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Purification and Characterization of a Novel Heparinase from *Bacteroides stercoris* HJ-15[1]

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A novel type of heparinase (heparin lyase, no EC number) has been purified from *Bacteroides stercoris* HJ-15, isolated from human intestine, which produces three kinds of heparinases. The enzyme was purified to apparent homogeneity by a combination of QAE-cellulose, DEAE-cellulose, CM-Sephadex C-50, hydroxyapatite, and HiTrap SP chromatographies with a final specific activity of 19.5 μmol/min/mg. It showed optimal activity at pH 7.2 and 45°C and the presence of 300 mM KCl greatly enhanced its activity. The purified enzyme activity was inhibited by Cu^{2+}, Pb^{2+}, and some agents that modify histidine and cysteine residues, and activated by reducing agents such as dithiothreitol and 2-mercaptoethanol. This purified *Bacteroides* heparinase is an eliminase that shows its greatest activity on bovine intestinal heparan sulfate, and to a lesser extent on porcine intestinal heparan sulfate and heparin. This enzyme does not act on acharan sulfate but de-*O*-sulfated acharan sulfate and *N*-sulfoacharan sulfate were found to be poor substrates. The substrate specificity of this enzyme is similar to that of *Flavobacterial* heparinase II. However, an internal amino acid sequence of the purified *Bacteroides* heparinase shows significant (73%) homology to *Flavobacterial* heparinase III and only 43% homology to *Flavobacterial* heparinase II. These findings suggest that the *Bacteroidal* heparinase is a novel enzyme degrading GAGs.

Key words: *Bacteroides stercoris* HJ-15, heparinase, heparan sulfate, purification.

Heparinases (or heparin lyases) are enzymes that can eliminatively cleave polysaccharides, heparin, or heparan sulfate glycosaminoglycans (GAGs), into disaccharide and oligosaccharide products (*1*). These enzymes are classified as: (i) heparinase I (heparin lyase I, EC 4.2.2.7), acting primarily at the →4)-α-D-GlcNS(6S or OH)(1→4)-α-L-IdoA2S-(1→linkages present in heparin; (ii) heparinase II (heparin lyase II or heparitinase II), acting at the →4)-α-D-GlcNS(6S or OH)(1→4)-α-L-IdoA(2S or OH) or -β-D-GlcA(1→linkages present in both heparin and heparan sulfate; and (iii) heparinase III (heparin lyase III or heparitinase, EC 4.2.2.8), acting on the →4)-α-D-GlcNS(or Ac) (1→4)-α-D-GlcA (or IdoA) (1→linkages found exclusively in heparan sulfate (*2–4*). Heparinases are very useful tools for studying the sequences within heparin and heparan sulfate responsible for their biological activities. In addition to the well-known anticoagulant activity (*5*) of these glycosaminoglycans, they also exhibit a wide array of additional activities, such as the potentiation of angiogenesis (*6*) and the modulation of cellular proliferation (*7*). Several heparinases of bacterial origin have been purified and characterized from various species including *Flavobacterium heparinum* (*2*), *Bacillus* sp BH 100 (*8*), and *Prevotella heparinolyticus* (formerly known as *Bacteroides heparinolyticus*) (*9*). Among bacteria producing heparinases, *Flavobacterium heparinum*, a gram-negative soil bacterium, is the most intensively studied and the three heparinases from the bacterium have been purified, characterized, cloned and expressed (*2*, *10–14*).

Acharan sulfate, a GAG isolated from the giant African snail *Achatina fulica*, has a structure closely related to heparin and heparan sulfate with a uniform repeating disaccharide structure of →4)-α-D-GlcNAc(1→4)-α-L-IdoA2S (1→ (*15*). Recently, an acharan sulfate degrading bacterium was isolated from human intestine and identified as *Bacteroides stercoris* HJ-15 (*16*). This organism also cleaves heparin, heparan sulfate, chondroitin sulfate A and chondroitin sulfate C (*16*). Despite reports of heparinase being present in *Bacteroides* from human intestinal flora (*17*), *B. thetaio-*

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Abbreviations: ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid; IdoA, iduronic acid; GlcA, glucuronic acid GlcN, glucosamine; S, sulfate; Ac, acetate; GAG, glycosaminoglycan; QAE, quaternary amino ethyl; DEAE, diethylaminoethyl; CM, carboxymethyl; SP, sulfopropyl; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

taomicron, B. ovatus, B. uniformis (*18*), and *B. stercoris* (*16*), there have been no reports of the purification of a *Bacteroides* heparinase. New heparinases are needed to study the fine structure of heparin and heparan sulfate as well as to study the novel structure of newly isolated glycosaminoglycans, such as acharan sulfate. We report the purification of a heparinase from *B. stercoris* HJ-15, which acts predominantly on heparan sulfate. The properties and substrate specificity of this heparinase are described.

MATERIALS AND METHODS

Materials—Heparin (porcine intestinal mucosa, 12 kDa), chondroitin sulfate A (bovine trachea), chondroitin sulfate B (bovine intestinal mucosa), chondroitin sulfate C (shark cartilage), thioglycolic acid (sodium salt), QAE cellulose fastflow, HA Ultrogel (microcrystalline hydroxyapatite, 4% beaded in agarose), and low molecular weight markers for gel filtration were obtained from Sigma Chemical. Porcine heparan sulfate (porcine intestinal mucosa, 11.7 kDa) was prepared as described by Griffin *et al.* (*19*). Bovine heparan sulfate (bovine kidney) was from Seikagaku (Tokyo). Acharan sulfate was prepared as described by Kim *et al.* (*15*). De-*O*-sulfated and *N*-sulfoacharan sulfate were prepared as described previously (*20*, *21*). CM-Sephadex C-50, HiTrap SP column, Sephacryl S-300 HR resins, and high molecular weight markers for gel filtration and protein electrophoresis were obtained from Amersham Pharmacia Biotech. DEAE cellulose resin was purchased from Wako Pure Chemical Industries. Protein assay reagent, sodium dodecyl sulfate, Coomassie Brilliant Blue R-250 and Model 111 Mini IEF Cell were from Bio-Rad Laboratories. Tryptic soy broth was provided by Difco. All other chemicals were of the highest grade available.

Bacterial Strains and Purification—B. stercoris HJ-15 was isolated and cultivated anaerobically (*16*) under an atmosphere of 90% nitrogen and 10% carbon dioxide at 37°C in 100 liters of tryptic soy broth (pH 7.2) containing heparin (0.15 g/liter) instead of glucose, 0.01% (w/v) sodium thioglycolate, and 0.1% (w/v) ascorbic acid. Cells were harvested in late exponential phase (11–12 h) by centrifugation at 5,000 rpm for 30 min at 4°C, and the resulting cell pellet was washed twice with saline containing 50 mM sodium phosphate (pH 7.0). The cell pellet was suspended in 600 ml of Buffer A (50 mM sodium phosphate buffer, pH 7.0). The cell suspension (30 ml at a time) was disrupted by 30-min periods of sonication at 1-s intervals on an ultrasonic processor (Eyela, Tokyo) at 80% output with cooling. Cell debris was removed by centrifugation at 18,000 rpm for 60 min at 4°C. All operations were done at 4°C unless otherwise noted. The cell extract (620 ml) was passed through a QAE cellulose column (5 × 40 cm) that had been pre-equilibrated with Buffer A. The column was washed with the same buffer until no further lyase activity was detectable in the effluent. The fractions that passed through the column were applied to a DEAE cellulose column (5 × 30 cm) equilibrated with Buffer A. The column was then eluted with the same buffer until no GAGs degrading lyase activity could be detected. The non-interacting protein that passed through the column was collected (1,800 ml). The eluate was loaded onto a CM-Sephadex column C-50 (3 × 30 cm) previously equilibrated with Buffer A. The column was washed with 1,000 ml of the same buffer and then eluted with a 2–l linear gradient of KCl from 0 to 0.6 M in Buffer A at a flow rate of 105 ml/h. All fractions obtained were assayed for heparinase activities. Three fractions (**a**, **b**, and **c**) containing heparin lyase activity were collected separately and assayed for the acharan sulfate and heparan sulfate degrading activities. Fraction **b** (149.5 ml) was loaded onto a hydroxyapatite column (2.5 × 10 cm) pre-equilibrated with Buffer A. The column was washed with 300 ml of Buffer A containing 0.5 M KCl, and then a 800-ml linear gradient from 0.5 M to 1.3 M KCl in Buffer A was conducted at a flow rate of 120 ml/h. The active fractions were pooled and dialyzed against Buffer A. The dialysate was applied to a HiTrap SP column (1 ml of SP Sepharose) equipped with GradiFrac (Amersham Pharmacia Biotech) and the column was washed with Buffer A at a flow rate of 2 ml/min until the absorbance at 280 nm reached the baseline. A 300-ml linear gradient of KCl from 0 and 0.5 M in Buffer A was used to elute the enzyme (flow rate, 2 ml/min) and heparinase-positive fractions were tested for purity by SDS-PAGE.

Enzyme Activity Assay—Lyase activities were assayed according to the following method. The standard assay mixture in a final volume of 700 μl contained 1 mg of substrate in 650 μl of 50 mM sodium phosphate buffer with 100 mM KCl (pH 7.2 , Buffer B). The mixture was placed in a 1-ml quartz cuvette thermally equilibrated in the spectrophotometer (Jasco V-530) at 40°C. The reaction was started by the addition of 50 μl of enzyme solution, allowed to proceed for 5 min, and the change in the absorbance at 232 nm was monitored. The activity was calculated from the change in absorbance per minute using an extinction coefficient of 3,800 M^{-1} for products (1 U = 1 μmol of ΔUA containing product formed/min) (*22*). The specific activity was calculated by dividing the micromoles of product produced per minute by the milligrams of protein in the cuvette. Protein concentration was measured by the Bradford assay using a bovine serum albumin standard curve.

Characterization of Purified Heparinase—SDS-PAGE was performed to determine molecular weight according to Laemmli's procedure (*23*). The gels were stained with Coomassie Brilliant Blue R-250 solution and further stained with silver. The pI value of heparinase was determined by IEF electrophoresis using a Model 111 Mini IEF Cell (from Bio-Rad) according to the manufacturer's instructions. The molecular weight of the native enzyme was estimated by gel-filtration using a Sephacryl S-300 HR column (1.6 × 70 cm) calibrated with a gel filtration low molecular weight calibration kit (from Sigma) and high molecular calibration kit (from Amersham Pharmacia Biotech). The pH optimum of heparinase was determined using 50 mM sodium phosphate buffer (pH 6.0 to 8.5). Temperature dependency of the enzyme was investigated by measuring enzyme activity at different temperatures (25–60°C). To investigate the effect of divalent metal ions and KCl on the lyase activity, divalent metal ion (final concentration, 100 μM) and KCl (0 to 500 mM) were added to the reaction mixture. Kinetic constants of heparinase were determined by measuring the initial rates at various substrate concentrations (200, 400, 600, 1,000, 2,000, and 3,000 μg) under the standard reaction conditions. The lyase activity toward other sulfated polysaccharides was also measured. One milligram of each substrate was added to the reaction mixture. Because of their low solubilities, 100 μg of acharan sulfate, de-*O*-sul-

fated acharan sulfate and *N*-sulfoacharan sulfate were used in this assay.

RESULTS AND DISCUSSION

Bacteroides stercoris HJ-15 was found to degrade a variety of GAGs including heparin, heparan sulfate and chondroitin sulfates (*16*). Although these *B. stercoris* HJ-15 heparinases are constitutive, when induced by heparin, the total heparinase activity increased by about 3-fold. Following ultrasonic disruption of *B. stercoris* HJ-15, the crude extract was subjected to a combination of QAE-cellulose and DEAE-cellulose column chromatographies to remove interacting proteins. Heparinase activity passed through these columns without binding. The effluent was then applied to a CM-Sephadex C-50 column to resolve three fractions efficiently (**a**, **b**, and **c** in Fig. 1) containing heparinase activities. Fraction **a** primarily degraded acharan sulfate, fraction **b** primarily degraded heparan sulfate and heparin to a lesser degree, and fraction c preferentially acted on heparin and on heparan sulfate to a lesser degree (Fig. 2). The heparinase found in fraction **b**, which most closely resembles *Flavobacterium* heparinase II based on its activity towards heparin and heparan sulfate, was present in sufficient amounts to be further purified to homogeneity by hydroxyapatite column chromatography and HiTrap Sp column chromatography. This purified enzyme, a heparin lyase acting eliminatively on heparan sulfate and heparin (Table I), was tentatively named *Bacteroidal* heparinase and its specific activity increased through purification from 0.029 to 19.5 μmol/min/mg, corresponding to a 672-fold enrichment (Table I). The *Bacteroidal* heparinase was apparently homogeneous and migrated as a 70 kDa protein on SDS-PAGE (Fig. 3). The molecular mass, determined by gel filtration, was identical to that measured by SDS-PAGE. The molecular masses of previously reported heparinases range from 41.7 to 116 kDa. The molecular mass of the *Bacteroidal* heparinase (70 kDa) is substantially lower than that of *Flavobacterium* heparinase II (84.1 kDa), but similar to that of *Flavobacterium* heparinase III (2). The optimum pH of the *Bacteroidal* heparinase is 7.0–7.2, similar to other heparinases having pH optima ranging from 6.5 to 7.6. The *Bacteroidal* heparinase showed less than 10% of its optimal activity below pH 6.0 or above pH 8.4 (Fig. 4), and was nearly inactive in Tris-HCl (pH 7.0 to 9.0) and glycine-NaOH (pH 8.0 to 10.0), suggesting that the enzyme activity is dependent on the presence of inorganic salts. The *Bacteroidal* heparinase activity increased 3.7-fold upon adding 300 mM KCl, but it was gradually inhibited at higher concentrations (>300 mM). The activity was slightly

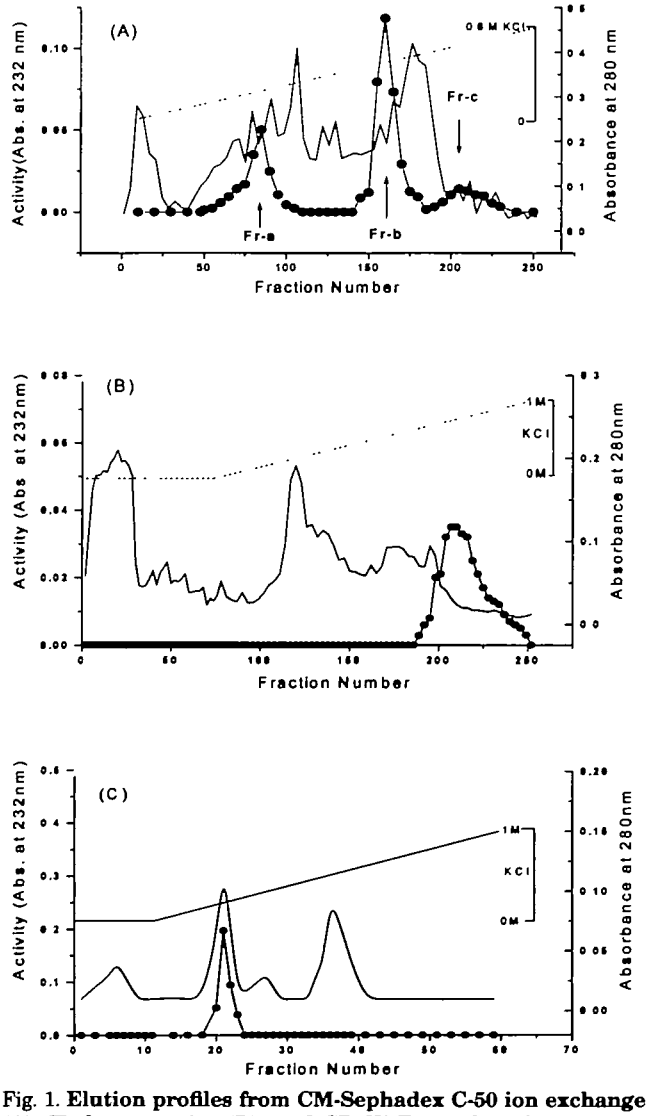

Fig. 1. **Elution profiles from CM-Sephadex C-50 ion exchange (A), Hydroxyapatite (B), and SP Hi-Trap (C) column chromatographies.** Solid circles, heparinase activity; simple line, absorbance at 280 nm.

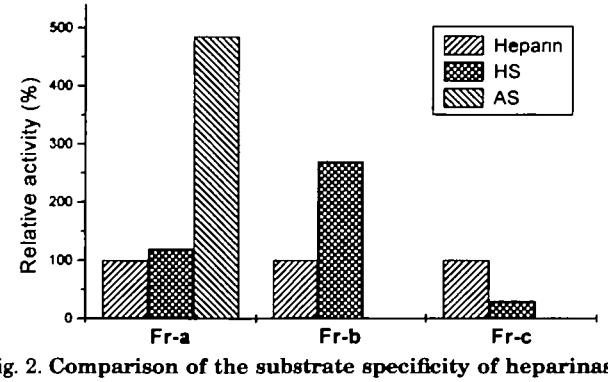

Fig. 2. **Comparison of the substrate specificity of heparinase activity fractions a, b, and c.** HS, porcine heparan sulfate; AS, acharan sulfate.

TABLE I. **Purification of the *Bacteroidal* heparinase from *B. stercoris* HJ-15.**

Step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)[a]	Overall yield (%)	Purification (fold)
Crude Extract	223.4	7,637	0.029	100	1
CM-Sephadex C-50	83.5	32.9	2.54	37.4	88
Hydroxyapatite	58.6	8.5	6.89	26.2	238
HiTrap SP (SP-Sepharose)	46.7	2.4	19.5[a] 51.1[b]	20.9	672

[a]Heparinase activity measured using heparin as substrate. [b]Heparinase activity measured using porcine heparan sulfate as substrate.

enhanced by the addition of Ca^{2+}, Mg^{2+}, and Ba^{2+}, whereas it was strongly inhibited by Cu^{2+} and Pb^{2+} (Table II). While Cu^{2+} appears to be an inhibitor of all heparinases (*2*), Ca^{2+} and Ba^{2+} inhibit *Flavobacterium* heparinase II and III but activate the *Bacteroidal* heparinase. The activation with KCl could well be an ionic strength effect. In contrast, the enhancement by Ca^{2+} requires additional study, as Ca^{2+} has been shown to be essential for the proper functioning of *Flavobacterial* heparinase I (*24*). The *Bacteroidal* heparinase shows optimal activity at 45°C, which is a little higher than in the case of *F. heparinum* heparinase II (40°C). When the *Bacteroidal* heparinase activity was assayed by adding heparin after a 3 min pre-incubation at various temperatures in the absence of substrate, the enzyme showed the highest activity at 40°C, but no activity was detectable at temperatures above 50°C. From this observation, the *Bacteroidal* heparinase appears to be very unstable above its optimal temperature.

Amino acid composition analysis revealed that the *Bacteroidal* heparinase contains a large proportion of lysine (Table III), consistent with its pI value of 8.7. The pI values of *Flavobacterial* heparinases range from 8.9 to 10.1 (*2*). Several attempts at N-terminal analysis failed to yield sequence information suggesting that the N-terminus is blocked. It is interesting to note that the N-termini in heparin lyases from *F. heparinum* are also blocked (*11*). Therefore, we analyzed the internal amino acid sequence of a peptide obtained by digestion with trypsin. The result of internal amino acid sequence analysis of *Bacteroidal* heparinase is shown in Table IV. The sequence of this internal peptide shows significantly greater (73%) homology to *Flavobacterial* heparinase III than to *Flavobacterial* heparinase II. Serine protease inhibitors (PMSF, paraoxon) and a cysteine protease inhibitor (iodoacetic acid) had little effect on the *Bacteroidal* heparinase activity. In contrast, another cysteine-directed reagent (*p*-chloromercuriphenylsulfonate) produced significant inhibition of the enzyme (Table II).

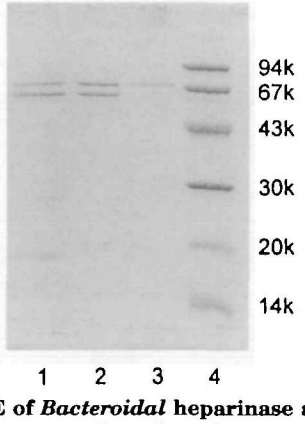

Fig. 3. **SDS-PAGE of *Bacteroidal* heparinase at various steps in the purification procedure.** Lane 1, preparation after elution from CM-Sephadex C-50 column; Lane 2, preparation after hydroxyapatite chromatography; Lane 3, purified heparinase II after Hi-Trap SP chromatography; Lane 4, molecular mass markers.

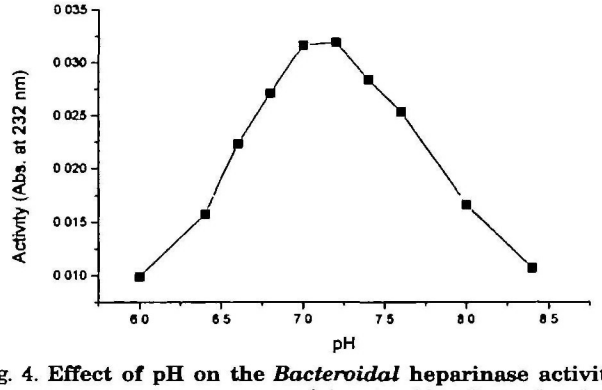

Fig. 4. **Effect of pH on the *Bacteroidal* heparinase activity.** The enzyme activity was assayed in 50 mM sodium phosphate buffer at the indicated pH.

TABLE II. **Effect of amino acid modifying reagents and divalent cations on the purified *Bacteroidal* heparinase activity.**

Inhibitors	Concentration (mM)	Residual activity[a] (%)
Control		100
Mg^{2+}	0.1	106
Ca^{2+}	0.1	111
Ni^{2+}	0.1	88
Co^{2+}	0.1	104
Cu^{2+}	0.1	8
Pb^{2+}	0.1	22
Mn^{2+}	0.1	91
Zn^{2+}	0.1	73
Cd^{2+}	0.1	93
Ba^{2+}	0.1	107
EDTA	0.1	96
PMSF	0.1	95
Diethyl *p*-nitrophenyl phosphate	0.1	98
TPCK	0.1	94
dl-Dithiothreitol	0.1	209
Iodoacetic acid	0.1	96
Carbodiimide	0.1	95
Butanediol	0.1	94
p-Chloromercuriphenyl sulfonic acid	0.05	0
TLCK	0.05	71
2-Mercaptoethanol	0.05	193

[a]0.03 unit of the homogenously purified enzyme activity was taken as 100%.

TABLE III. **Amino acid composition analysis of the *Bacteroidal* heparinase.**

Amino acid	pmol	Mol%
Cys	N.D.[b]	0.00
Asx[a]	368.56	9.28
Glx[a]	439.19	11.06
Ser	127.62	3.21
Gly	162.00	4.08
His	110.28	2.78
Arg	173.72	4.38
Thr	196.72	4.96
Ala	278.25	7.01
Pro	214.34	5.40
Tyr	209.24	5.27
Val	294.53	7.42
Met	113.59	2.86
Ile	158.99	4.00
Leu	378.59	9.54
Phe	321.02	8.09
Trp	N.D.[b]	0.00
Lys	423.15	10.66
Total	3,969.79	100.00

[a]Asx and Glx indicate the sum of asparagine and aspartic acid, glutamine, and glutamic acid, respectively. [b]Not determined.

Reducing agents such as *dl*-dithiothreitol and 2-mercaptoethanol enhanced the enzyme activity. Interestingly, one trypsin inhibitor, TPCK, did not affect the enzyme activity, while another related inhibitor, TLCK, had a moderately inhibitory effect on the *Bacteroidal* heparinase activity. *Flavobacterium* heparinase II has been reported to have histidine and cysteine residues in its active site (12). The results of our studies on the *Bacteroidal* heparinase suggest that these amino acids may also play an important role in catalysis by this enzyme.

Michaelis-Menten constants were determined using a Hanes plot (Fig. 5). K_m and V_{max} values for heparin and porcine heparan sulfate were calculated as 9.05×10^{-6} M, 38.2 μmol/min/mg and 1.53×10^{-5} M, 58.4 μmol/min/mg, respectively (Table V). The *Bacteroidal* heparinase was most active towards bovine heparan sulfate but was inactive towards acharan sulfate and chondroitin sulfates (Table VI). The *Bacteroidal* heparinase acted slowly on acharan sulfate derivatives, including de-2-*O*-sulfated acharan sulfate and *N*-sulfoacharan sulfate. The *Bacteroidal* heparinase acts preferentially on the undersulfated sequences →4)-Δα-D-GlcNAc(1→4)-β-D-GlcA(1→, →4)-α-D-GlcNS(1→4)-β-D-GlcA(1→ and →4)-α-D-GlcNAc6S(1→4)-β-D-GlcA-(1→, comprising heparan sulfate affording the expected unsaturated unsulfated disaccharide (ΔUA-GlcNAc) and monosulfated disaccharide products (ΔUA-GlcNS and ΔUA-GlcNAc6S) (data not shown). Its preference for bovine intestinal heparan sulfate over porcine intestinal heparan sulfate might be the result of its preference for this lower molecular weight substrate or some other as yet undefined structural feature. The *Bacteroidal* heparinase also acted well on heparin, which is largely comprised of the trisulfated sequence →4)-α-D-GlcNS6S(1→4)-α-L-IdoA2S(1→ affording an unsaturated disaccharide product (data not shown). This result demonstrates that the *Bacteroides* enzyme has a broad specificity acting at either iduronic or glucuronic acid containing linkages and in both low and

TABLE IV. **Internal amino acid sequences of *Bacteroidal* heparinase.**

Enzyme	Internal amino acid sequence	Homology (%)
Bacteroidal heparinase	-----MADEALQHTFFA----	
Flavobacterial heparinase II	329 -----YKDEYLNYEFLK-----	48
Flavobacterial heparinase III	98 -----MADKALVHQFQP---	73

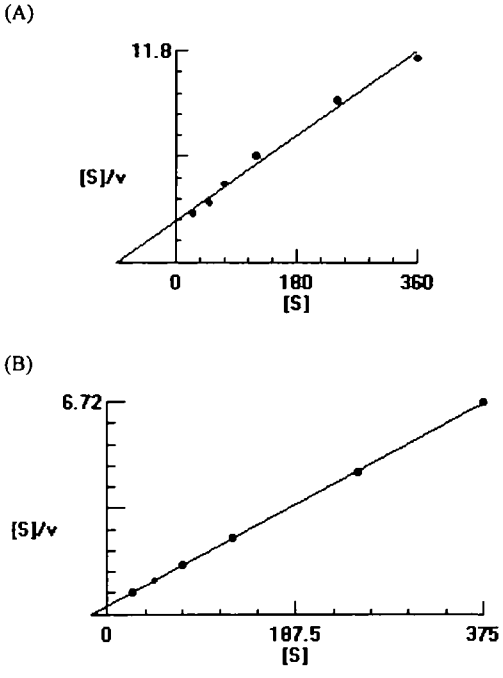

Fig. 5. **Hanes plot for the determination of kinetic constants of *Bacteroidal* heparinase.** (A) heparin; (B) heparan sulfate.

TABLE V. **Kinetic constants of purified *Bacteroidal* heparinase.**

Enzyme	V_{max} (μM)		K_m (μM)	
	Heparin	Heparan sulfate	Heparin	Heparan sulfate
Bacteroidal heparinase	38.2	58.4	90.5	15.3
Flavobacterial heparinase I[a]	219	—[b]	17.8	—
Flavobacterial heparinase II	16.7	28.6	57.4	11.2
Flavobacterial heparinase III	—	141	—	29.4

[a]Data from Ref. *2*. [b]Not determined.

TABLE VI. **Substrate specificity of heparin lyases.**

Substrates	Relative activity (%)[a]			
	Bacteroidal heparinase	*Flavobacterial* heparinase[b]		
		I	II	III
Heparin	100	100	100	0
Heparan sulfate (porcine)	262	nd[c]	nd	nd
Heparan sulfate (bovine)	610	30	172	100
Acharan sulfate	0	0	100[d]	0
De-*O*-sulfated acharan sulfate	3	nd	nd	nd
N-Sulfoacharan sulfate	2	nd	nd	nd
Chondroitin sulfate A	0	0	0	0
Chondroitin sulfate B	0	0	0	0
Chondroitin sulfate C	0	0	0	0

[a]Activity on heparin (or heparan sulfate in *Flavobacterial* heparinase III) as the substrate was set at 100%. [b]Data from Refs. *2*, *4*, *15*, and *26*. [c]Not determined. [d]Unpublished data.

high sulfated domains. The *Bacteroidal* heparinase shows weak action on *N*-sulfoacharan sulfate and de-*O*-sulfated acharan sulfate suggesting that it can also act on →4)-α-D-GlcNS(1→4)-α-L-IdoA2S(1→ and →4)-α-D-GlcNAc(1→4)-α-L-IdoA(1→ linkages, respectively. *Flavobacterium* heparinase II has demonstrated activity on all of these substrates as well (*2–4*, *25*, *26*). Surprisingly, the *Bacteroidal* heparinase fails to act on acharan sulfate, which is comprised entirely of →4)-α-D-GlcNAc(1→4)-α-L-IdoA2S(1→ linkages, although acharan sulfate serves as an excellent substrate for *Flavobacterium* heparinase II (*26*).

This is the first report of the purification of a heparinase from an anaerobic *Bacteroides*. The major difference in specificity between the *Bacteroidal* heparinase and the *Flavobacterium* heparinase II is the capability of the *Flavobacterium* enzyme to act on acharan sulfate (*26*). Substrate specificity studies employing new, structurally defined substrates, such as acharan sulfate and its derivatives, suggest new guidelines for the classification of heparinases. However, an internal amino acid sequence in the purified *Bacteroides* heparinase shows significantly greater (73%) homology to *Flavobacterial* heparinase III than to *Flavobacterial* heparinase II. These findings suggest that *Bacteroides* heparinase is a novel enzyme degrading GAG. Although the biochemical properties and substrate specificity of *Bacteroidal* heparinase are similar to those of heparinase II previously purified from *Flavobacterium heparinum*, its structure appears more closely to resemble that of *Flavobacterial* heparinase III.

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