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Rapid Purification, Characterization and Substrate Specificity of Heparinase from a Novel Species of *Sphingobacterium*

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A type of heparinase (heparin lysase, no EC number) was isolated from the periplasmic space of a novel species of *Sphingobacterium* by three-step osmotic shock. It was further purified to apparent homogeneity by a combination of SP-sepharose and Source 30S chromatographies with a final specific activity of 17.6 IU/mg protein and purification factor of 13-fold. MALDI-TOF mass spectrum of the purified heparinase gave a molecular mass of 75,674 Da of the native enzyme. Peptide mass spectrum showed poor homogeneity with the database in the peptide bank. Inhibition of the enzyme activity by N-acetylimidazole indicated that tyrosine residues were necessary for enzyme activity. K_m and V_{max} of the heparinase for de-*o*-sulfated-*N*-acetyl heparin were 42 μM and 166 μM/min/mg protein, respectively. The heparinase showed similiar activity on both heparin and heparan sulfate, except for the heparin from bovine lung. The heparinase exhibited only 8.3% of the activity when de-*N*-sulfated heparin was used as the substrate, but *N*-acetylation of the de-*N*-sulfated heparin restored the activity to 78.4%. Thus modification of *N*-site in heparin structure was favorable for heparinase activity. On the other hand, de-*o*-sulfation in heparin showed positive effects on the heparinase activity, since the enzyme activity for *N*-acetyl-de-*o*-sulfated heparin was increased by 150%. Based on the present findings, the sphingobacterial heparinase differed from flavobacterial and other reported heparinases in molecular mass, composition, charge properties, active site, substrate specificities and other important characteristics, suggesting that it a novel heparin lysase distinct from those from other sources.

Key words: heparinase, rapid purification, *Sphingobacterium* sp., substrate specificity.

Abbreviations: NAI, *N*-acetylimidazole; NBS, *N*-bromosuccinimide; DTT, *dl*-dithiothreitol; DEPC, diethylprocarbonate; EDC, 1-ethyl-3(3-dimethyaminopropyl) carbodimide; IEF, isoelectric focusing; HA, hydroxylapatite.

Heparin is a naturally occurring sulfated polysaccharide composed of a mixture of chains of different length, made up of alternating residues of uronic acid (L-iduronic acid, IdoA; D-glucuronic acid, GlcA) and a hexosamine (D-glucosamine). 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. A typical example is the specific pentasaccharide sequence of the active site for antithrombin (*1*). Moreover, heparin has been used for over 60 years as an anticoagulant. Its derivatives are being examined for a number of therapeutic applications, including anti-inflammatory, anti-viral, antiatherosclerotic, and regulation of cell growth (*2*). Some of these activities seemed to reside within the complex fine structure of heparin. Thus it is helpful to understand the molecular mechanism of heparin's biological functions. Structural studies on heparin have relied on spectroscopic, chemical, and enzymatic methods (*3*). Enzymatic methods for heparin and heparan sulfate depolymerization are specific and require mild conditions giving oligosaccharide products that closely resemble the glycosaminoglycans from which they were derived. Two types of enzymes that degrade heparin and heparan sulfate glycosaminoglycans are the polysaccharide lysases from prokaryotic sources that mainly act through an eliminative mechanism, and the glucuronidases (hydrolases) from eukaryotic sources that act through a hydrolytic mechanism (*4*, *5*). Several heparinases of prokaryotic origin have been purified and characterized from various species including *Flavobacterium heparinum* (*5*), *Bacillus* sp. BH 100 (*6*), *Prevotella heparinolyticus* (formerly known as *Bacteriodes heparino- lyticus*) (*7*), and *Bacteriodes stercoris* HJ-15 (*8*). Among heparinase-producing bacteria, *Flavobacterium heparinum*, a gram-negative soil bacterium, is the most intensively studied and its three heparinases have been purified, characterized, cloned and expressed (*5*, *9–13*).

The three heparinases from *Flavobacterium heparinum* are distinguished on the basis of their size, charge properties, substrate specificities and other properties (*14*). Heparinase I, a 42-kDa protein with a pI of 8.5–9.3, primarily cleaves HLGAGs at sites with an o-sulfated L-iduronic acid linkage (*i.e.*, heparin-like regions). Heparinase III, with a pI of about 10, requires primarily an unsulfated D-glucuronic acid moiety (heparan sulfate–

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like regions). Heparinase II is the largest of the heparinases and has the broadest substrate specificity. The 84-kDa protein has a pI of around 9 and cleaves both heparin and heparan sulfate–like regions of HLGAGs (*5*). Thus, unlike heparinase I and heparinase III, which distinguish between the C5 epimers of L-iduronic acid and D-glucuronic acid, heparinase II is catalytically active toward both (*14*). Extensive biochemical and site-directed mutagenesis revealed that three amino acid residues, cysteine 135, histidine 203 and lysine 199, are critical for enzymatic functions (*9*, *10*, *15*). Cysteine 348 and histidine 451 are both present in the active site of heparinase II (*16*).

A strain that could produce heparinase was isolated from effluent of a food mill and identified as a novel species of *Sphingobacterium* in our lab. Three heparinase components were isolated and the molecular mass were approximately 96.8, 68.0, and 70.1 kDa on SDS-PAGE. Primary tests showed that the 70.1 kDa heparinase could produce larger oligosaccharide fragments. This paper focuses on the rapid purification of the 70.1-kDa heparinase, its various properties compared with those of heparinase reported previously, and its substrate specificities for heparin-like and heparin-derived polysaccharides.

MATERIALS AND METHODS

Chemicals—Heparin (porcine intestinal mucosa) used for activity determination and cultivation of the strain was from Dongbao Biochemical Ltd (Yantai, China). Heparin-like polysaccharides and heparin-derived polysaccharides used for substrate specificity tests were the following: heparin (porcine intestinal mucosa), heparin (bovine lung), heparan sulfate (bovine lung), heparan sulfate (porcine intestinal mucosa), chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, hyaluronic acid, de-*N*-sulfated heparin, deaminated heparin, de-*N*-sulfated acetyl heparin, *N*-acetyl heparin, *N*-acetyl-de-*o*-sulfated heparin, and low-molecular-weight heparin (average molecular weight of 6,000), all were purchased from Sigma. SP-sepharose and Source-30S resins were obtained from Pharmacia. Protein marker was from Bio-Rad. BSA was obtained from Shanghai (China). Other reagents were either biological or analytical grade.

Strain and Cultivation—The strain used in this work was isolated from effluent of a food mill. It was short-rod shaped, gram-negative and aerobic. The cells showed shallow red-purple pigments. 16s rDNA sequence indicated that it was a novel species of *Sphingobacterium* (Accession number AF492000). The cultivation medium was as follows (g/liter): Na_2HPO_4 2.5, K_2HPO_4 2.5, NaCl 1.0, $MgCl_2$ 0.5, heparin 2.0, Yeast extract 4, tryptone 3, soya peptone 12.5, sucrose 20. pH was adjusted to 7.5–7.8 with NaOH. The organism was grown at 30°C for 40 h in 500-ml flasks containing 100 ml of medium.

Analytical Methods—Heparinase was assayed by observing the changes of A_{232nm}. In a 0.2 ml reaction volume, containing 1% heparin, 20 mM sodium phosphate buffer, pH 7.4, reaction was started by addition of appropriate enzyme. After incubation for 3min at 37°C, 1.8 ml 0.05 M hydrochloric acid was added to stop the reaction. Control was performed by adding 0.05 M hydrochloric acid before incubation. Activity was calculated from the change of absorbance/min using an extinction coefficient of 3,800 M^{-1} for products (1 IU = 1 μmol of ΔUA containing product formed/min) (*5*). The specific activity was calculated by dividing by the weight of protein in micrograms. Protein concentration was assayed by the Bradford assay using a bovine serium albumin standard curve (*17*).

Osmotic Shock Procedure—A modified three-step osmotic shock procedure was applied to isolate the heparinase according to Joseph *et al.* (*18*). About 21 g of cell paste was obtained from 1,000 ml of culture fluid and subjected to the following operations sequentially: (a) wash with 20 mM phosphate buffer, pH 7.4, twice with a total volume of 200 ml; (b) suspend in 200 ml of 20% sucrose in the above buffer; (c) suspend in 100 ml of 20 mM phosphate buffer, pH 7.4, (low-salt buffer); (d) suspend in 100 ml of 300 mM sodium chloride in 20 mM phosphate buffer, pH 7.4, (high-salt buffer). Cell pellets were removed by centrifugation (6,000 rpm, 20 min). All procedures were performed at 4°C. The supernatants from low-salt and high-salt buffer suspensions were combined as the crude enzyme for chromatographic purification after dialysis against 20 mM phosphate buffer, pH 7.4.

Chromatography—Both SP-sepharose and Source 30S resins were preequilibrited with 20 mM sodium phosphate buffer, pH 7.4. Crude enzyme was applied to the SP-sepharose column and eluted with a linear gradient of 0.12–0.18 M sodium chloride in the above buffer. Fractions with activity were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.4. This sample was then applied to the Source 30S column and eluted with a linear gradient of 0.05–0.15 M sodium chloride in the same buffer. All operations were done at 4°C unless otherwise mentioned.

Characterization of Purified Heparinase—Discontinuous SDS-PAGE was performed according to Laemmli's procedure (*19*). The enzyme was applied to 12% gel, stained with Coomassie Brilliant Blue R-250 solution, then destained with 7% acetic acid. Nonreducing SDS-PAGE was performed under the same conditions except that the protein was not treated with β-mercaptoethanol. To determine the molecular weight of the native enzyme by MALDI-TOF mass spectrometry, the heparinase was desalted exhaustively and concentrated to approximately 1 mg/ml protein by ultrafiltration. Sinapic acid was used as the matrix. Analysis was performed on BIFLEX III (Bruker). For peptide mass spectrum by MALDI-TOF mass spectrometry, the protein band was cut from the SDS-PAGE gel after Coomasie Blue R-250 staining and destaining. The sample was prepared according to the instructions provided with the Perspective Biosystem Voyager-DE™PRO (America). Amino acid composition was determined on the 2690 Automatic amino acid analyzer (Waters). To estimate the pI, a marker of the broad range pI marker kit (pH 3–10, Pharmacia Biotech) was loaded alongside the purified enzyme, electrophoresis was conducted on Flat Bed Apparatus FBE-3000 (Pharmacia Fine Chemicals).

To investigate the effects of different compounds on the activity, calcium chloride, sodium chloride, ammonium sulfate in the reaction system were tested at final concentrations of 0–1 mM, 0–0.6 M, and 0–0.7 M, respectively.

Table 1. **Extraction of heparinase activity from the periplasmic space of *Sphingobacterium* sp.** Heparinase activity at each step was assayed by the A_{232nm} method.

Treatment	Washing I	Washing II	20% Sucrose	Low-salt buffer	High-salt buffer
Volume (ml)	100	100	200	100	100
Total activity (IU)	2.4	0.4	2.1	16	25
Proportion in total activity (%)	5.3	0.9	4.6	35.2	54.1

Table 2. **Summary of purification of the sphingobacterial heparinase.** Heparinase activity in each step was assayed by the A_{232nm} method. The step "low–high salt osmotic shock" indicates the total activity from low salt buffer and high salt buffer.

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg protein)	Purification factor (fold)	Recovery (%)
Low-High salt osmotic shock	27.3	37.4	1.4	1	100
SP-sepharose chromatography	2.0	14.6	7.4	7	39.0
Source 30S chromatography	0.7	12.8	17.6	13	34.1

The final concentration of BSA was 1–7 mg/ml. For stability tests, the heparinase containing different stabilizers [0.1 M NaCl, 0.2 M $(NH_4)_2SO_4$, and 2 mg/ml BSA respectively] was stored at 4°C. Activities were determined every two days.

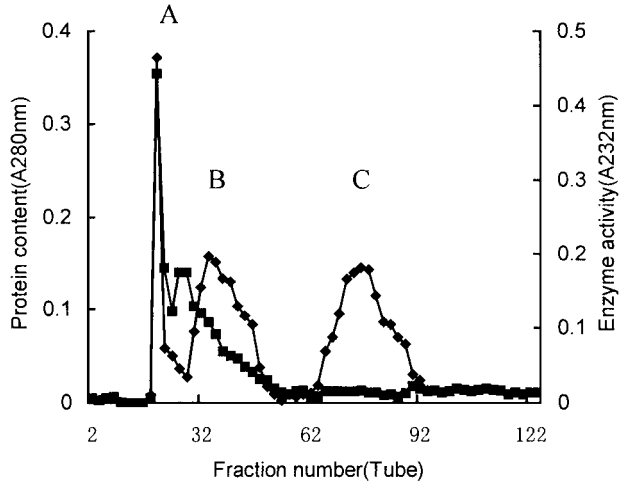


Fig. 1. **SP-sepharose chromatography of heparinase.** Squares represent protein content, diamonds represent enzyme activity.

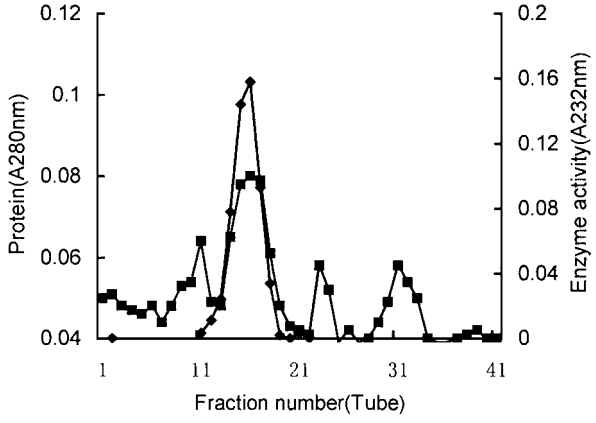


Fig. 2. **Source 30S chromatography of heparinase.** Squares represent protein content, diamonds represent enzyme activity.

In chemical modification tests, NAI, NBS, pCMB, PMSF, DEPC, and EDC were added to a final concentration of 1 mM in the reaction system, and 2-mercaptoethanol and DTT were added to 0.1 mM. NAI at a concentration of 0–5 mM was also used in the reaction system to observe its inhibition of the activity.

Denaturing effects of urea on the activity were investigated by adding urea to a final concentration from 0 to 2.8 M. For renaturing test, the partially or completely denatured enzyme was diluted sequentially by 0, 2, 4, 6, 8, 10, 20, and 30 times with 20 mM sodium phosphate, pH 7.4, and changes at A_{232nm} were monitored.

In substrate specificity tests on the heparinase, the substrates were dissolved in 20 mM phosphate buffer, pH 7.4. Other operations followed the analytical methods.

RESULTS

Purification of the Heparinase—Isolation and purification of the heparinase were conducted as described in "MATERIALS AND METHODS." Heparinase activity was selectively released from cell periplasm of the novel species of *Sphingobacterium.* by the three-step osmotic shock procedure. The enzyme was recovered mainly in the low-salt buffer and high-salt buffer. Loss of enzyme activity in the two washings was below 10% (Table 1). The crude enzyme collected from both buffers was applied to SP-sepharose chromatography. Three peaks were found containing activity (peaks A, B, and C) on the elution profile (Fig. 1). Primary tests showed that peak C degraded heparin to larger fragments, which was helpful to understand the fine structure and biological function of heparin (unpublished data). Thus peak C was pooled and further purified by Source 30S chromatography (Fig. 2). The purification results was summarized in Table 2. Specific activity increased from 1.4 to 17.6 IU/mg protein with the recovery of 34.1%, and the purification factor was 13-fold.

properties of the Heparinase—The heparinase was apparently homogeneous on SDS-PAGE (Fig. 3) with the estimated molecular mass of 70.1 kDa. Nonreducing SDS-PAGE revealed the same banding pattern suggesting that no subunits were present. MALDI-TOF mass spectrometry gave three characteristic peaks of the hepa-

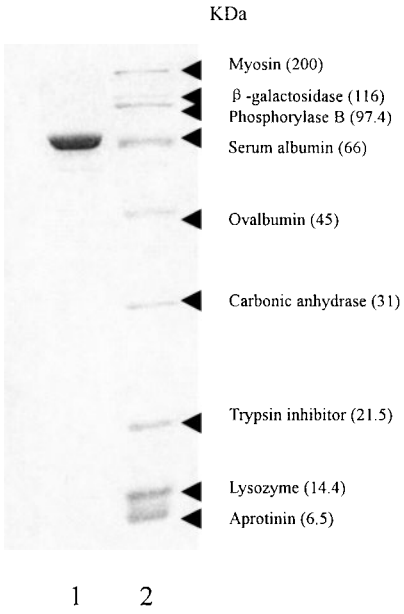


Fig. 3. **SDS-PAGE of the purified heparinase.** Lane 1 was the heparinase sample after Source 30S chromatography. Lane 2 was protein marker.

rinase (Fig. 4), 75,674, 37,894, and 25,346, showing the molecular mass of 75,674 Da, which was in accordance with that measured by SDS-PAGE, meaning a monomer of the native heparinase. It was close to bacteroidal heparinase (70 kDa) and flavobacterial heparinase III (70,800 Da), but substantially different from the heparinase II (84,100 Da) and heparinase I (42,800 Da) from *Flavobacterium heparinum* (*5*, *8*).

Table 3. **Amino acid composition analysis of the sphingobacterial heparinase.** Amino acid composition for sphingobacterial heparinase was determined under the conditions described in "MATERIALS AND METHODS," and expressed as mol%. Asx and Glx indicate the sum of asparagine and aspartic acid, and glutamine and glutamic acid, respectively. Trp was not determined.

Amino acid	pmol	mol%
ASX[1]	224.94	9.30
SER	167.06	6.91
GLX[1]	142.13	5.88
GLY	280.79	11.61
HIS	68.13	2.82
ARG	146.71	6.06
THR	179.12	7.40
ALA	237.19	9.81
PRO	189.69	7.84
CYS	5.86	0.24
TYR	61.53	2.54
VAL	180.05	7.44
MET	12.25	0.51
LYS	97.80	4.04
ILE	131.54	5.44
LEU	192.43	7.95
PHE	101.81	4.21
TRP	N.D.	0.00
Total	2,419.02	100.00

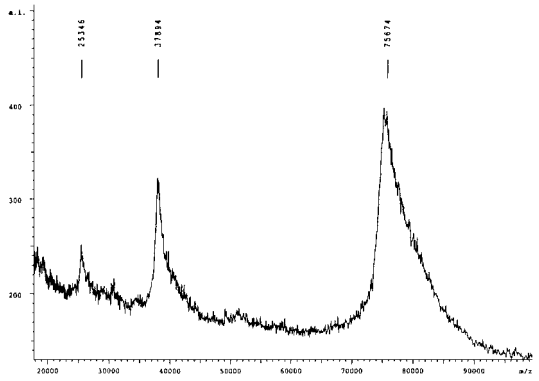


Fig. 4. **MALDI-TOF spectrum of the purified heparinase.**

The amino acid composition of the heparinase is shown in Table 3. Compared with flavobacterial heparinase II, it had similiar individual amino acids, including high proportions of glycine and alanine, and a similar proportion of cysteine, which was not found in flavobacterial heparinase III. The proportion of lysine in sphingobacterial heparinase was lower than in heparinases from other sources. Its pI value was 9.2–9.6.

The heparinase was hydrolysed with trypsin and analysed by MALDI-TOF mass spectrometry (Fig. 5). A series of peptide mass spectra was obtained ranging mainly from 610 to 2,916. MS-Fit search results showed that it has poor homology with the database in the peptide bank. The most homologous peptides were a yeast protein-tyrosine phosphatase 2 (19%) [EC 3.1.3.48], phosphoenlopyruvate carboxykinase (19%) [EC 4.1.1.49] and ATP-dependent protease LA2 (19%) [EC 3.4.21.53].

Michaelis-Menten constants of the heparinase were determined with de-*o*-sulfated-*N*-acetyl heparin as substrate. The values of K_m and V_{max} were 42 μM and 166 μM/min/mg protein respectively.

In the presence of a final concentration of 0.6 mM calcium chloride, 0.2 M sodium chloride, 0.2 M ammonium sulfate or 1 mg/ml BSA, the activity of the heparinase increased by 134.7, 112.6, 130, and 187.6%, respectively. In addition, the heparinase remained in an activated state over a broad final concentration range of $(NH_4)_2SO_4$

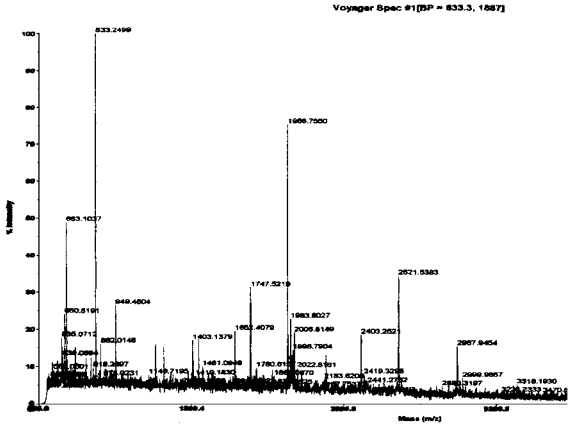
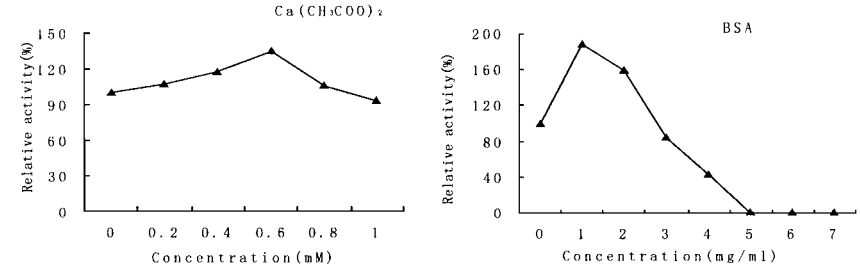


Fig. 5. **Peptide mass spectrum of the purified heparinase after hydrolysis by trypsin.**



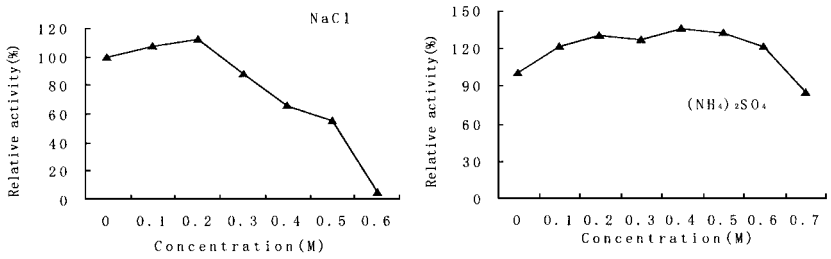


Fig. 6. **Effects of some chemicals on the heparinase activity.**

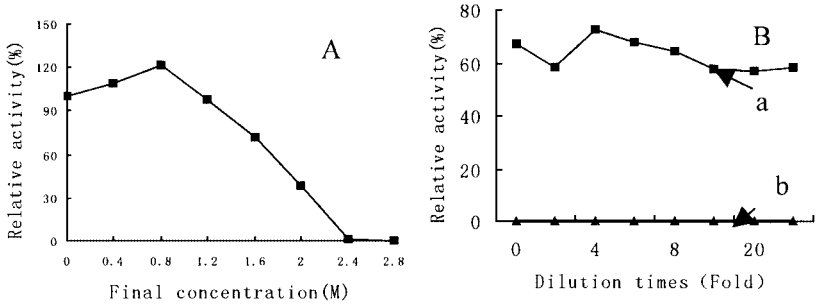


Fig. 7. **Denaturation and renaturation tests on the heparinase.** A. Denaturation; B. Renaturation under the condition of partial-denaturation (a) and complete-denaturation (b).

from 0.1 to 0.6 M (Fig 6). About 55% of residual activity was detected when the concentration of sodium chloride was increased to 0.5 M. The heparinase was unstable when stored at 4°C. Addition of BSA and $(NH_4)_2SO_4$ increased the stability greatly with about 130% and 106% retention of activity after 20 days storage at 4°C. Sodium chloride could also partially retain the activity.

The effect of urea on the heparinase activity was also investigated (Fig. 7). Curiously, urea at concentrations up to 1 M activated the enzyme. The activity was inactivated completely at the final concentration of 2.4 M. Reactivation was not observed after either partial or complete inactivation.

Table 4. **Effects of amino acid modifying reagents on the heparinase activity.** Heparinase activity was determined under the conditions described in "MATERIALS AND METHODS." The activity on heparin from porcine intestinal mucosa was taken as 100%.

Modifying reagents	Final concentration/mM	Residual activity/%
NAI	1	22.0
NBS	1	69.0
pCMB	1	75.5
2-mercaptoethanol	0.1	94.4
DTT	0.1	97.8
PMSF	1	74.1
DEPC	1	89.6
EDC	1	100.9
Control	/	100

NAI, *N*-acetylimidazole; NBS, *N*-bromosuccinimide; DTT, *dl*-dithiothreitol; DEPC, diethylprocarbonate; EDC, 1-ethyl-3(3-dimethyaminopropyl) carbodimide.

The effects of amino acid modifiers on the activity are shown in Table 4 and Fig. 8. NAI inhibited the activity greatly, indicating the necessity of tyrosine residues in the active site. NBS, pCMB, and PMSF resulted in moderate loss of the activity, while the histidine-directed reagent DEPC produced little inhibition. Other reducing agents such as 2-mercaptoethanol and DTT did not affect the enzyme activity.

Substrate Specificity of the Heparinase—The substrate specificity of sphingobacterial heparinase was examined using various heparin-like polysaccharides and heparin-derived polysaccharides. The degree of breakdown of the substrates was monitored by measuring the increase in the absorbance at 232 nm, and the absorbance for heparin (porcine intestinal mucosa) was taken as 100%. Results are summarized in Table 5. The purified heparinase was equally active on the heparin from porcine intestinal mucosa and heparan sulfates from both porcine intestinal mucosa and bovine lung, but less active on the bovine lung heparin. The heparinase showed 2.0% and 1.0% activity on chondroitin sulfate B and chondroitin sulfate C, respectively, which might be caused by slight contamination of the chondroitin sulfate lysase B and C. No activities were observed on chondroitin sulfate A, colominic acid and hyaluronic acid.

The heparinase activity decreased by 27.6% for low-molecular-weight heparin (average molecular weight

Table 5. **Activity of the heparinase toward heparin-like and heparin derived polysaccharides.** Heparinase activity was determined under the conditions described in "MATERIALS AND METHODS." The activity on heparin from porcine intestinal mucosa was taken as 100%.

Heparin-like polysaccharides	Relative activity (%)	Heparin-derived polysaccharides	Relative activity (%)
Heparin (porcine)	100	*N*-Acetyl-heparin	78.4
Heparin (bovine)	58.3	Deaminated heparin	53.8
Heparan sulfate (porcine)	97.0	de-*N*-Sulfated heparin	8.3
Heparan sulfate (Bovine)	91.6	de-*N*-Sulfated acetyl heparin	108.3
Hyaluronic acid	0	*N*-Acetyl-de-*o*-sulfated heparin	250
Chondroitin sulfate A	0	Heparin (low molecular weight 6,000)	72.4
Chondroitin sulfate B	2.0		
Chondroitin sulfate C	1.0		
Colominic acid	0		

6,000) as substrate. The heparinase exhibited only 8.3% of the activity when de-*N*-sulfated heparin was used as the substrate, but the activity toward *N*-acetyl of the de-*N*-sulfated heparin recovered to 78.4%. de-*N*-Sulfated acetyl heparin, closely related to natural heparan sulfate in its *N*-acetyl content, was thus a better substrate for heparinase. Thus modification of the *N*-site in heparin structure was decisive for heparinase activity. On the other hand, heparinase showed high activity for *N*-acetyl-de-*o*-sulfated heparin, indicating the positive effect of de-*o*-sulfation on the heparinase activity.

DISCCUSSION

Heparin lysases are a kind of cell-associated enzymes, probably residing in the periplasmic space. They are generally released by sonication, resulting in a complex procedure to resolve and purify the different components of the enzymes (*5*, *20*, *21*). This study adopted the three-step osmotic shock to isolated heparinase from the periplasmic space of *Sphingobacterium* without causing leakage of the cytoplast. After the osmotic shock treatment, specific activity of the heparinase increased remarkably (unpublished result), allowing for rapid purification with two steps of chromatography.

Reducing agents such as DTT and 2-mercaptoethanol have been reported to enhance the enzymatic activity of heparinases from different sources (*5–8*), but PCMB, 2-mercaptoethanol and DTT did not show such effects on the heparinase in this study. Since it was hypothesized that heparinase required a cysteine to depolymerize heparin and no cysteine was necessary for the depolymerization of the heparan sulfate, it is understandable that cysteine was not crucial in sphingobacterial heparinase activity. Another amino acid residue, histidine was identified to be critical for the catalytic activity of flavobacterial heparinase II. In contrast, the histidine residue was not important in sphingobacterial heparinase, since the histidine-directed reagent DEPC produced little inhibition of the enzyme. However, tyrosine residues were shown to be necessary for the active site of the sphingobacterial heparinase, which was not found in previous work on the active site of heparinases.

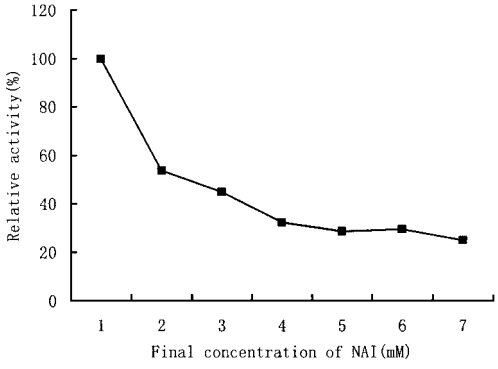


Fig. 8. **Modification of heparinase by NAI.**

The substrate specificity of sphingobacterial heparinase was investigated using both heparin-like and heparin-derived polysaccharides. The results indicated that it had broad substrate specificity on both heparin and heparan sulfate, just like flavobacterial heparinase II and bacteriodal heparinase, but they showed different catalytic levels (*8*, *22*). Another semi-synthetic heparin derived polysaccharide, de-*N*-sulfated acetyl heparin, was closely related to natural heparan sulfate in its *N*-acetyl content, and was also a better substrate. Modification of the *N*-site in heparin structure was decisive for sphingobacterial heparinase activity. *N*-Acetyl-de-*o*-sulfated heparin seemed to be the best substrate for the heparinase, indicating the positive effect of de-*o*-sulfation on heparinase activity.

Our studies showed that several characteristics of sphingobacterial heparinase resembled those of flavobacterial heparinase II, but the two enzymes differed in molecular weight, peptide mass spectrum, active site and tolerance to salt concentration.

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