Purification and Characterization of Mucopolysaccharidase from an Oral Strain of *Bacteroides* sp.

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A mucopolysaccharidase in the cell extract of an oral strain of *Bacteroides* sp. was purified to homogeneity by ammonium sulfate precipitation, DEAE-cellulose column chromatography, gel filtration on Sephadex G-200, and isoelectric focusing. Specific activity increased 110-fold and recovery was 2%. The molecular weight was determined to be 89,000 by gel filtration, and the isoelectric point was 7.0. The optimum pH for the activity was 6.5. The enzyme was inactivated by heating at 60°C for 5 min. The purified mucopolysaccharidase degraded hyaluronic acid more rapidly than chondroitin and chondroitin sulfate A and C. However, it had no activity against chondroitin sulfate B, heparin, and heparan sulfate. Since unsaturated disaccharides were derived from the enzyme substrate, this enzyme was considered to be a mucopolysaccharidase lyase.

Evidence has accumulated that the primary etiology of periodontal diseases is bacteria (3, 25, 30). In particular, anaerobic gram-negative rods, which are indigenous organisms in the oral cavity, are thought to be associated with periodontal diseases (23, 26, 27, 29).

These bacteria elaborate a few enzymes which damage gingival tissue (9, 11, 24, 25, 30). Collagenase produced by *Bacteroides* spp. was noted because of its possible role in destruction of major tissue components (4, 12, 35).

On the other hand, Nakamura et al. (13) and Nakamura et al. (14) demonstrated production of mucopolysaccharidases by *Bacteroides* sp. These enzymes may also degrade other major constituents of connective tissue. They discussed the relation of these enzymes to the pathogenetic factor of the strain producing them. However, precise characterization of these enzymes has not been determined. Therefore, we undertook to purify the mucopolysaccharidase from an oral strain of heparinase-producing *Bacteroides* sp. for an investigation of its properties.

**MATERIALS AND METHODS**

**Bacterial strain and cultivation.** A producer strain of mucopolysaccharidase, *Bacteroides* sp. strain 26, was isolated from the pus around experimentally introduced crevice materials in guinea pigs (13). Identification of the species level of the strain is still impossible. The properties of the strain are as follows. The strain is a gram-negative rod, nonsporeforming, obligately anaerobic, and nonmotile. Black pigment was not produced on blood agar. It could not grow when 10% bile was present in the medium. Growth was enhanced by hemin. The strain fermented glucose, maltose, fructose, raffinose, galactose, lactose, sucrose, arabinose, xylose, and starch. Mannitol and sorbitol were not fermented. Indole and H₂S were not produced. It did not liquefy gelatin. Nitrates were not reduced. Cultivation was performed anaerobically at 37°C in a glove box for 3 days in a medium containing (per liter): Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 17 g; yeast extract (Difco Laboratories, Detroit, Mich.), 3 g; NaCl, 5 g; KH₂PO₄, 2.5 g; sodium thioglycolate, 0.5 g; hemin, 5 mg; and menadione, 0.5 mg (22).

**Enzyme assay.** Mucopolysaccharidase activity was measured colorimetrically by assaying the released N-acetylgalactosamine (GlcNAc) end group from hyaluronic acid. The reaction mixture contained 0.8 ml of 1.25-mg/ml hyaluronic acid solution in 0.05 M Tris-hydrochloride buffer (pH 7.2) and 0.2 ml of a suitably diluted enzyme in the same buffer. After incubation at 37°C for 15 min, 0.2 ml of 0.8 M sodium tetraborate (pH 9.2) was added. The GlcNAc end group was quantitatively analyzed by the Morgan-Elson method (28), using GlcNAc as a standard. One unit of enzyme activity was defined as the amount that catalyzed the release of 1 μmol of GlcNAc per min.

**Protein determination.** Protein was estimated by the method of Lowry et al. (10), using bovine serum albumin as a standard.

**Polyacrylamide disc gel electrophoresis.** To determine the purity of the samples, polyacrylamide disc gel electrophoresis was carried out with 7.5% acrylamide at pH 8.0, by the method of Williams and Reisfeld (33). Protein bands were stained with 0.25% Coomassie brilliant blue G-250.

**Determination of molecular weight.** The molecular weight of the mucopolysaccharidase was estimated by gel filtration on a Sephadex G-200 column (2.6 by 100 cm) by the method of Andrews (1). The standard proteins aldolase, bovine serum albumin, chymotrypsinogen, and cytochrome c were used.
Preparation of crude extract. The following experiments were conducted at 4°C, unless otherwise specified. The cells from 45 liters of a culture of *Bacteroides* sp. strain 26 were harvested by centrifugation at 10,000 x g in 0.05 M Tris-hydrochloride buffer (pH 7.2). The washed cells were suspended in 3 volumes of the same buffer and then disrupted by ultrasonic treatment at 9 kHz for 30 min. A clear supernatant fluid was obtained by centrifugation at 100,000 x g for 1 h.

Isoelectric focusing. Electrofocusing was carried out at about 4°C, using an LKB 110-ml-capacity column with 1% (vol/vol) amphetamine (pH range 5 to 8), by the method of Vesterberg et al. (32). Focusing was done for 48 h under constant voltage (600 V). Fractions of 3 ml were collected, and the pH of each fraction was determined.

Optimal pH. Enzyme activity against various mucopolysaccharides was measured at various pH values to estimate the optimal pH. Hyaluronic acid, chondroitin, chondroitin sulfate A (ChS-A), chondroitin sulfate B (ChS-B), chondroitin sulfate C (ChS-C), heparin, and heparan sulfate were used as substrates. Activity was measured by the method of Linker (7) with some minor modifications. Each reaction mixture contained 0.1 ml of 0.0035 U of purified enzyme (20 µg of protein) and 0.02 ml of the substrate (10 mg/ml). Buffers used were 0.1 M acetate buffer (pH 4.0 to 6.0), 0.1 M potassium phosphate buffer (pH 6.0 to 7.5), and 0.1 M Tris-Cl buffer (pH 7.5 to 9.0). After incubation for 1 h at 37°C, the reaction was stopped by the addition of 0.9 ml of 3% perchloric acid. These mixtures were then centrifuged at 10,000 x g for 10 min, and the increase of absorbance at 232 nm of the supernatants was measured.

Substrate specificity. Substrate specificity of the purified preparation was determined against various mucopolysaccharides, including hyaluronic acid, chondroitin, ChS-A, ChS-B, ChS-C, heparin, and heparan sulfate. Each reaction mixture consisted of 0.1 ml of 0.0035 U of enzyme (20 µg of protein) in 0.05 M Tris-hydrochloride buffer (pH 7.2) and 0.02 ml of the substrate solution in the same buffer. The concentration of each substrate was adjusted to the same level (0.2 µmol) with respect to uronic acid or hexosamine in the substrate molecule. Incubation was carried out at 37°C and stopped at the times indicated in Fig. 3 by the addition of 0.9 ml of 3% perchloric acid. The subsequent assay procedures were the same as in determining the optimal pH.

Paper chromatography. Identification of the reaction products obtained from the above-described mucopolysaccharides by the action of the enzyme was done by using paper chromatography. Reaction mixtures of the enzyme and substrates were incubated for 8 h, and supernatant solutions, obtained by centrifugation, were spotted onto Toyo no. 50A filter paper (60 cm long). The chromatogram was developed in a descending solvent system of isobutyric acid-1 M NH₄OH (5:3 [vol/vol]) (6) or n-butanol-acetic acid-water (50:12:25 [vol/vol/vol]) (8). Detection of unsaturated disaccharides in the enzymatic digestions of the substrates was carried out by using a UV lamp (6) and staining with aniline hydrogen phthalate reagent (18).

Identification of the disaccharides was carried out by comparing their migration rates under the same chromatographic system with authentic standards of ΔDi-4S (2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose) (34), ΔDi-6S (2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-6-O-sulfo-D-galactose) (34), ΔDi-OS (2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-galactose) (34), GlcNAc, and N-acetylgalactosamine (GalNAc).

Chemicals. Hyaluronic acid, ChS-A, and ChS-C were purchased from Sigma Chemical Co., St. Louis, Mo. The ChS-A used in this work contained as much as 25% ChS-C (21). Faltynek and Silbert have suggested that it may not be possible to obtain a pure preparation of chondroitin sulfate polymers (2). Chondroitin, ChS-B, heparan sulfate, and the authentic standards of ΔDi-4S, ΔDi-6S, and ΔDi-OS were obtained from Seikagaku Kogyo Ltd., Tokyo, Japan. Heparin was supplied by Wako Pure Chemical Co., Tokyo, Japan. GlcNAc and GalNAc were purchased from Nakarai Chemical Co., Kyoto, Japan. Aldolase, bovine serum albumin, chymotrypsinogen, and cytochrome c standards were obtained from Boehringer Mannheim, Mannheim, West Germany. DEAE-cellulose (DE-32) was from Whatman Chemical Separation Ltd., Kent, England.

RESULTS

Purification of mucopolysaccharidase. The purification of mucopolysaccharidase from *Bacteroides* sp. strain 26 is summarized in Table 1. To 270 ml of the crude extract, 20 ml of a 2% protamine sulfate solution was added with stirring. After standing for 20 min, the suspension was centrifuged at 10,000 x g for 20 min and the precipitate was discarded.

(i) Ammonium sulfate precipitation. Solid ammonium sulfate was added slowly to the supernatant of protamine sulfate-treated crude extract to give 50% saturation. The suspension was allowed to stir for about 1 h and was then centrifuged at 10,000 x g for 20 min to remove the precipitate. The concentration of ammonium sulfate of the supernatant was raised to 70% saturation. The resulting precipitate was collect-

<table>
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<tr>
<th>Table 1. Purification of mucopolysaccharidase</th>
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ed by centrifugation and dissolved in 50 ml of 0.05 M Tris-hydrochloride buffer (pH 7.2) and then dialyzed for 24 h against 2 liters of the same buffer with several changes of the buffer.

(ii) DE-32 column chromatography. The dia-
lized sample (74 ml) was applied to a DE-32 column (2.6 by 40 cm) previously equilibrated with 0.02 M Tris-hydrochloride buffer (pH 7.2) and was washed with 800 ml of this buffer. The column was then eluted with a linear gradient created by mixing 300 ml of buffer containing 0.4 M NaCl at a flow rate of 40 ml/h. Fractions of 5 ml were collected. In this chromatography, mucopolysaccharidase emerged from the column as a single peak of activity at about 0.1 M NaCl. The active fractions were combined and concentrated with ammonium sulfate (70% saturation) to 3 ml.

(iii) Gel filtration. The active fraction from the DE-32 column was applied to a Sephadex G-200 column (2.6 by 100 cm) equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.2) containing 0.15 M NaCl. Active fractions were collected as described above and dialyzed overnight against 1% glycine solution.

(iv) Isoelectric focusing. In isoelectric focusing, the peak of the activity was found at pH 7.0 (Fig. 1). The active fractions (fractions 23 to 26) were pooled, concentrated, and dialyzed against 0.05 M Tris-hydrochloride buffer (pH 7.2). This sample was the purified mucopolysaccharidase. It showed homogeneity in polyacrylamide gel electrophoresis (Fig. 2).

The yield was about 2%, and the enzyme was purified about 110-fold compared with crude extract.

Properties of the purified mucopolysaccharidase. In the subsequent experiments, the enzyme used was from the final step of purification.

(i) Molecular weight. The molecular weight of the enzyme was estimated to be 89,000 by gel filtration on Sephadex G-200.

(ii) Effects of various ions on activity. Ca\(^{2+}\) and Mg\(^{2+}\) ions at a concentration of 1 mM activated the enzyme by ca. 50% (Table 2). The activity was slightly inhibited by Mn\(^{2+}\) and was 70 to 90% inhibited by the other ions listed in Table 2. When NaCl was added to the standard assay component, the enzymatic activity increased up to a NaCl concentration of 0.15 M. At this concentration, 305% of the activity in a control
TABLE 2. Effects of metal ions on mucopolysaccharidase activity

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<th>Relative activity</th>
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<tr>
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<tr>
<td>Mg²⁺</td>
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<tr>
<td>Cu²⁺</td>
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</table>

a Each ion was added to standard assay components at the indicated concentration. The activity is expressed as the percentage of activity of a control (no ions added). Incubation was carried out for 30 min with 0.0035 U of purified (Table 1, step 7) enzyme (20 μg of protein).

measuring the increase of absorbance at 232 nm. Hyaluronic acid was the most rapidly degraded substrate, moderately rapid breakdown was seen with chondroitin, and lower degradation rates were seen with ChS-A and ChS-C (Fig. 3). ChS-B, heparin, and heparan sulfate were resistant to this enzyme. Degradation of these mucopolysaccharides was not seen at pH 4 to 10.

(v) Paper chromatography. Products derived from mucopolysaccharides by the action of the purified enzyme were analyzed by paper chromatography. The migration rates of unsaturated disaccharides from hyaluronic acid and chondroitin coincided with that of ADi-0S in the two-solvent system described above. Fig. 4 shows a diagram of paper chromatography with isobutyric acid—1 M NH₄OH. Hyaluronic acid consists of a repeating unit of a dimer of D-glucuronic acid and GlcNAc, and chondroitin is an epimer of hyaluronic acid in which GlcNAc is replaced by GalNAc. Therefore, the unsaturated disaccharides from hyaluronic acid and chondroitin were tentatively identified as ADi-GlcNAc(β) [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-ene)pyranosyluronic acid]-D-glucose] (8), which is an epimer of ADi-0S, and ADi-0S, respectively. On the other hand, ADi-4S and ADi-6S were detected

FIG. 3. Comparison of rate of degradation of various mucopolysaccharides by the purified mucopolysaccharidase (Table 1, step 7). Incubation was carried out with 0.0035 U of enzyme (20 μg of protein). The other conditions are described in the text. Symbols: •, hyaluronic acid; ○, chondroitin; ▲, ChS-A; △, ChS-C; □, ChS-B, heparin, and heparan sulfate.

FIG. 4. Tracing of paper chromatogram of the disaccharides obtained from digestion of hyaluronic acid, ChS-A, ChS-B, ChS-C, and chondroitin with the purified mucopolysaccharidase (Table 1, step 7). Each reaction mixture contained 0.05 U of enzyme (280 μg of protein) and 1 mg of each substrate in 1 ml of 0.05 M Tris-hydrochloride buffer (pH 7.2). Incubation was carried out for 8 h. Chromatography was done for 48 h in isobutyric acid—1 M NH₄OH as described in the text. 1, Digestion of hyaluronic acid; 2, digestion of ChS-A; 3, digestion of ChS-B; 4, digestion of ChS-C; 5, digestion of chondroitin; 6, authentic standard disaccharides: ADi-6S, ADi-4S, and ADi-0S, from top to bottom.
and ΔDi-0S, GlcNAc, and GalNAc were not detected in the digestion of ChS-A and ChS-C (Fig. 4).

DISCUSSION

Mucopolysaccharidase from a cell extract of Bacteroides sp. strain 26 was purified to homogeneity. The specific activity increased about 110-fold compared with that of the crude extract.

The molecular weight of the enzyme was estimated to be 89,000. This value is close to that of hyaluronidases from Staphylococcus aureus, Streptococcus equisimilis, and Propionibacterium acnes; they were reported to have molecular weights of 84,000 (19), 90,000 (17), and 85,110 (5), respectively. However, the molecular weight of the chondroitinase of Proteus vulgaris was reported to be significantly higher. It was roughly estimated to be 150,000 (34).

The optimum pH value of 6.5 was similar to those of the hyaluronidases obtained from Propionibacterium acnes (5), Proteus vulgaris (34), and Flavobacterium heparinum (7, 34). However, Staphylococcus aureus hyaluronidase had a lower optimum pH, between 4.8 and 6.0 (31).

The isoelectric point was determined to be 7.0. The same pl value was reported for Propionibacterium acnes hyaluronidase (5, 16). Vesterberg (31) demonstrated heterogeneity in isoelectric points of Staphylococcus aureus hyaluronidase at pH 7.4 and 7.9. He found that the pl of this enzyme converted from the former value to the latter with prolongation of the culture period. Recently, Ozegowski et al. (17) obtained a more acidic pl value of 4.95 from streptococcal hyaluronidase.

Although the enzymatic activity was stimulated 50% by Mg2+ and also by Ca2+, this enzyme was found to have no essential requirements for the divalent ions. Fe2+ significantly inhibited the enzyme activity. Fe3+ also inhibited the activity to the same degree as Fe2+. Almost all activity was lost when Zn2+ or Hg2+ was added. Cu2+ inhibited the enzyme completely.

About threefold activation of the enzyme was found in a NaCl concentration range of 0.05 to 0.15 M. These findings suggest that appropriate ionic strength has an apparent effect on the activity. A similar effect of increasing buffer molarity on the activity of Propionibacterium acnes hyaluronidase was reported by Ingham et al. (5).

ΔDi-GlcNAc(B), ADi-0S, ΔDi-4S, and ΔDi-6S were derived by the enzymatic digestion of hyaluronic acid, chondroitin, ChS-A, and ChS-C, respectively. Neither ΔDi-0S, which is produced from either ΔDi-4S or ΔDi-6S by chondrosulfatase, nor GlcNAc and GalNAc produced by glucuronidase (34) was detected in the enzymatic products from the various mucopolysaccharides tested. These findings suggest that our enzyme released unsaturated disaccharides from hyaluronic acid through an elimination reaction. Therefore, this enzyme may be a lyase.

Considering the molecular organization of ChS-A and ChS-C, it is quite unlikely that ΔDi-6S and ΔDi-4S were derived from the respective chondroitin sulfate molecules by enzymatic digestion. These contradictory results may be partly due to the possible contamination of one type of chondroitin sulfate with other types of chondroitin sulfate (see above).

The substrate specificity of this mucopolysaccharidase resembled that of Propionibacterium acnes hyaluronidase (5). Both preparations were active on hyaluronic acid, ChS-A, and ChS-C but did not degrade Chs-B (dermatan sulfate).

When heparin was added to the growth medium at a concentration of 0.6 mg/ml, the specific activity (units of enzymatic activity per milligram of the bacterial protein in crude extract) of hyaluronate degradation was higher by four times than that without heparin (data not shown). According to Salyers and Kotarski (20), production of chondroitin sulfate lyase by Bacteroides thetaiotaomicron was stimulated 10-fold by the addition of Chs-A to the growth medium. Therefore, it would be interesting to investigate precisely the stimulatory mechanism in our strain to compare with their results.

LITERATURE CITED

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