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Purification and Properties of *Bacteroides heparinolyticus* Heparinase (Heparin Lyase, EC 4.2.2.7)

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Heparinase (heparin lyase, EC 4.2.2.7) was isolated from the cell extract of an oral bacterium, *Bacteroides heparinolyticus*. It was a basic protein with an isoelectric point of 9.5. Its molecular weight was 63,000. The enzyme was the most active against heparin among the tested mucopolysaccharides. Catalytic properties may be similar to those of heparinase of *Flavobacterium heparinum*, since the enzymatic degradation products obtained by using the two enzymes were the same on the basis of paper chromatography.

It is widely accepted that anaerobic gram-negative rods predominate in the flora of periodontitis lesions (7). In particular, black-pigmented *Bacteroides* spp. are suspected as the most likely etiological agent of periodontitis (5); *Bacteroides forsythus* has also been associated with periodontitis (1, 15). However, nonpigmented *Bacteroides* spp. are also recovered frequently from the subgingival lesions of patients with periodontitis (11). A heparinolytic *Bacteroides* sp. was first isolated by Nakamura from the oral cavity (9). This organism is saccharolytic and is the only one with heparinolytic activity that has been isolated from the oral cavity to date. Recently, this organism has been proposed as a new species, *Bacteroides heparinolyticus* (12). This species may be pathogenic since it has been implicated in abscess formation in experimental mixed infections of guinea pigs (13). However, the actual role of the nonpigmented *Bacteroides* spp., including *B. heparinolyticus*, in the genesis of periodontitis is still largely unknown. It is also known that *B. heparinolyticus* produces acid mucopolysaccharidases which are partially responsible for tissue destruction (10); one of them, a hyaluronidase, was purified and characterized (14). We describe, in this report, the purification and properties of another acid mucopolysaccharidase, a heparinase (heparin lyase, EC 4.2.2.7).

B. heparinolyticus ATCC 35895 was cultured in 2 liters of general anaerobic medium (Nissui Seiyaku Co., Tokyo, Japan) (2) supplemented with hemin (5 mg/liter) and heparin (2 g/liter; Nakarai Chem. Ltd., Kyoto, Japan). The organism was cultivated at 37°C for 3 days in an anaerobic glove box filled with a mixture of gases (N_2-H_2-CO_2; 85:10:5, vol/vol/vol). Heparinase activity was assessed by the method of Linker (3). One unit of enzyme activity was defined as the amount that increased the A_{232} by 1.0 after 30 min.

The enzymatic degradation products of mucopolysaccharides generated by the purified enzyme were identified by the method of Linker and Hovingh (4). The enzymatic degradation products were analyzed by paper chromatography with a solvent consisting of *n*-butanol–acetic acid–water (10:3:7, vol/vol/vol). The enzymatic degradation products of heparin generated by the purified *B. heparinolyticus* enzyme and by commercially available heparinase from *Flavobacterium heparinum* (Seikagaku Kogyo Co., Tokyo, Japan) and authentic ΔDi-tris [2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyl uronic acid)-4,6-bis-*O*-sulfo-D-galactose, Seikagaku Kogyo Co.] were simultaneously developed on the same paper (Toyo no. 50A filter paper; Advantec Toyo Co., Tokyo, Japan). The *B. heparinolyticus* ATCC 35895 cells were harvested by centrifugation at 10,000 × *g* for 20 min, washed twice in 0.05 M phosphate buffer (pH 7.0), and then sonicated at 9 kHz for 10 min. The sonicate extract was clarified by centrifugation at 100,000 × *g* for 30 min. The purity of the proteins and their molecular weights were determined by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The proteins were stained with silver by the method of Morrissey (8). Proteins were quantitated by the method of Lowry et al. (6), with bovine serum albumin as the standard.

Most heparinolytic activity was detected in the cell extract prepared by sonication, and only a negligible amount of the activity (<10% the activity in the sonic extract) was found in the culture supernatant. For enzyme production, addition of heparin to the culture medium was required. Its optimum concentration for enzyme production was around 2 g/liter. Therefore, heparin was added to the culture medium at 2 g/liter prior to inoculation with the strain.

Heparinase was purified by the following procedure. The cell extract was applied to a column (2.6 by 25 cm) of DEAE-cellulose equilibrated with 0.05 M phosphate buffer (pH 7.0) and was washed with this buffer. Since heparinolytic activity was detected in the wash solution from the column, it was concentrated and dialyzed against 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. The concentrated active material was applied to a column (2.6 by 90 cm) of Sephacryl S-300 (Pharmacia AB, Uppsala, Sweden) and eluted with 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. The active fractions from this column were combined, concentrated, and dialyzed against 0.07 M phosphate buffer (pH 7.0). This sample was then applied to a column (1.6 by 7 cm) of hydroxyapatite which was previously equilibrated with the same buffer. After the column was washed, the proteins were eluted with NaCl in 0.07 M phosphate buffer (pH 7.0); the concentration of NaCl was increased stepwise. Heparinase was found in the fraction eluted with 0.1 M NaCl. Finally, the enzyme fraction was electrophoresed after thorough dialysis against a 1% glycine solution on an isoelectric focusing column (110-ml capacity) by using ampholyte (pH 3.5 to 10) for 48 h at about 4°C with a constant current of 400 V (Fig. 1). The enzyme activity, concentrated at pH 9.5, was collected and dialyzed against 0.05 M Tris hydrochloride buffer (pH 7.2).

Heparinase was purified from the crude extract 1,920-fold, with a recovery of 8.9%. The purified preparation was

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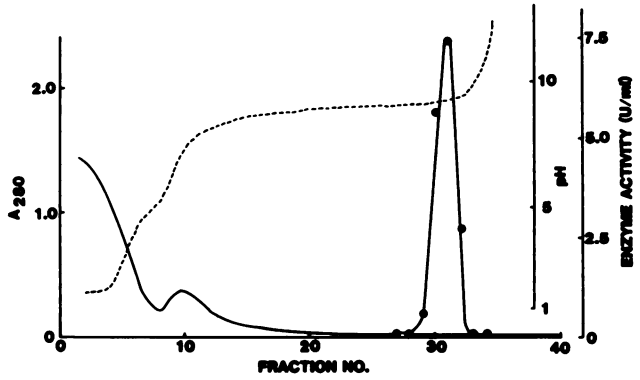

FIG. 1. Isoelectric focusing of *B. heparinolyticus* heparinase. Symbols: ●, enzyme activity – – –, pH; ———, A_{280}.

homogeneous on polyacrylamide gel electrophoresis (Fig. 2). Its molecular weight was calculated to be 63,000 from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimum pH for enzyme activity was 6.5. The heparinase was rather thermolabile; it was completely inactivated by heating at 45°C for 5 min. Heparinolytic activity was activated about twice by 1 mM Fe^{2+}, but Cu^{2+} and Hg^{2+} were quite inhibitory. The purified preparation was most active against heparin and moderately active against heparan sulfate, but hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C were not degraded by this enzyme (Fig. 3). When the enzymatic digestions of heparin by the purified enzyme were analyzed by paper chromatography, two unsaturated oligosaccharide spots appeared on the paper. One was a major spot, and the other was a minor spot. The positions of these spots coincided with the major spot (unsaturated tetrasaccharide) and minor spot (ΔDi-triS) which appeared in paper chromatography of digestions of heparin obtained by treatment with *F. heparinum* heparinase. Furthermore, the positions of the minor spots coincided with that of authentic ΔDi-triS. These findings suggest that the catalytic properties of the *B. heparinolyticus* heparinase are similar to those of the heparinase of *F. heparinum*.

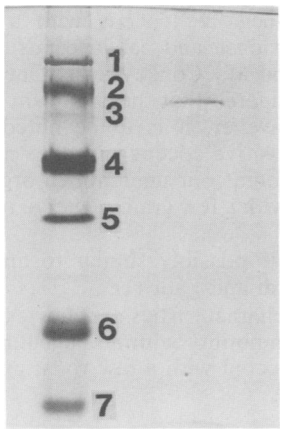

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The left lane contains the purified heparinase sample and standard proteins, as follows: 1, phosphorylase *b*; 2, bovine serum albumin; 3, heparinase; 4, ovalbumin; 5, carbonic anhydrase; 6, soybean trypsin inhibitor; 7, α-lactalbumin. The right lane contains the purified heparinase sample.

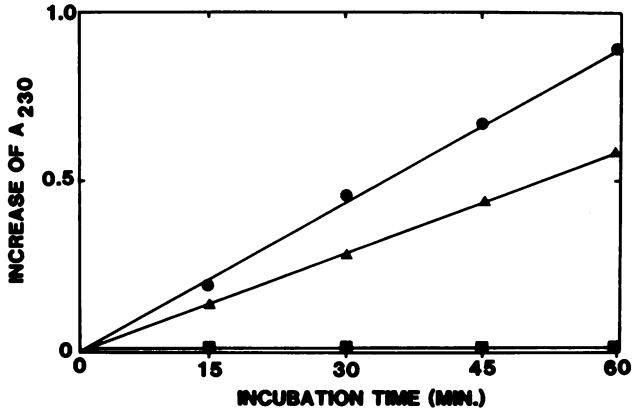

FIG. 3. Comparison of the degradation rate of mucopolysaccharides by heparinase. Symbols: ●, heparin; ▲, heparan sulfate; ■, hyaluronic acid, chondroitin, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C.

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