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# Characterization of Heparinase from an Oral Bacterium *Prevotella heparinolytica*[1]

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**Heparinase was purified to homogeneity from the cell extract of an oral bacterium, *Prevotella heparinolytica*, by a combination of anion exchange chromatography, gel filtration chromatography, and hydroxyapatite chromatography. Properties of the purified *P. heparinolytica* heparinase (*P.* heparinase) were investigated. The enzyme exhibited a maximum activity in 50 mM Tris-HCl buffer, pH 7.5-8.0, containing 75 mM sodium acetate, 0.1 M NaCl, and 1 mM $CaCl_2$. Optimum conditions for the maximum activity of *P.* heparinase were similar to those of the heparinase from *Flavobacterium heparinum* (*F.* heparinase). The two enzymes also yielded similar digestion profiles of various glycosaminoglycans and heparin tetrasaccharides, suggesting that they have a similar substrate specificity. Kinetic study of the *P.* heparinase reaction using porcine intestinal heparin as substrate gave a $K_m$ value of $3.8\times10^{-5}$ M and a $V_{max}$ value of 11.4 $\mu$mol/min·mg protein. The Michaelis constant of *P.* heparinase was slightly larger than but not significantly different from that of *F.* heparinase. The amino acid composition of *P.* heparinase was also similar to that of *F.* heparinase, but its N-terminal sequence of 20 amino acid residues was different and hitherto unreported. These results together indicate that these heparinases are different proteins with closely similar enzymatic properties. Since *F. heparinum* produces not only heparinase but also heparitinase II, which has a broad substrate specificity, *F.* heparinase may be contaminated with this enzyme. In contrast, *P. heparinolytica* does not produce heparitinase II, and *P.* heparinase should prove a useful tool for degrading heparin without the risk of contamination with heparitinase II.**

**Key words: heparin, heparin-lyase, oral bacterium, sulfated glycosaminoglycan, sulfated oligosaccharide.**

Heparin exhibits a variety of biological and biochemical activities, such as inhibition of blood coagulation (*1*), potentiation of angiogenesis (*2*), and modulation of cellular proliferation (*3, 4*). Some of these activities seem to reside within the complex fine structure of heparin. Heparin is a highly sulfated polysaccharide that is composed of alternating 1-4 linked glucosamine and hexuronic acid. Although the principal structure, called the regular region, is composed of major trisulfated disaccharide units, →4GlcN-(*N*,6-disulfate)α1-4IdoA(2-sulfate)α1→, substantial structural variability is observed in the irregular region, which is less but variably sulfated. The resulting structural variability is the basis of a wide variety of domain structures with biological activities. Specific enzymes are important tools for structural analysis. Commercially available enzymes that can be used for structural analysis of heparin are restricted to the three bacterial eliminases, heparinase and heparitinases I and II, isolated from *Flavobacterium heparinum*, which is a Gram-negative, non-pathogenic soil bacterium (*5*). Heparin lyases from *F. heparinum* convert heparin and/or heparan sulfate into disaccharides through eliminative cleavage of the α1-4 bond between glucosamine and hexuronic acid residues in the heparin and heparan sulfate backbone. The gene encoding heparinase (heparinase I; EC 4.2.2.7) has been cloned, and a functional, recombinant heparinase has been expressed in *Escherichia coli* without its predicted signal sequence (*6*). Recently, the genes encoding heparitinases I (heparinase III, EC 4.2.2.8) and II (heparinase II; no EC number), which are also produced by *F. heparinum*, have been cloned (*7, 8*). These genes were not contiguous with each other or with the heparinase gene.

*Prevotella heparinolytica* (*Bacteroides heparinolyticus* in previous nomenclature) is a Gram-negative, anaerobic,

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Abbreviations: GlcN, 2-deoxy-2-amino-D-glucose; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; ΔHexA, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; 2S, 2-*O*-sulfate; 6S, 6-*O*-sulfate; NS, 2-*N*-sulfate.

rod-shaped bacterium isolated from the oral cavity (*9, 10*). Nakamura *et al.* found that some strains of this bacterium produced an enzyme that cleaves heparin in an eliminative fashion and isolated the heparinase from the cell extract of an oral strain (*11*). In this study, we investigated various properties of the heparinase from *P. heparinolytica* in comparison with the heparinase from *F. heparinum*. In particular, its substrate specificity was studied using structurally defined oligosaccharides as substrates (*12*).

## MATERIALS AND METHODS

*Materials*—Bovine kidney heparan sulfate was purchased from Seikagaku, Tokyo. Heparin from bovine lung, bovine intestine, and ovine intestine, and heparan sulfate from bovine intestine were obtained from Sigma, St. Louis, MO, USA. Porcine intestinal heparin was from Nacalai Tesque, Kyoto. Bovine lung heparan sulfate was provided by Dr. Martin Mathews, University of Chicago. The heparin tetrasaccharides were prepared by enzymatic digestion with heparinase from *F. heparinum* as reported (*12*).

*Heparinase Preparations*—Heparinase from *F. heparinum* (*F.* heparinase) was obtained from Seikagaku. Heparinase from *P. heparinolytica* (*P.* heparinase) was purified in principle as described (*11*) but with a slight modification. Briefly, *P. heparinolytica* ATCC35895 was cultured in general anaerobic medium supplemented with heparin sodium salt at 37°C for 3 days. The cell extract was applied to a Q-Sepharose column equilibrated with a 0.05 M phosphate buffer (pH 7.1). The flow-through solution from the column contained a heparinolytic activity and was applied to a Sephacryl S-300 column, which was eluted with the above buffer containing 0.15 M NaCl. The active fractions from this column were then applied to a hydroxyapatite column. Elution was performed by stepwise increase of the NaCl concentration. The heparinase activity was found in the fraction eluted with 0.1 M NaCl. The purified preparation of *P.* heparinase gave a homogeneous protein band on SDS-polyacrylamide gel electrophoresis in the presence of 5% 2-mercaptoethanol.

*Enzyme Assay*—*P.* heparinase activity was determined based on the absorption of reaction products at 232 nm. Digestion was conducted using porcine intestinal heparin (1.2-3.3 mg/ml) as substrate at 37°C in a total volume of 30-100 $\mu$l of 50 mM Tris-HCl buffer (pH 8.0). Aliquots (30 $\mu$l) were withdrawn at 30-90 min intervals, mixed with 70 $\mu$l of 0.01 M HCl, and the absorbance was measured at 232 nm.

*Effects of pH on Heparinase Activities*—The optimum pH for *P.* heparinase was examined using the following buffers: 50 mM acetate (pH 3.5-7.0), 50 mM phosphate (pH 5.5-7.5), 50 mM Hepes-NaOH (pH 6.5-8.0), 50 mM MES-NaOH (pH 5.5-7.5), and 50 mM Tris-HCl (pH 7.0-9.0). *P.* heparinase (0.58 mIU) and *F.* heparinase (0.84 mIU) were individually incubated with 3.3 mg/ml of porcine intestinal heparin in each buffer at 37°C for 3 or 0.5 h, respectively. The activities were determined by measuring the increase in the absorbance at 232 nm.

*Effects of Salt Concentrations on the P. Heparinase Activity*—Effects of salt concentrations on the enzyme activity were investigated as follows. Firstly, heparinase (0.87 mIU) was incubated with 180 $\mu$g of porcine intestinal heparin in a total volume of 180 $\mu$l of 50 mM Tris-HCl buffer (pH 8.0) containing varying concentrations of sodium acetate for 3 h at 37°C (A). Secondly, heparinase was incubated with heparin as in (A) except that the buffer contained varying concentrations of NaCl and 75 mM sodium acetate (B). Thirdly, heparinase (0.57 mIU) was incubated with heparin as in (A) except that the buffer contained 75 mM sodium acetate, 0.1 M NaCl, 2.5 mM cysteine, and varying concentrations (0-10 mM) of $CaCl_2$ (C). The activities were calculated based on the absorbance at 232 nm.

*Substrate Specificities of Heparinases on Various Heparins and Heparan Sulfates*—Various heparin and heparan sulfate preparations (180 $\mu$g) were digested with heparinase from *P. heparinolytica* or *F. heparinum* under the optimized assay conditions below and each reaction was monitored by absorbance at 232 nm. For digestion with *P.* heparinase, 0.57 mIU of the enzyme was incubated with an appropriate substrate in a total volume of 100 $\mu$l of 50 mM Tris-HCl buffer (pH 8.0) containing 75 mM sodium acetate, 0.1 M NaCl, 3 mM $CaCl_2$, and 2.5 mM cysteine at 37°C for 3 h. For digestion with *F.* heparinase, 0.10 mIU of the enzyme was incubated in a total volume of 100 $\mu$l of 20 mM acetate buffer (pH 7.0) containing 3 mM $CaCl_2$ (*13, 14*). The substrates used were the following: porcine intestinal heparin, ovine intestinal heparin, bovine lung heparin, bovine intestinal heparin, bovine lung heparan sulfate, bovine intestinal heparan sulfate, and bovine kidney heparan sulfate.

*Digestion of Heparin Tetrasaccharides with P. Heparinase*—Various individual tetrasaccharides (0.3 or 0.5 nmol) isolated from heparin (*12*) were digested with 0.86 or 1.43 mIU of *P.* heparinase in a total volume of 30 $\mu$l of a 20 mM acetate buffer (pH 7.0) containing 3 mM calcium acetate for 90 min at 37°C. Reactions were terminated by boiling for 1 min. Enzyme digests were diluted in 400 $\mu$l of 16 mM $NaH_2PO_4$ and analyzed by HPLC on an amine-bound silica PA03 column (YMC, Kyoto) as reported (*15*).

*Kinetic Analysis of the P. Heparinase Reaction*—Kinetic constants of the heparinase reaction were determined at 37°C by measuring the initial rates of porcine intestinal heparin degradation as a function of heparin concentration in the range from 0.1 to 1.0 mg/ml under the optimized incubation conditions. Initial rates were measured by monitoring the increase in the absorbance at 232 nm. Incubations were conducted at a protein concentration of 0.08 $\mu$g/ml in a total volume of 2.5 ml of 50 mM Tris-HCl buffer (pH 8.0). Aliquots (0.8 ml) were withdrawn at 90-min intervals, added to 80 $\mu$l of 0.1 M HCl and the absorbance at 232 nm was measured.

*Amino Acid Analysis*—The amino acid composition was determined after acid hydrolysis in 6 M HCl at 110°C for 20, 37, and 48 h in a Beckman 6300E amino acid analyzer (*15*). The N-terminal sequence of purified *P.* heparinase (9.2 $\mu$g) was analyzed in an Applied Biosystems protein sequencer model 492.

## RESULTS

Purification of *P.* heparinase to homogeneity was accomplished by column chromatographies on Q-Sepharose, Sephacryl S-300, and hydroxyapatite. The physical and kinetic characteristics and substrate specificity of the

heparinase were compared with those of *F.* heparinase.

*Characterization of Optimal Catalytic Activity for the Heparinase*—The optimal reaction conditions for *P.* heparinase were determined by a series of experiments. The first parameter examined was the pH optimum with heparin as substrate using various buffers (pH 3.5-9.0). The buffer that gave optimum activity was a Tris-HCl buffer in the pH range of 7.5-8.0 (Fig. 1). The pH optimum was similar to that of *F.* heparinase, but the former enzyme displayed a broader pH profile, indicating that *P.* heparinase activity is less influenced by pH than *F.* heparinase.

The effects of concentrations of sodium acetate, sodium chloride and calcium ion on *P.* heparinase activity were examined making reference to a prior study on *F.* heparinase activity (*14*). *P.* heparinase showed increasing activity in response to increasing concentrations of sodium acetate, sodium chloride or calcium ion with the optimum activity at 75 mM, 0.1 M, or 1 mM, respectively (Fig. 2). In the presence of 2 mM EDTA, *P.* heparinase activity was greatly reduced (data not shown), in good agreement with the finding that the addition of calcium ion increased the activity. The effects of the optimum salt concentrations were very similar to those of *F.* heparinase (*14*).

Since the importance of the cysteine residue for *F.* heparinase activity has been documented (*16*), the effect of cysteine on *P.* heparinase activity was investigated. The enzymatic activity was activated in the presence of 2.5 mM cysteine (data not shown), indicating that the added cysteine may have protected the heparinase from inactivation.

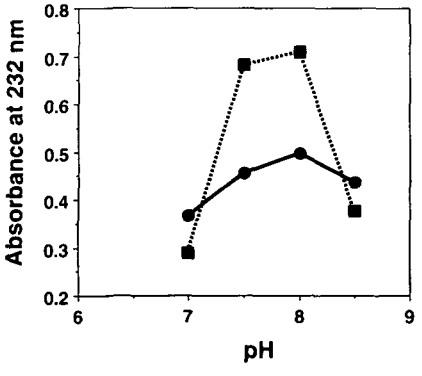


Fig. 1. **Effects of pH on heparinase activities.** Heparinase activities were assayed in 50 mM Tris-HCl buffer with the indicated pHs, and the reactions were monitored by absorbance at 232 nm. The solid and a dashed line represent the data obtained with heparinases from *P. heparinolytica* and *F. heparinum*, respectively.

*P.* heparinase seems to have a reactive cysteine residue near the active site, as does *F.* heparinase (*16*).

*Substrate Specificity of P. Heparinase*—Substrate specificity of *P.* heparinase was examined using various heparin and heparan sulfate preparations as substrates and compared with that of *F.* heparinase (Fig. 3). The degree of breakdown of the substrates was monitored by measuring the increase in the absorbance at 232 nm. Both heparinases were highly active against heparin preparations but inert to heparan sulfate preparations. The profile of the *P.* heparinase action towards heparin and heparan sulfate preparations was similar to that of *F.* heparinase, suggesting close similarities in their substrate specificities.

To better understand the substrate specificity of *P.* heparinase, it was investigated using structurally-defined tetrasaccharides that contain only one potentially susceptible glucosaminidic linkage. The results obtained with seven tetrasaccharides are summarized in Table I together with those of *F.* heparinase (*12*). *P.* heparinase was active on Fractions XI, XII, and XIII only. The cleavage of Fraction XII by *P.* heparinase demonstrated that the enzyme recognizes glucosaminidic bonds linked to the 2-sulfated glucuronic acid residue as well as those linked to a 2-sulfated iduronic acid. Hence, *P.* heparinase was concluded to act on a *N*-sulfated glucosaminidic linkage in the trisaccharide sequence, GlcN(NS)-IdoA(2S)-GlcN(NS, 6S) and GlcN(NS)-GlcA(2S)-GlcN(NS,6S).

*Determination of the Michaelis-Menten Constants for P. Heparinase*—The kinetics of *P.* heparinase action are shown in a Michaelis-Menten curve (Fig. 4, inset) and a Lineweaver-Burk plot (Fig. 4). The $K_m$ and $V_{max}$ values, determined from the average of two separate experiments conducted at 37°C and pH 8.0, were $3.8 \times 10^{-5}$ (M) and 11.4 ($\mu$mol/min·mg protein), respectively. An average molecular weight of 10,000 Da was used for heparin to calculate its molar concentration. Compared with an apparent $K_m$ value of $1.8 \times 10^{-5}$ (M) reported for *F.* heparinase (*14, 17*), that of *P.* heparinase was slightly larger but not significantly different. The subtle differences are likely to be due to variations in experimental conditions. The $V_{max}$ value of 219 ($\mu$mol/min·mg protein) reported for *F.* heparinase (*14, 17*) was approximately 20 times larger than that of *P.* heparinase. The highly purified *P.* heparinase is not stable and its activity tends to decrease during storage. Thus, it is likely that the $V_{max}$ value of *P.* heparinase was overestimated. The kinetic data support the similarity between these heparinases.

*Amino Acid Composition and Terminal Sequence Analyses*—The amino acid composition of *P.* heparinase is

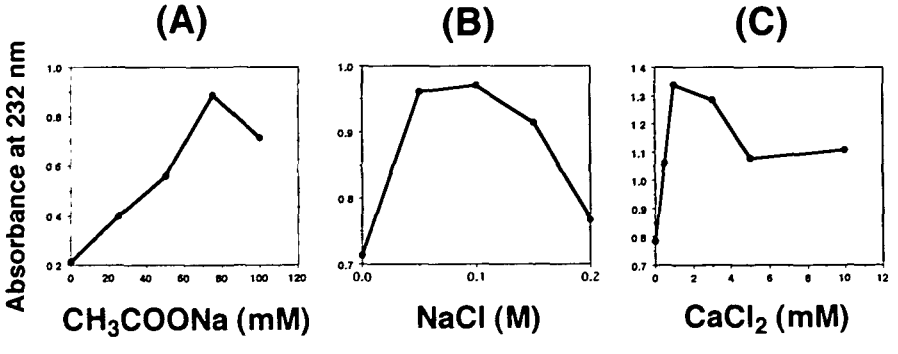


Fig. 2. **Effects of salt concentrations on *P. heparinolytica* heparinase activity.** Heparinase activity was assayed by the UV 232 nm method. Effects of sodium acetate (A), sodium chloride (B), or $CaCl_2$ (C) concentrations on the heparinase activity were investigated. Reaction mixtures in B contained 75 mM sodium acetate and those in C contained 75 mM sodium acetate, 0.1 M NaCl, and 2.5 mM cysteine.

shown in Table II along with that of *F.* heparinase. The two have a similar composition. Computer modeling, using the amino acid composition of *F.* heparinase, demonstrated that the high proportion of lysine residues contributes to the high isoelectric point of 9.2 (*14*). *P.* heparinase was also revealed to contain a high proportion of lysine, and this might contribute to the high isoelectric point of 9.5 (*11*).

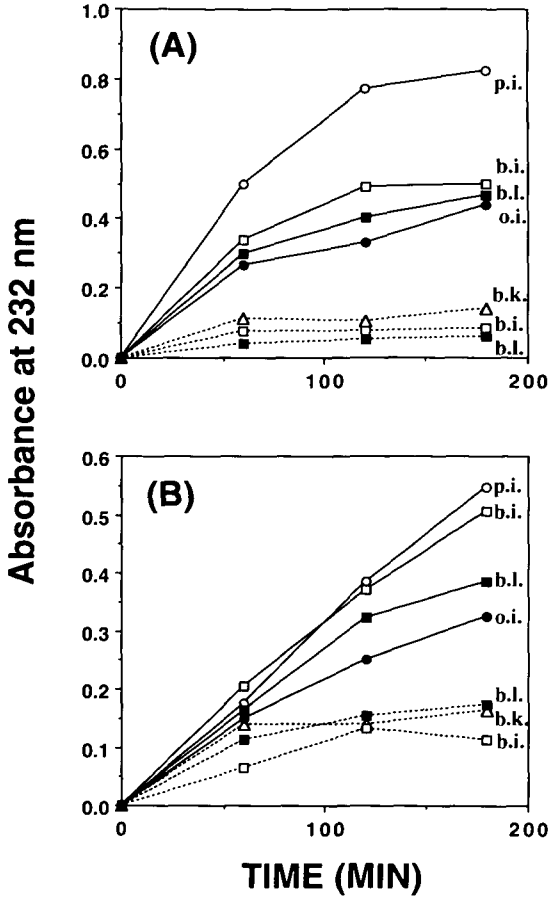


Fig. 3. **Substrate specificities of heparinases on various heparin and heparan sulfate.** Substrate specificities of the heparinases from *F. heparinum* (A) and *P. heparinolytica* (B) were compared using the following heparin (solid lines) and heparan sulfate (dashed lines) preparations as substrates: porcine intestinal (p.i.) heparin (open circle), ovine intestinal (o.i.) heparin (closed circle), bovine lung (b.l.) heparin and heparan sulfate (closed square), bovine intestinal (b.i.) heparin and heparan sulfate (open square), bovine kidney (b.k.) heparan sulfate (open triangle).

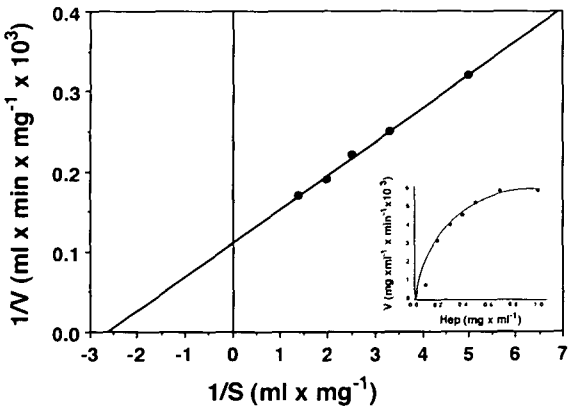


Fig. 4. **Kinetic analysis of heparin degradation by *P. heparinolytica* heparinase.** Porcine intestinal heparin at various concentrations was incubated with *P.* heparinase under the conditions described in "MATERIALS AND METHODS." $K_m$ and $V_{max}$ were determined using the Lineweaver-Burk plots of initial rates ($V$) based on the absorbance at 232 nm produced by the enzymatic reactions, and the Michaelis-Menten plots of initial rates ($V$) are shown in the inset.

TABLE II. **Amino acid composition of the heparinases.** Amino acid composition for *P.* heparinase was determined under the conditions described in "MATERIALS AND METHODS," and expressed as mol%. Values obtained after hydrolysis for 20 h are shown. Values for *F.* heparinase were taken from Ref. *14*. Asx and Glx represent the sum of Asp and Asn and that of Glu and Gln, respectively. Tryptophan was not determined.

| Amino acids | *P.* heparinase | *F.* heparinase |
|---|---|---|
| Asx | 11.5 | 12.2 |
| Glx | 9.5 | 9.7 |
| Ser | 5.3 | 6.5 |
| Gly | 8.0 | 8.2 |
| His | 1.6 | 1.6 |
| Arg | 2.9 | 3.5 |
| Thr | 6.5 | 5.4 |
| Ala | 9.4 | 7.0 |
| Pro | 5.0 | 5.4 |
| Tyr | 3.0 | 7.3 |
| Val | 5.5 | 4.9 |
| Met | 2.0 | 0.5 |
| Ile | 7.3 | 5.4 |
| Leu | 4.4 | 4.6 |
| Phe | 7.1 | 4.6 |
| Lys | 9.9 | 12.7 |
| Cys | 1.1 | 0.5 |
| Total | 100% | 100% |

TABLE I. **Substrate specificities of the heparinases towards structurally-defined heparin oligosaccharides.** Purified *P.* heparinase was incubated with each structurally-defined tetrasaccharide (*12*) under the conditions described in "MATERIALS AND METHODS" and the reaction products were identified by HPLC. A sensitive or resistant nature of tetrasaccharides to the enzymes is indicated as + or −, respectively. The sensitivity of each tetrasaccharide to *F.* heparinase was based on our previous data (*12*). 2S, 6S, and NS in the structures represent 2-*O*-, 6-*O*-, and 2-*N*-sulfate, respectively.

| Fr. | Structure | *P.* heparinase | *F.* heparinase |
|---|---|---|---|
| VI | ⊿HexA(2S)α1-4GlcN(NS) α1-4IdoA(2S) α1-4GlcN(NS) | − | − |
| VIII | ⊿HexA(2S)α1-4GlcN(NS,6S) α1-4GlcA β1-4GlcN(NS,6S) | − | − |
| IX | ⊿HexA(2S)α1-4GlcN(NS,6S) α1-4GlcA(2S) β1-4GlcN(NS) | − | − |
| X | ⊿HexA(2S)α1-4GlcN(NS,6S) α1-4IdoA(2S) α1-4GlcN(NS) | − | − |
| XI | ⊿HexA(2S)α1-4GlcN(NS) α1-4IdoA(2S) α1-4GlcN(NS,6S) | + | + |
| XII | ⊿HexA(2S)α1-4GlcN(NS,6S) α1-4GlcA(2S) β1-4GlcN(NS,6S) | + | + |
| XIII | ⊿HexA(2S)α1-4GlcN(NS,6S) α1-4IdoA(2S) α1-4GlcN(NS,6S) | + | + |

TABLE III. **Amino terminal sequences of the heparinases.** N-Terminal twenty amino acid residues of the purified *P.* heparinase were unambiguously identified by automated Edman degradation. N-Terminal sequence analysis of *F.* heparinase was not conducted, rather the sequence was deduced from the cDNA reported by Sasisekharan *et al.* (*6*).

| Residue number | 1 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| *P.* heparinase | A | N | L | D | L |
| *F.* heparinase | Q | Q | K | K | S |

| | | | | | |
|---|---|---|---|---|---|
| S | K | Y | G | I | K |
| G | N | I | P | Y | R |

| | | | | | |
|---|---|---|---|---|---|
| D | V | K | E | I | I |
| V | N | V | Q | A | D |

| | | | | | |
|---|---|---|---|---|---|
| H | N | P | | | |
| S | A | K | | | |

The results of N-terminal sequence analysis of *P.* heparinase are shown in Table III along with the N-terminal amino acid sequence deduced from the *F.* heparinase gene (*6*). In striking contrast with the composition data, their N-terminal sequences were totally different, indicating that *P.* heparinase is a distinct protein from *F.* heparinase. The twenty amino acid residues at the N-terminus of *P.* heparinase did not have a significant homology with any reported proteins.

## DISCUSSION

In this study we characterized the heparinase from *P. heparinolytica*, an oral cavity bacterial strain with heparinolytic activity. The potential role of this enzyme in the pathogenesis of periodontal disease has been suggested to be to increase epithelial permeability by the degradation of heparan sulfate located in intercellular spaces and basement membranes of the junctional epithelium (*18*). Thus, *P.* heparinase may be involved in the initial permeation of the gingival epithelium, permitting the ingress of further microbial virulence factors.

Although several bacteria produce heparinase, only the heparinase from *F. heparinum* has been commercialized. In the present study, we compared a variety of properties of *P.* heparinase and *F.* heparinase. Their optimum catalytic properties, substrate specificities, kinetic constants, and amino acid compositions were not significantly different. However, *P.* heparinase differed from *F.* heparinase in its N-terminal amino acid sequence and molecular weight. The molecular weights of *F.* heparinase and *P.* heparinase have been reported as 43,000 and 63,000 Da, respectively. Though *Prevotella* and *Flavobacteria* are phylogenetically related, Southern blotting experiments using the heparinase gene did not detect any cross-hybridizing material in the DNA from *P. heparinolytica* (*6*). Therefore, it seems that *P.* heparinase is a distinct enzyme from *F.* heparinase, but that its active site is similar to that of *F.* heparinase. The similarity in their substrate specificity might have resulted from convergent evolution.

The structural variability observed in heparin/heparan sulfate is often the basis of a wide variety of domain structures with a number of biological activities. Recent structural studies of the binding domains to antithrombin III (*19, 20*) and basic fibroblast growth factor (*21-24*) are the best known examples showing the relationships between the complicated fine structures and biological functions. Specific enzymes are essential for microanalyzing such biologically active domains. The three heparin-lyases, heparinase and heparitinases I and II, isolated from *F. heparinum* have been used for preparation and structural analysis of heparin/heparan sulfate oligosaccharides (*12, 25-32*), some of which may interact with various functional proteins. *P.* heparinase may also prove a valuable tool for elucidating the structure-function relationship of heparin/heparan sulfate.

The substrate specificity of *P.* heparinase was investigated using structurally-defined tetrasaccharides and compared with that of *F.* heparinase. The results indicated that they have the same specificity at least towards the tetrasaccharides examined. When commercial *F.* heparinase is used in a high concentration for structural analysis, it sometimes acts on the glucosaminidic linkages that are known to be resistant to the enzyme. Since *F. heparinum* produces not only heparinase but also heparitinase II, which has a broad substrate specificity (*33*), the above observation is presumably due to contamination by heparitinase II (*29*). Therefore, we examined whether *P. heparinolytica* produced heparitinase II, using $\Delta$HexA(2S)-GlcN(NS)-IdoA(2S)-GlcN(NS) as substrate, which is cleaved by heparitinase II. No heparitinase II activity was detected in the *P. heparinolytica* cell homogenate (results not shown), indicating that *P.* heparinase could prove a useful tool for degrading heparin without the risk of contamination with heparitinase II.

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