *S. aureus* S8440 grown under anaerobic conditions with the release of hydroxyproline from reconstituted collagen reveals some differences (Fig. 2-4). Degradation of collagen occurred during the logarithmic-growth phase of *C. tetani*. With *Bacteroides* B5891, measurable lysis became evident only by the third day after maximal bacterial density had already been achieved. In the case of *S. aureus* S8440, collagenolysis started with the onset of the logarithmic-growth phase and continued after the growth curve had leveled off.

### Table 1. Collagenolytic activity of anaerobic bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of Strains Tested</th>
<th>No. positive</th>
<th>No. negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>C. histolyticum</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>C. tetani</em></td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><em>C. botulinum</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>C. butyricum</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Bacteroides melaninogenicus</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>B. succinogenes</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>B. ruminicola</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Bacteroides species</em></td>
<td>12</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Anaerobic <em>Streptococcus</em></td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Clinical isolates of Bacteroides species other than *B. melaninogenicus*.

*Each strain cultured under anaerobic and aerobic conditions; lysis of collagen occurred only with one strain and then under anaerobic conditions only.

### Table 2. Digestion of collagen gel after 5 days of incubation with organisms showing collagenolytic activity under anaerobic conditions

<table>
<thead>
<tr>
<th>Organism</th>
<th>Collagenolyzed*%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium tetani</em> (HMS)</td>
<td>66</td>
</tr>
<tr>
<td><em>Bacteroides melaninogenicus</em></td>
<td>53</td>
</tr>
<tr>
<td>(ATCC 15032)</td>
<td></td>
</tr>
<tr>
<td><em>Bacteroides species</em> (B8440)*</td>
<td>74</td>
</tr>
<tr>
<td><em>Bacteroides species</em> (B891)*</td>
<td>44</td>
</tr>
<tr>
<td><em>Bacteroides species</em> (BHP)*</td>
<td>40</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> S8440*</td>
<td>28</td>
</tr>
<tr>
<td>(anaerobic)*</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> S8440 (aerobic)*</td>
<td>0</td>
</tr>
<tr>
<td><em>C. perfringens</em> collagenase*</td>
<td>30 µg</td>
</tr>
<tr>
<td><em>C. perfringens</em> collagenase*</td>
<td>5 µg</td>
</tr>
</tbody>
</table>

*Expressed as amount of hydroxyproline released from culture residues after 5 days of incubation, as compared with uninoculated controls.

*Refers to *Bacteroides* species other than *B. melaninogenicus*.

*Grown in thioglycolate medium.

*Grown in Nutrient Broth and measured against uninoculated nutrient broth.

*From Worthington Biochemical Corp.
As denatured collagen (gelatin) is attacked by proteolytic enzymes such as trypsin and chymotrypsin, the specificity of a collagenase assay depends on the use of natural or reconstituted, pure collagen as a substrate. With the assay reported here, collagenolytic activity has been demonstrated only with certain anaerobic organisms: some strains of Bacteroides other than B. melaninogenicus, all strains of C. tetani tested, and one strain of S. aureus (when grown under anaerobic conditions). Similar bacterial collagenolytic factors have previously been obtained with C. perfringens (16), C. histolyticum (10), and B. melaninogenicus (4) by use of either native or reconstituted collagen as a substrate. Our experiments failed to demonstrate any collagenolytic activity in aerobic organisms, particularly in P. aeruginosa. Our results do not corroborate those of others (11, 19) who found a collagenolytic activity in many strains of Pseudomonas when Azocoll was used as a substrate. The specificity of such an assay has been questioned (8), since hide powder consists mostly of denatured protein and is attacked by a variety of proteolytic enzymes (15).

Schoellman and Fisher (18) have shown that a purified fraction from P. aeruginosa is able to split the synthetic polypeptide Cbz-Gly-Pro-Gly-Gly-Pro-Ala, a hexapeptide with an amino acid sequence very similar to that of collagen. Such an enzyme, however, may be without activity on the natural collagen molecule. Our experiments indeed indicate that the same P. aeruginosa extracellular fraction which releases free amino groups when incubated with the synthetic hexapeptide has no lytic activity when tested with the collagen gel method. Moreover, incubation of this same fraction with 14C-labeled collagen fails to release significant radioactivity. These results are in keeping with those of Morihara (15), who showed that a P. aeruginosa protease was unable to attack collagen, whereas it lysed gelatin promptly. It seems, therefore, that certain peptide bonds of the collagen molecule are susceptible to the Pseudomonas protease, but that these bonds are protected in native and reconstituted collagen. The presence of a weak collagenolytic activity of this protease cannot be absolutely excluded, since proteolytic enzymes like trypsin and chymotrypsin (7) have been shown to liberate polypeptides from reconstituted collagen.

All aerobic organisms tested failed to produce lysis of collagen. Identical results were obtained by McLennan, Mandl, and Howes (14) with native collagen, and by Evans with decalcified dentine (2). The discovery of collagenolytic activity in three additional organisms growing under anaerobic conditions raises the question of whether collagen is altered by such experimental conditions. This does not seem to be the case, since neither lysis nor release of hydroxyproline from reconstituted collagen can be observed with trypsin under anaerobic conditions.

The occurrence of collagenolytic activity among staphylococcal strains is very rare. The observation of collagenolytic activity under anaerobic conditions only is similar to the findings of Hausmann, Courant, and Arnold (9) with B. melaninogenicus collagenase, since this enzyme seems to be inactivated by the oxygen tension of room air.

The presence of a collagenolytic factor in C. tetani emphasizes the caution that must be exercised in relating the presence of an extracellular enzyme to the pathogenicity of the organism; e.g., the collagenase of C. perfringens has been implicated in the rapidly spreading nature of clinical gas gangrene. Quite in contrast, however, is the noninvasive nature of infections due to C. tetani, which raises some question as to the importance of bacterial collagenase in tissue breakdown in invasive infections.

ACKNOWLEDGMENT

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LITERATURE CITED

COLLAGENOLYTIC ACTIVITY