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Purification and Characterization of Heparin Lyase I from *Bacteroides stercoris* HJ-15

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Heparin lyase I was purified to homogeneity from *Bacteroides stercoris* HJ-15 isolated from human intestine, by a combination of DEAE-Sepharose, gel-filtration, hydroxyapatite, and CM-Sephadex C-50 column chromatography. This enzyme preferred heparin to heparan sulfate, but was inactive at cleaving acharan sulfate. The apparent molecular mass of heparin lyase I was estimated as 48,000 daltons by SDS-PAGE and its isoelectric point was determined as 9.0 by IEF. The purified enzyme required 500 mM NaCl in the reaction mixture for maximal activity and the optimal activity was obtained at pH 7.0 and 50°C. It was rather stable within the range of 25 to 50°C but lost activity rapidly above 50°C. The enzyme was activated by Co^{2+} or EDTA and stabilized by dithiothreitol. The kinetic constants, K_m and V_{max} for heparin were 1.3×10^{-5} M and 8.8 μmol/min·mg. The purified heparin lyase I was an eliminase that acted best on porcine intestinal heparin, and to a lesser extent on porcine intestinal mucosa heparan sulfate. It was inactive in the cleavage of *N*-desulfated heparin and acharan sulfate. In conclusion, heparin lyase I from *Bacteroides stercoris* was specific to heparin rather than heparan sulfate and its biochemical properties showed a substrate specificity similar to that of *Flavobacterial* heparin lyase I.

Keywords: *Bacteroides stercoris* HJ-15, Heparin, Heparin lyase, Heparan sulfate, Purification

Introduction

Heparin-like glycosaminoglycans (HLGAGs) play an important role in the extracellular matrix, and regulate a wide variety of biological functions (Jackson *et al*., 1991; Lindahl *et al*., 1994). HLGAGs are highly sulfated acidic polysaccharides with disaccharide units of uronic acid (L-iduronic or D-glucuronic acid) and D-glucosamine residues connected through 1 → 4 linkages (Conrad, 1989). HLGAGs are a heterogeneous group due to varying degrees of modification of the functional groups in the disaccharide unit (Jackson *et al*., 1991; Griffin *et al*., 1995). Heparin, heparan sulfate, chondroitin sulfate, hyaluronic acid, and keratan sulfate are typical examples; they are obtained from animal tissues.

Recently, a new glycosaminoglycan named acharan sulfate, was isolated and characterized from the African giant snail *Achatina fulica* (Kim *et al*., 1996). This GAG is neither a heparin nor a heparan sulfate, but instead represents an unusual repeating sequence, → 4)-2-acetamido-2-deoxy-α-D-glucopyranose(1 → 4)-2-sulfo-α-L-idopyranosyluronic acid (1 → [→ GlcNAc → IdoA2S →].

Heparin lyases (heparinases) have aided our understanding of the heterogeneous structures related to the important physiological roles of heparin and heparan sulfate, which include anticoagulation, the potentiation of angiogenesis (Folkman and Shing, 1992) and the modulation of cellular proliferation (Castellot *et al*., 1986), and have clinical applications such as, in the monitoring of heparin levels and the neutralization of heparin in blood (Heres *et al*., 2001). They are also useful for preparing low molecular weight heparins. Heparin lyase I and II were applied as potent neovascularization inhibitors (Sasisekharan *et al*., 1994). Three types of heparin lyase from *Flavobacterium heparinum* that degrade HLGAGs (heparin and heparan sulfate) have been studied; 1) heparin lyase I (heparinase 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; 2) heparin lyase II (heparinase 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 3) heparin lyase III (heparinase III or heparitinase I, EC 4.2.2.8), acting on the → 4)-α-D-GlcNS(or Ac) (1 →

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4)-β-D-GlcA(or IdoA) (1 → linkages found exclusively in heparan sulfate (Linhardt *et al*., 1990).

Several heparin lyases of bacterial origin have been purified and characterized from various species including *Flavobacterium heparinum* (Yang *et al*., 1985; Lohse and Linhardt, 1992), *Bacillus circulans* (Yoshida *et al*., 2002), *Prevotella heparinolyticus* (formerly known as *Bacteroides heparinolyticus*) (Watanabe *et al*., 1998). Of the bacteria producing heparinases, *F. heparinum*, a gram-negative soil bacterium, has been most intensively studied and three heparin lyases have been purified, characterized, cloned and expressed (Lohse and Linhardt, 1992; Sasisekharan *et al*., 1993; Ernst *et al.*, 1996; Godavarti *et al*., 1996a; Godavarti *et al*., 1996b). Recently, we purified heparinase (heparin lyase III) (Kim *et al*., 2000) and two acharan sulfate lyases I and II from *Bacteroides stercoris* HJ-15 (Kim *et al*., 2001). The previously purified heparinase from *Bacteroides stercoris* HJ-15 predominantly cleaved heparan sulfate and heparin in part and has similar properties to *F. bacterium* heparin lyase III (Kim *et al*., 2000). However, heparin lyase I has not been characterized from this species. In this study, we purified heparin lyase I from *Bacteroides stercoris* HJ-15 and characterized its biochemical properties.

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), DEAE-Sepharose Fast Flow, HA Ultrogel (microcrystalline hydroxyapatite, 4% beaded in agarose), and low molecular weight markers for gel-filtration, sodium dodecyl sulfate and Coomassie brilliant blue R-250 were obtained from the Sigma Chemical Co (St. Louis, USA). Porcine heparan sulfate from intestinal mucosa, prepared as described by Griffin (Griffin *et al*., 1995), was a gift from Prof. Robert J. Linhardt (Renssealer Polytech Institute, USA). Bovine heparan sulfate (bovine kidney) was purchased from Seikagaku Co (Tokyo, Japan). *N*-desulfated heparin was purchased from Sigma. 2-*O*-desulfated heparin was prepared previously reported (Ishihara *et al*., 1997). Acharan sulfate was prepared as described by Kim (Kim *et al*., 1996). Authentic unsaturated disaccharides derived heparin and heparan sulfate (ΔUA2S-GlcNS6S, ΔUA-GlcNAc and ΔUA-GlcNS6S) were purchased from Sigma. CM-Sephadex C-50 and high molecular weight markers for gel-filtration were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Bio-Gel P-100 (medium), protein electrophoresis, protein assay reagent and Model 111 Mini IEF Cell were from Bio-Rad Laboratories (Hercules, CA, USA). Tryptic soy broth was purchased from Difco Co (Sparks, MD, USA), and all other chemicals were of the highest grade available.

Bacterial strains and heparinase I purification *Bacteroides stercoris* HJ-15 was isolated and cultivated as previously described (Ahn *et al*., 1998; Kim *et al*., 2000). It was cultured anaerobically in a 90% nitrogen and 10% carbon dioxide atmosphere at 37°C in 50 liters of tryptic soy broth (pH 7.2) containing 0.15 g/l of heparin instead of glucose, 0.01 (w/v)% sodium thioglycolate, and 0.1 (w/v)% ascorbic acid. Cultured cells were harvested by centrifugation at 4,500 × *g* for 30 min at 4°C. The resulting cell pellets were washed twice with saline containing 50 mM sodium phosphate buffer (pH 7.0) and then suspended in the same buffer. The suspension was disrupted by a 30 min period of sonication at 1 s interval on an ultrasonic processor (Cole-Parmer Instruments, Vernon Hills, USA) at 80% output with cooling. Cell debris was removed by centrifugation at 36,000 × *g* for 60 min at 4°C. All operations were done at 4°C unless otherwise stated. Three hundred ml of the supernatant was passed through a DEAE-Sepharose column (5 × 30 cm) that had been pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was then eluted with the same buffer until no heparin lyase I activity could be detected. Fractions that passed through the column were pooled and concentrated by precipitation with ammonium sulfate (30~70%). This precipitate was dissolved in 50 mM sodium phosphate buffer (pH 7.0), dialyzed, and applied to a column packed with Bio-Gel P-100 (2.6 × 88 cm) previously equilibrated with the same buffer. The active fractions from this column were then applied to a hydroxyapatite column (2.6 × 27 cm), active fractions were pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). Elution was performed using a linear gradient of NaCl from 0 to 1.2 M NaCl in 50 mM sodium phosphate buffer (pH 7.0). Active fractions were collected and dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and the dialysate was applied onto a CM-Sephadex C-50 column (1.8 × 29 cm). A 400 ml linear gradient of NaCl from 0 to 0.6 M in 50 mM sodium phosphate buffer (pH 7.0) was used to elute the enzyme and the purified preparation of heparin lyase I gave a homogeneous protein band on SDS-PAGE.

Enzyme activity assay The activities of GAG lyases including heparin lyase I, were determined as previously described (Lohse and Linhardt, 1992). Heparin lyase I activity was determined based on reaction product absorption at 232 nm; the reaction used heparin as a substrate at 45°C. The spectrophotometer (Jasco Co. V-550, Tokyo, Japan) was adjusted to 45°C and a 1.0 ml quartz cuvette (path length 1 cm) containing 1.0 mg of heparin in 650 μl of 50 mM sodium phosphate buffer containing 500 mM NaCl (pH 7.0) was thermally equilibrated. Fifty micro liters of the enzyme solution were added and the cuvette contents were gently mixed. The cuvette was then immediately returned to the spectrophotometer and absorbance change at 232 nm was measured at 1 sec interval over 5 min.

Enzyme activity was calculated using a molar extinction coefficient of 3,800 $M^{-1} \cdot cm^{-1}$ for unsaturated oligosaccharide products formed by heparin lyase I (1 U = 1 μmol of ΔUA containing product formed/min) (Lohse and Linhardt, 1992). Specific activity was calculated by dividing the number of micromoles of products produced per minute by the quantity of protein (mg) in the cuvette. Protein concentrations were determined using the Bradford assay and a BSA standard curve (Bradford, 1976).

Polyacrylamide gel electrophoresis SDS-PAGE was performed to determine apparent molecular mass according to the procedure previously described by Laemmli (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 solution.

Effect of NaCl concentration and pH on heparin lyase I activity The optimum pH for heparin lyase I activity was determined using a 50 mM sodium phosphate buffer at different pHs (5.8~8.0). Heparin lyase was individually incubated with 1 mg of heparin at each pH at 45ºC for 5 min. Enzyme activity was also examined at various salt concentrations (0~1.0 M), and enzyme activity was determined by reassuring absorbance increases at 232 nm.

Effect of temperature on heparin lyase I activity Temperature dependence of the enzyme was investigated by measuring enzyme activity at different temperatures (25~60ºC). Heparin lyase I was pre-incubated with 50 mM sodium phosphate buffer (pH 7.0) for 3 min at each temperature without substrate. The substrate was added to the mixture and activities were determined by measuring absorbance increases at 232 nm.

Substrate specificity of heparin lyase I on various substrates Various substrates were incubated with *Bacteroides* heparin lyase I under the optimal assay conditions as described above and each reaction was monitored at 232 nm. The substrates used were the following: heparin, heparan sulfate, 2-*O*-desulfated heparin, *N*-desulfated heparin, acharan sulfate, 2-*O*-desulfated acharan sulfate, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C. Some of reaction mixtures were analyzed by SAX-HPLC as described below.

SAX-HPLC analysis of the enzymatic reaction HPLC was performed to analyze the reaction products formed by treatment with heparin lyase I, which were injected on an analytical strong anion-exchange chromatography (SAX)-HPLC column to monitor the reaction. The system was equipped with an AKTA™ Purifier 10 controlled by Unicorn software 3.1 (Amersham Pharmacia Biotech., Uppsala, Sweden). The column was a 5 μm Phenosphere strong-anion exchange (SAX) column from Phenomenox (Torrells, USA) of dimension 0.46 × 25 cm. A linear NaC1 gradient of 0.1-1.6 M, pH 3.5, at a flow rate of 1.0 ml/min was used and detection was performed at 232 nm. Authentic unsaturated disaccharides (ΔUA2S-GlcNS6S, ΔUA-GlcNAc and ΔUA-GlcNS6S, 5 μg each) were also injected and their retention times were compared to identify reaction products.

Determination of kinetic constants The kinetic constants of *Bacteroidal* heparin lyase I were determined at 45ºC by measuring the initial rates of porcine intestinal mucosa heparin degradation as a function of heparin concentration in the range 3.57 to 357 μM under standard reaction conditions. Initial rates were measured by monitoring the increase of absorbance at 232 nm.

Results

Purification of heparin lyase I Heparin lyase I was purified to homogeneity by sequential chromatographic steps on DEAE-Sepharose, gel-filtration, hydroxyapatite, and CM-Sephadex C-50. Heparin lyase I passed through the DEAE-Sepharose column without binding to the matrix. The effluent was further purified to homogeneity using Bio-Gel P-100 column chromatography, hydroxyapatite column chromatography, and finally CM-Sephadex C-50 column chromatography (Fig. 1). The specific activity and total activity of each purification step are listed in Table 1. The molecular weight of heparin lyase I was determined to be 48,000 by SDS-PAGE (Fig. 2) and its isoelectric point was measured as 9.0 by IEF (data not shown). Because of a blockage at the *N*-terminus of the protein, the *N*-terminal sequence could not be obtained.

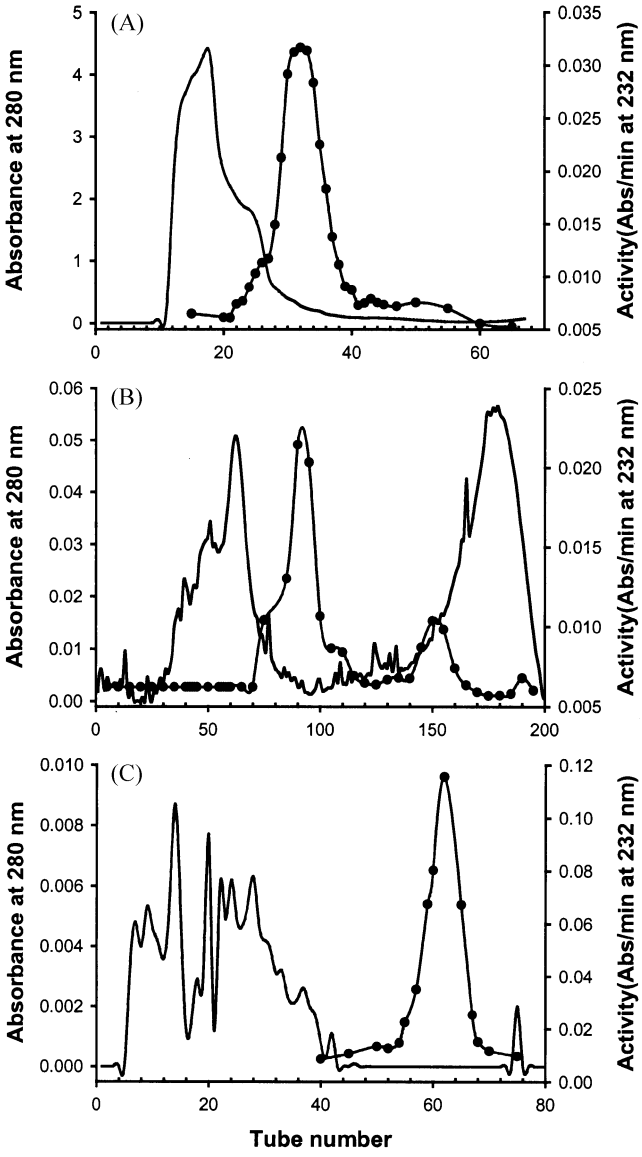

Fig. 1. Purification of heparin lyase I from *Bacteroides stercoris* HJ-15 by a combination of (A) Bio-Gel P-100; (B) hydroxyapatite; and (C) CM-Sephadex C-50 ion-exchange chromatography. Simple line, absorbance at 280 nm; solid circle, heparin lyase I activity.

Characterization of the optimal catalytic activity of heparin lyase I The optimal reaction conditions for *Bacteroidal* heparin lyase I were determined by a series of experiments. The optimal pH of heparin lyase I was

Table 1. Purification summary of heparin lyase I

Step	Total Protein (mg)	Total Activity* (U)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Crude extract	3818.4	49.6	6.5×10^{-3}	100	1.0
Bio-Gel P-100	41.4	9.8	2.4×10^{-1}	19.7	36.3
Hydroxyapatite	7.5	7.1	9.4×10^{-1}	14.2	144.0
CM-Sephadex C-50	0.033	0.5	16.0	1.1	2461.5

*One unit (U) was defined as the amount required to form 1 μmol of ΔUA product per min.

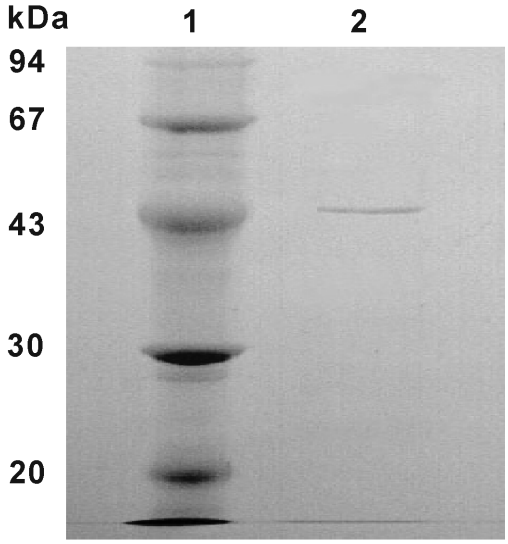

Fig. 2. SDS-PAGE of the purified heparin lyase I. SDS-PAGE was done using a 10% polyacrylamide gel at pH 7.0 using Lammlis method. The gel was stained with Coomassie brilliant blue R-250. Lane (1), molecular mass markers; lane (2), purified heparin lyase I.

determined to be 7.0 for heparin (Fig. 3B). Heparin lyase I was relatively stable within the range of 25 to 50°C but its activity was lost rapidly above 50°C (Fig. 4). Enzyme activity increased with increasing NaCl concentration. Maximal activity was obtained at 500 mM NaCl (Fig. 3A). The effect of salt concentration was marked compared to its effect on *Flavobacterial heparinase,* which has an optimum salt concentration of 100 mM. Enzyme activity was activated by Co^{2+} and by EDTA, but slightly inhibited by Pb^{2+}, Cd^{2+}, $Mg,^{2+}$ and Mn^{2+} (Table 3).

Substrate specificity of heparin lyase I The substrate specificity of heparin lyase I was examined using various HLGAGs as substrates. The degree of substrate breakdown was monitored by measuring the increase of absorbance at 232 nm. Heparin lyase I degraded heparin and heparan sulfate. When heparin lyase I activity for heparin was taken as 100%, heparin lyase I activity for heparan sulfate was 15% (Table 2), but heparin lyase I did not degrade any type of chondroitin sulfate or acharan sulfate or 2-*O*-desulfated acharan sulfate.

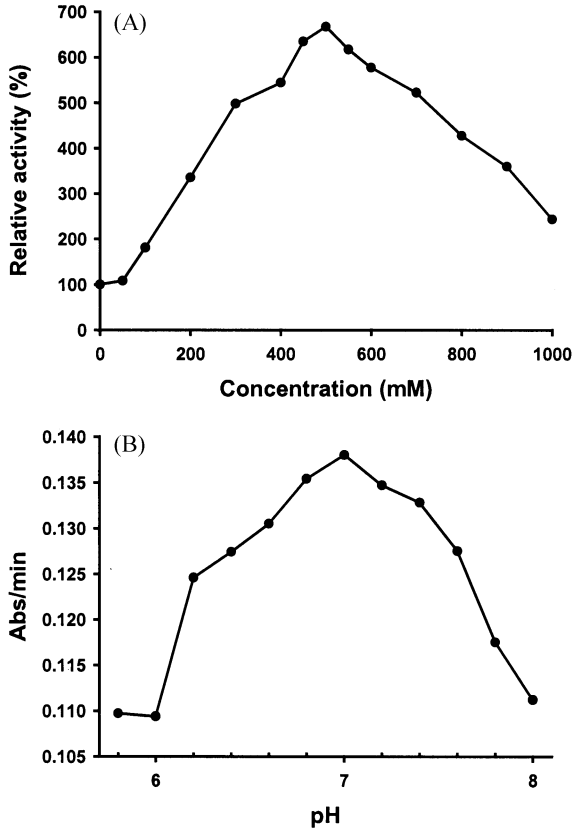

Fig. 3. Effect of NaCl concentration (A) and pH (B) on the activity of *Bacteroidal* heparin lyase I.

Bacteroidal heparin lyase I acts preferentially on the trisulfated sequence → 4)-α-D-GlcNS6S(1 → 4)-α-L-IdoA2S (1 →, comprising heparin affording the expected unsaturated trisulfated disaccharide (ΔUA2S-GlcNS6S) (Fig. 5A), and acted minimally on heparan sulfate, suggesting that it can also act on → 4)-α-D-GlcNS(1 → 4)-α-L-IdoA2S(1 → and → 4)-α-D-GlcNS6S (1 → 4)-α-L-IdoA2S(1 → linkages in heparan sulfate, respectively (Fig. 5B).

Kinetic constants of heparin lyase I Michaelis-Menten constants were determined using a Hanes plot. The K_m and V_{max} of heparin lyase I towards heparin were calculated as 1.3×10^{-5} M and 8.8 μmol/min · mg, respectively. The K_m value of the *Bacteroidal* heparin lyase I was similar to the apparent K_m (1.8×10^{-5} M) of *Flavobacterial* heparin lyase I

Table 2. Substrate specificity of heparin lyase I

Substrate	Relative activity (%)[a]	
	Bacteroidal heparin lyase I	*Flavobacterial* heparin lyase I
Heparin (porcine mucosa)	100	100
Heparan sulfate (porcine mucosa)	17	18
Heparan sulfate (bovine kidney)	15	30
2-*O*-desulfated heparin	26	34
N-desulfated heparin	0	0
Chondroitin sulfate A	0	0
Chondroitin sulfate B	0	0
Chondroitin sulfate C	0	0
Acharan sulfate	0	0

[a]The activity on heparin as substrate was taken as 100%.

Table 3. Effect of amino acid modifying reagents and divalent cations on heparin lyase activity

Inhibitors	Concentration (mM)	Residual activity (%)
Control	-	100
Co^{2+}	0.1	223
Cu^{2+}	0.1	114
Ni^{2+}	0.1	106
Ba^{2+}	0.1	111
Zn^{2+}	0.1	109
Pb^{2+}	0.1	94
Mg^{2+}	0.1	83
Ca^{2+}	0.1	99
Cd^{2+}	0.1	78
Mn^{2+}	0.1	91
EDTA	0.1	204
PMSF	0.1	93
Paraoxon	0.1	93
TPCK	0.01	104
Iodoacetic acid	0.1	153
Carbodiimide	0.1	73
Butanediol	0.1	90
p-Chloromercuriphenyl sulfonic acid	0.01	0
TLCK	0.01	16

(Yang *et al.*, 1985; Lohse and Linhardt, 1992), but the V_{max} value (219 μmol/min·mg) reported earlier for *Flavobacterial* heparin lyase I was approximately 24.8-fold higher than that measured for this enzyme.

Discussion

Heparin-degrading enzymes from *Flavobacterium heparinum* have been fully characterized. In the present paper, we purified and characterized a number of the biochemical properties of *Bacteroidal* heparin lyase I, and compared these

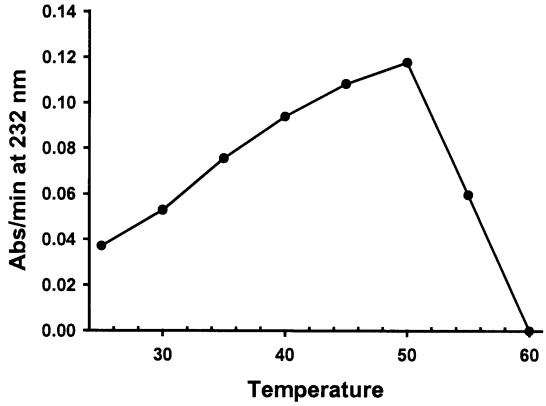

Fig. 4. Optimization of *Bacteroidal* heparin lyase I activity with respect to temperature.

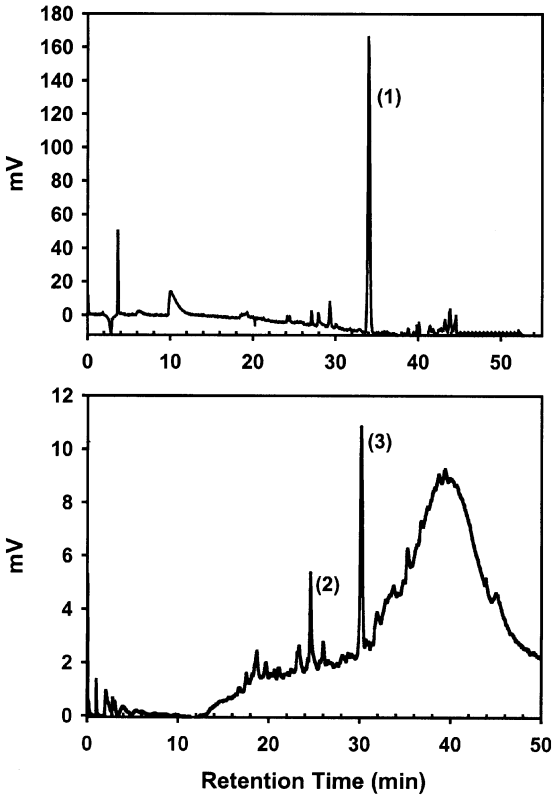

Fig. 5. Analysis of the reaction products using SAX-HPLC after incubating heparin and heparan sulfate with *Bacteroidal* heparin lyase I. Heparin and heparan sulfate were incubated with heparin lyase I in 50 mM phosphate buffer, pH 7.0 containing 500 mM NaCl overnight. The reaction mixture was then subjected to HPLC using a Phenosphere 5 μm SAX analytical (250 × 4.6 mm) column and a 0.1~1.6 M NaCl salt gradient in 16 column volumes. Elution was monitored at 232 nm. Panel (A) shows the product profile of heparin degradation by heparin lyase I. Peak (1) indicates heparin-derived unsaturated trisulfated disaccharide (ΔUA2S-GlcNS6S); panel (B) shows the product profile of heparan sulfate degradation by heparin lyase I. The two peaks (2) and (3) are unsaturated disulfated disaccharide (ΔUA2S-GlcNS) and trisulfated disaccharide (ΔUA2S-GlcNS6S), respectively.

Table 4. K_m and V_{max} values of heparin lyase I*

Substrates	Heparin lyase I	
	K_m (M)	V_{max} (μmol/min · mg)
Heparin (porcine mucosa)	1.3×10^{-5}	8.8
Heparan sulfate (porcine mucosa)	2.1×10^{-4}	7.4
Heparan sulfate (bovine kidney)	3.9×10^{-4}	11

*The kinetic constants of *Bacteroidal* heparin lyase I were determined at 45°C as described in Materials and Methods.

Table 5. Comparison of the biochemical properties of *Bacteroidal* heparin lyase I with *Flavobacterial* heparin lyase I

Properties	*Bacteroidal* heparin lyase I	*Flavobacterial* heparin lyase I*
Optimum pH	7.0	7.15
Isoelectric point (pI)	9.0	9.1 to 9.2
Molecular weight (Da)	48,000	43,000
Optimum temperature (°C)	50	35
Optimum salt concentratiom (mM)	500	100

*Reference: Yang *et al*., 1985; Lohse and Linhardt, 1992.

with those of *Flavobacterial* heparin lyase. The molecular weight of *Bacteroidal* heparin lyase I was 48,000 Da, which is higher than the 43,000 Da of *Flavobacterial* heparin lyase I. Their biochemical properties are compared and illustrated in Table 5. On the basis of these results *Bacteroidal* heparin lyase I differs from *Flavobacterial* heparin lyase I on several points, but the active sites and catalytic specificities of the two heparin lyases were found to be similar. The isoelectric points and optimum pHs were also not significantly different. However, the two differed in terms of the optimum temperature, optimum salt concentration, and kinetic constants. Moreover, *Bacteroidal* heparin lyase I was remarkably more stable at high temperature. In the activity inhibition test, serine protease inhibitors (PMSF and paraoxon) and a cysteine inhibitor (iodoacetic acid) had little effect on heparin lyase I activity. In contrast, another cysteine-directed reagent (*p*-chloromercuriphenyl sulfonic acid) significantly inhibited the enzyme. Reducing agents such as DTT (dithiothretol) and 2-mercaptoethanol enhanced its enzyme activity (data not shown). Interestingly, one trypsin inhibitor, TPCK, did not affect *Bacteroidal* heparin lyase I activity, while another related inhibitor, TLCK, significantly inhibited its activity. The *Flavobacterial* heparin lyase I has been reported to have cysteine, histidine and lysine residues at its active site (Godavarti and Sasisekharan, 1998). The results of our studies on *Bacteroidal* heparin lyase I suggest that cysteine residues play an important role in the activity of this enzyme.

In conclusion, we purified and characterized heparin lyase I from the human intestinal bacteria *Bacterides stercoris* HJ-15. The physicochemical properties of this enzyme were found to differ somewhat from those of *Flavobacterial* heparin lyase I. Heparin lyase I purified from *Bacteroides stercoris* HJ-15 may be a useful tool for neutralizing heparin overdoses and for the structural characterizations of heparin and heparan sulfate. Currently, the molecular cloning of *Bacterioidal* heparin lyases are underway.

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