Purification and Characterization of a Novel Heparinase*

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A unique heparinase was isolated from a recently discovered Gram-negative soil bacterium. The enzyme (heparinase III) was purified by hydroxyapatite chromatography, chromatofocusing, and gel permeation chromatography. The enrichment was 48X, and the specific activity of catalytically pure heparinase was 127 IU/mg of protein. Similar to the heparinase I from Flavobacterium heparinum, heparinase III also degrades heparin to mainly disaccharide fragments. It is specific for heparin and also breaks down heparan sulfate, but not hyaluronic acid and chondroitin sulfate. Heparinase III is quite different from that of heparinase I.

Enzymes which degrade heparin find a wide range of applications. They may be used to characterize the structure (1-5) or endogenous activity (6-9) of heparin and related compounds. There is also great interest in the preparation of antagulant reagents with less side effects (10-17) and as antitumor reagents (18, 19). Heparinase is furthermore used to normalize prothrombin and thromboplastin times of heparin-containing plasma samples (20). Recently, heparinase has also been reported the isolation of a novel Gram-negative soil bacterium (26). Production of the enzyme by this organism requires the inclusion of heparin as an inducer (27-29), at fairly high costs for the commercial available heparinase is prepared from Flavobacterium heparinum (26). Production of the enzyme by this organism requires the inclusion of heparin as an inducer (27-29), at fairly high costs.

**EXPERIMENTAL PROCEDURES**

**Materials**

Sigma was the supplier of heparinase I (from F. heparinum), heparin (sodium salt, from porcine intestinal mucosa), heparan sulfate (sodium salt, from bovine kidney), hyaluronic acid (potassium salt, from human umbilical cord), chondroitin sulfate A (sodium salt, from whale cartilage), PVS* (potassium salt), GlcNAc, p-nitroacetanilide, sodium phosphate, 1 mM mercaptoethanol, pH 7.0. Five ml of cell suspension was sonicated on ice for 8 x 15 s with a MSE Soniprep 150 at maximum power (2±0 kHz) with a 45-s cooling after each pulse and 4-min cooling after the pulse.

**Cultivation of the Organism—Isolate 114 was generously supplied by J. J. Joubert (Dept. of Medical Microbiology, University of Stellenbosch, Stellenbosch, South Africa). The organism was stored at room temperature on agar slants which contained the following per liter of water: 28 g of trypticase soy broth, 8 g of glucose, 1 g of yeast extract, 1 g of NaCl, 0.5 g of MgCl2, and 12 g of agar. This medium was autoclaved for 15 min at 121 °C. The bacteria were transferred to fresh media every 14 days. Suspension cultures were grown in a 10-liter fermentor (Magnaferm, New Brunswick Scientific Co.) with a medium containing the following per liter: 10 g of casein peptone, 19 g of sucrose, 1 g of yeast extract, 1 g of NaCl, 0.5 g of MgCl2, and 0.2 ml of antifoaming agent. The medium (sucrose separately) was sterilized at 121 °C for 2.5 h. Inocula (10% v/v) of exponential phase cells were used. Cultures were incubated at 30 °C, 180 rpm, for 18 to 30 h with an aerating rate of 100% (80% O2, 20% N2). Growth was measured by turbidity at 640 nm with a Bausch and Lomb Spectronic 20 colorimeter. Cells were harvested by centrifugation in a Beckman J-6 centrifuge with a J-2.2 rotor at 1,570 x g for 90 min at 4 °C and washed with 10 mM sodium phosphate, 1 mM mercaptoethanol, pH 7.0, and sedimented on a Beckman J-26 centrifuge with rotor 30 at 15,000 x g for 15 min at 4 °C.

**Preparation of the Crude Extract—Washed cells were resuspended to 0.2 g of cells/mL of buffer in 10 mM sodium phosphate, 1 mM mercaptoethanol, pH 7.0. Five ml of cell suspension was sonicated on ice for 8 x 15 s with a MSE Soniprep 150 at maximum power (2±0 kHz) with a 45-s cooling after each pulse and 4-min cooling after the pulse.

*Portions of this paper (including parts of "Experimental Procedures" and "Results" and Figs. I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

† The abbreviations used are: PVS, polyvinyl sulfate; SDS, sodium dodecyl sulfate; GPC, gel permeation chromatography; PAGE, polyacrylamide gel electrophoresis; SC, Sephadex G-25; CF, chromatofocusing.
FIG. 1. Purification profile of crude extract on hydroxylapatite. Crude extract (4.67 g of protein in 432 ml of 10 mM sodium phosphate, 1 mM mercaptoethanol, pH 7.0; 60.5 units/mg of protein) was loaded onto a hydroxylapatite column (Pharmacia C26/40) pre-equilibrated with the same buffer. The flow rate was 27 ml/h, and 30-min fractions were collected. Heparinase activity (ΔA280/min > 0.2) was found in the shaded area.

FIG. 2. SDS-PAGE of active fractions. a, HA2; b, SC2.2 (+dithiothreitol); c, SC2 (-dithiothreitol); d, standards; e, heparinase I; f, CF4; g, crude extract.

Table I
Summary of heparinase III purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Milligrams of protein</th>
<th>IU</th>
<th>IU/mg of protein</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4,665.6</td>
<td>13,996.8</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>HA2</td>
<td>594.0 (12.7)</td>
<td>9,028.8 (64.5)</td>
<td>15.2</td>
<td>5.0</td>
</tr>
<tr>
<td>CF4</td>
<td>80.0 (1.7)</td>
<td>5,272.0 (37.7)</td>
<td>65.9</td>
<td>24.0</td>
</tr>
<tr>
<td>SC2</td>
<td>29.4 (0.6)</td>
<td>3,722.0 (26.6)</td>
<td>156.6</td>
<td>47.0</td>
</tr>
<tr>
<td>SC2.2</td>
<td>27.0 (0.6)</td>
<td>3,439.8 (24.6)</td>
<td>127.4</td>
<td>47.5</td>
</tr>
</tbody>
</table>

Protein Assay—Protein concentrations were measured according to the method of Bradford (31).

Enzyme Assays—Heparinase activity in the crude extract and hydroxylapatite-purified fractions was determined from the disappearance of heparin (1 unit = 1 mg of heparin degraded/h). The Azure A assay of Galliher et al. (27) was adapted for microdeterminations with the Titertek Multiskan MC (see Miniprint Supplement). Purer preparations (after chromatofocusing and GPC) were assayed by the increase in absorbance of heparin at 232 nm (32). One IU is defined as the amount of enzyme which causes 1 pmol of 4,5-uranic acid to be formed/min, based on a molar extinction coefficient of 5.1 × 10^5 M⁻¹ cm⁻¹ at 232 nm (33). The procedure of Linhardt et al. (32) was modified for direct determination of initial rates in a temperature-controlled spectrophotometer (see Miniprint Supplement). Heparanase, chondroitinase, and hyaluronidase activities were determined similarly, using heparan sulfate, chondroitin sulfate, and hyaluronic acid, respectively, as substrates. Glycuronidase activity was measured by the decrease of absorbance at 232 nm using heparin fragments (prepared by heparinase digestion as described below) as substrate. Sulfatase activity was assayed by the procedure of Dodgon and Spencer (34) using p-nitrocatechol as substrate.

Polyacrylamide Gel Electrophoresis—SDS-PAGE was carried out on 10-15% gels with a Phast System according to the procedure of Olsson et al. (35, 36).

Isoelectric Focusing—The pI of purified heparinase III was established by isoelectric focusing using a Pharmacia FBE-3000 apparatus according to the directions of the manufacturer.

Amino Acid Analysis—The amino acid composition of purified...
heparinase III was determined after HCl hydrolysis by reversed phase high performance liquid chromatography with a Waters Pico-Tag System using pre-column derivatization with phenylisothiocyanate according to the method of Bidlingmeyer et al. (37). Tryptophan, which is destroyed by HCl hydrolysis, was determined spectrophotometrically according to the procedure of Edelhoch (38), as well as chromatographically after hydrolysis with methanesulfonic acid by the method of Simpson et al. (39). Cysteine was determined after alkylation with [3H]iodoacetic acid according to the procedure of Anfinsen and Haber (40) (see Miniprint Supplement). Corrections were made for the destruction of Ser, Thr, and Tyr.

N-Terminal Analysis—The N-terminal amino acid was determined by the Edman technique (41, 42) with a spinning cup Beckman apparatus (Model 890) converted to a vapor liquid solid phase instrument according to the method of Brandt et al. (43). The phenylthiohydantoin amino acid was identified on a Hewlett-Packard high performance liquid chromatography instrument (Model 1084B) and a 3.9 x 300-mm µBondapack C18 column, using a gradient of 8-58% methanol in 0.01 M NaAc, pH 4.5, at a flow rate of 1.5 ml/min.

Preparation of Antibodies—A New Zealand white rabbit was immunized intramuscularly with an emulsion (2:1) of complete Freund's adjuvant and highly purified heparinase (1.5 mg/ml phosphate-buffered saline). Three boosters (containing incomplete instead of complete Freund's adjuvant) were administered intramuscularly, intradermally, or subcutaneously at 14-day intervals thereafter. The total amount of heparinase injected ranged from 70 to 160 µg. The rabbit was bled from the ear on day 69, and the blood was stored overnight at 4 °C to clot. The serum was decanted, centrifuged at 2500 rpm for 15 min, and stored at -30 °C.

Immunodiffusion—Immunodiffusion was carried out in agar (1 g/100 ml phosphate-buffered saline, 0.05% Tween 20, pH 7.0) according to the procedure of Ouchterlony and Nilsson (44). The plates were
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FIG. 5. Rechromatography of SC2 on Sephacryl S200 sf. SC2 (4.8 mg of protein in 1 ml of phosphate-buffered saline, 1 mM mercaptoethanol, pH 7.0; 97.4 IU/mg of protein) was rechromatographed under reducing conditions on a Sephacryl S-200 sf column (Pharmacia C26/100) equilibrated with phosphate-buffered saline, 1 mM mercaptoethanol, pH 7.0. The flow rate was 8 ml/h, and fractions of 15 min were collected.

RESULTS

Purification—Heparinase III was enriched 5x with respect to the crude extract by hydroxylapatite chromatography, the first purification step (Fig. 1 and Table I). The complex mixture of proteins in the crude extract (Fig. 2a) was thereby reduced to about 25 main components (Fig. 2a), with proteins of $M_r = 94,000$ and 72,000 being the most prominent. Further purification was achieved by chromatofocusing with PBE 118 (Fig. 3). Heparinase activity was found in the major component, CF4, which eluted at a pH of 9.2. This suggests that the $p_I$ of heparinase III may be significantly higher than that of heparinase I (8.5, Ref. 45). Besides heparinase activity, CF4 also contained heparitinase, chondroitinase, and hyaluronidase activity. Sulfatase activity was found in CF1 while glycuronidase activity was observed in CF8. The presence of

![Fig. 6. Isoelectric focusing of heparinase III (a) and standards (b).](image)

Free protein was removed by five successive washes with phosphate-buffered saline at room temperature over 48 h. Immunoprecipitin lines were stained with 0.5% Paragon blue, 5% acetic acid for 1 h at room temperature. The gel was destained at room temperature with 5% acetic acid, 30% methanol, 20% acetic acid, and 5% acetic acid (each for 1 h).

Characterisation of Heparin Fragments—Heparin (2.5 mg in 0.1 M sodium phosphate, 1 mM mercaptoethanol, pH 7.0) was digested by either heparinase I or III (both at 0.025 IU) for 96 h at 30 °C. The reaction was stopped by acidifying with acetic acid. Heparin fragments were separated on a Sephadex G-25 column (Pharmacia C16/100) equilibrated and eluted with 0.5 M NH$_4$Ac.

![FIG. 6. Comparison of the amino acid compositions of heparinase I and III](image)

### TABLE II

Comparison of the amino acid compositions of heparinase I and III

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Heparinase III</th>
<th>Nearest integer</th>
<th>Mole percent</th>
<th>Heparinase I (44)</th>
<th>Nearest integer</th>
<th>Mole percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>106</td>
<td>12.1</td>
<td>52</td>
<td>12.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>61</td>
<td>6.9</td>
<td>41</td>
<td>10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>79</td>
<td>9.0</td>
<td>25</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>96</td>
<td>10.9</td>
<td>35</td>
<td>8.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>12</td>
<td>1.4</td>
<td>11</td>
<td>2.7</td>
<td></td>
<td></td>
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<tr>
<td>Arginine</td>
<td>24</td>
<td>2.7</td>
<td>10</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>37</td>
<td>4.2</td>
<td>23</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>101</td>
<td>11.5</td>
<td>50</td>
<td>12.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>42</td>
<td>4.8</td>
<td>21</td>
<td>5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>26</td>
<td>3.0</td>
<td>16</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>48</td>
<td>5.5</td>
<td>15</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>16</td>
<td>1.8</td>
<td>3</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1</td>
<td>&lt;0.1</td>
<td>4</td>
<td>0.9</td>
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<td></td>
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<tr>
<td>Isoleucine</td>
<td>40</td>
<td>4.6</td>
<td>16</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>70</td>
<td>8.0</td>
<td>28</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>31</td>
<td>3.4</td>
<td>20</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>70</td>
<td>8.0</td>
<td>37</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>18</td>
<td>2.1</td>
<td>ND*</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>878</td>
<td>100</td>
<td>407</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND, not determined.
more than one protein in CF4 was also demonstrated by SDS-PAGE (Fig. 2f). Bands were found at $M_r = 94,000$ (main band), 72,000, 49,000, and 14,000. Significantly, no band was seen at 43 kDa, the size of heparinase I. These proteins were separated on Sephacryl S-200 (Fig. 4). Heparinase activity coincided with the high molecular mass fraction SC2. Chondroitinase and hyaluronidase activity were found in SC3, while heparanase activity was associated mainly with SC3 but to a lesser extent also with SC2. After rechromatography of SC2 under reducing conditions (Fig. 5), more than 90% of the material was found in a symmetrical peak SC2.2, with the same $K_m$ value as SC2 in Fig. 4 and corresponding to a molecular mass of 93.9 kDa. SDS-PAGE of SC2 and SC2.2 gave similar results: purified heparinase (SC2.2) showed a single band (Fig. 2c) with an electrophoretic mobility corresponding to a molecular mass of 94 kDa. The reducing agent, dithiothreitol, did not affect the migration position (compare Fig. 2, b and c). These results indicate that heparinase III does not possess interchain disulfides and is most likely a single chain protein with a molecular mass of 94 kDa. This is about twice the size of heparinase I. Heparinase III was purified 48-fold over the crude extract to a specific activity of 127 IU/mg of protein (Table I), which is substantially higher than the specific activity of purified heparinase I (26.6 IU/mg of protein, Ref. 45). Recovery of protein (0.6%) and enzyme activity (18.9%) were also higher than the values determined for heparinase from F. heparinum (0.003% and 0.8%, respectively, Ref. 45).

Isoelectric Focusing—Purified heparinase III focused at pH 9.2 (Fig. 6), which is in agreement with the pH determined by chromatofocusing (Fig. 3). This value differs from the pH of 8.5 of heparinase I (45).

Amino Acid Analysis—The amino acid compositions of heparinase I and III are compared in Table II and differ significantly, confirming that heparinase III is not a dimer of heparinase I. The lower content of the basic amino acids, lysine and arginine of heparinase III (10.7 mol%), in comparison to heparinase I (11.5 mol%, Ref. 45) was noticeable. On the other hand, the former contains less acid and acid amide amino acids: 19.0 versus 22.9 mol% (45), which may contribute to the higher pH of heparinase III versus I (see above).

The low content of sulfur-containing amino acids in hepa-
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Effect of ionic strength, pH, and temperature on heparinase III activity. Initial rates were measured with the ΔAννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννννν

FIG. 9. Effect of ionic strength, pH, and temperature on heparinase III activity. Initial rates were measured with the ΔAννννννννννννννννννννννννννννν

condition varied between 0 and 0.223 M in 50 mM sodium phosphate, 1 mM mercaptoethanol, pH 7.6. I, citrate-phosphate-borate buffer, pH 5.3 to 9.7 was used. Δ, Temperatures ranged from 5–65°C.

Fig. 10. Immunodiffusion plate. Well a contains rabbit antiserum against heparinase III (20 µl undiluted); well b, heparinase III (108 µg); well c, heparinase I (4.4 µg); and well d, heparinase III (8.6 µg).

Heparinase III was conspicuous. Only one cysteine/enzyme could be determined after alklylation with [3H]iodoacetic acid (see Miniprint Supplement). Heparinase I, on the other hand, contains 4 residues of cysteine per molecule (45). Good agreement was found between the Trp determinations obtained by methanesulfonic acid hydrolysis (17.5 residues/molecule of protein) and spectroscopy (19 residues/molecule of protein).

The molar (ε) and specific (Ε 0.1%) extinction coefficients of heparinase III at 280 nm were calculated from the observed Trp and Tyr contents and were 1.36 × 10⁵ M⁻¹ cm⁻¹ and 1.44, respectively.

N-Terminal Analysis—Just one type of N-terminal amino acid, viz. alanine, was found after Edman degradation. Results from SDS-PAGE, GPC, and amino acid analysis had already shown that heparinase III is not a disulfide-bonded dimer of heparinase I. Covalent linkage of two subunits by, e.g. Lys and Glu, as is found in cross-linked fibrin (46), has not been ruled out. However, if this were the case, heparinase III most likely would have shown two different N-terminal amino acids. The results therefore strongly indicate that heparinase III is a single chain protein.

Kinetics and Substrate Specificity—The kinetic constants of heparinase III were determined by Lineweaver-Burk analysis (Fig. 7). The Kₐ for heparin was found to be 3.4 µM and the Vₐₐₐₐₐ as 36.8 µmol/min at a total enzyme concentration of 0.18 µg/ml. The corresponding values reported for heparinase I activity were 8.0 µM and 98.5 µmol/min, respectively, at an enzyme concentration of 0.5 µg/ml. Heparinase III thus shows a greater affinity toward heparin than heparinase I. The kₐ value of heparinase I (4.23 × 10⁻⁵ min⁻¹, Ref. 45) is also lower than that of heparinase III (1.93 × 10⁻⁴ min⁻¹). This is in agreement with the higher specific activity of heparinase III (127.4 versus 26.6 IU/mg of protein).

Heparinase III was very specific, acting only on heparin and to a lesser extent on heparan sulfate (11% of the activity on heparin), but not on chondroitin sulfate nor on hyaluronic acid. The latter two, as well as GlicNAc, also did not inhibit heparin degradation when present at 22 mg/100 ml PVS, on the other hand, was found to be a competitive inhibitor of heparinase III activity. The Kᵢ was determined to be 1.7 × 10⁻⁸ M by the new Dixon method (Fig. 8). Heparinase I was found to bind with a higher affinity to PVS (Kᵢ of 3.0 × 10⁻⁸ M, Ref. 23), suggesting that PVS is a tight binding inhibitor (47) for heparinase I.

Conditions for Optimal Activity—The effects of ionic strength, pH, and temperature on heparinase III are shown in Fig. 9. Optimal activity was found at 0.03 M NaCl, 50 mM sodium phosphate, 1 mM mercaptoethanol (i.e. Γₐ = 0.15), pH 7.6 and 45 °C. The activation energy was calculated to be 15.63 kcal/mol and the temperature coefficient (Qₐ) as 2.48. Heparinase I activity was optimal under quite different conditions: 0.1 M NaCl, 0.25 M sodium acetate, 2.5 mM calcium acetate, 10 mM sodium phosphate (i.e. Γₐ = 0.4), pH 6.5 and 37 °C (45). The activation energy as well as the temperature coefficient were also lower: 6.3 kcal/mol and 1.45, respectively (45). The presence of calcium (0.2 mM) in the reaction mixture did not affect the activity, neither did EDTA (0.1 mM) or mercaptoethanol (1 mM). On the other hand, the enzyme was
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**Conditions for Optimal Stability**—Heparinase III was found to be most stable at pH 7.0, just as heparinase I (45). The presence of glycerol (0.8 M) or NaCl (0.5 M) stabilized the enzyme when stored as a diluted solution. A higher residual activity was also found if 1 mM mercaptoethanol was included in the buffer as a reducing agent. Preliminary results suggest that heparinase III is more stable than heparinase I. The former enzyme could be exposed to a higher temperature (37 versus 30 °C) with the retention of a comparable amount of enzyme activity (87 versus 93%) after incubation for 96 h under optimal conditions (45).

**Immunodiffusion**—No immunoprecipitation of heparinase I was observed after Ouchterlony double diffusion with rabbit antiserum against heparinase III. The latter antigen gave a strong precipitin band (Fig. 10).

**Degradation Products**—Heparin fragments produced by heparinase III were compared by GPC to those prepared with heparinase I (Fig. 11). According to the known profile of heparinase I-derived fragments (48), SD1 can be identified as the disaccharide-, SD2 the tetrasaccharide-, and SD3 as the hexasaccharide-containing fraction. The chromatogram clearly shows that heparinase III also produced mainly disaccharide fragments from heparin. The results, however, also reveal that the reaction with heparinase III proceeded further than with heparinase I, since more disaccharide and less higher molecular mass fragments were observed. This was also found to be the case with heparinase II (49). Subtle differences in the specificities of the enzymes thus seem to exist.

**DISCUSSION**

A heparin lyase was purified to homogeneity according to SDS-PAGE and GPC. In comparison with the properties of the known heparinase I from *F. heparinarum* (45), this enzyme proved to be unique. It has a molecular mass of about twice that of heparinase I: 94 versus 43 kDa, but is not a dimer of heparinase I. Molecular mass determinations were done under reducing conditions and just one type of N-terminal amino acid, alanine, could be determined.

The unique structure of heparinase III was also evident from its different amino acid composition compared to heparinase I (45). Most conspicuous was the occurrence of only 1 cysteine in heparinase III compared to the 4 in heparinase I (45). Retention of the reduced form of Cys proved to be of great importance for the reactivity of the enzyme, since HgCl₂ was shown to completely inhibit heparinase III, while mercaptoethanol preserved activity during prolonged storage. The low content of sulfur-containing amino acids of heparinase I (1.6 mol%) and III (1.8 mol%) might be related to their functions, *viz.* the degradation of heparin in order to replenish the sulfur pool of the bacteria when sulfur becomes limiting. Taking the synthesis of the enzymes under limiting sulfur concentrations into account, natural selection most probably put heparinases with high contents of methionine or cysteine at a disadvantage.

Interestingly, the two enzymes also differed immunologically as was evident from Ouchterlony double diffusion analysis which showed no cross-reactivity between heparinase I and anti-heparinase III serum. Heparinase I and III not only differed structurally but also with regard to kinetic parameters: heparinase III showed a lower $K_m$ (3.4 versus 8.0 μM) and a higher $k_{cat}$ (1.9 × 10⁴ versus 4.2 × 10³ min⁻¹) than heparinase I (45). This means that not only does heparinase III bind more strongly to heparin, but it also degrades it more effectively. The higher efficacy of heparinase III is clearly demonstrated in its specificity constant, $k_{cat}/K_m$, which is an order of magnitude higher than that of heparinase I (5.7 × 10⁵ versus 5.3 × 10⁴ M⁻¹ min⁻¹, Ref. 45).

In addition to the $K_m$ and $k_{cat}$, other parameters also differed between heparinase I and III. The energy of activation for example was higher, 15.6 versus 6.3 kcal/mol (45). Ca²⁺ was found not to influence heparinase III activity, whereas it was shown to activate heparinase I (26). Furthermore, optimal activity was found under different conditions: pH 7.6, $\Gamma_2 = 0.16$ and 45 °C for heparinase III versus pH 6.5, $\Gamma_2 = 0.4$ and 37 °C for heparinase I (45). Since conditions for optimal heparinase III activity are nearer to plasma values (0.154 M...
NaCl, pH 7.4, 37 °C, Ref. 50) and heparinase III is more effective and heat-stable than heparinase I, the former enzyme should be superior for the deheparinization of blood. Although heparinase III differs structurally and kinetically from heparinase I, both react with the same glycosidic bond in heparin, cleaving it nonhydroxylamically by elimination (51). In both cases, disaccharide units are the main end products (49). On the basis of these findings, heparinase III should also be classified as a heparin lyase, class 4.2.2.7, according to the system of the Enzyme Commission. In order to distinguish it from the known flavobacterial enzymes (I and II), the designation heparinase IV is recommended.

Recently, a fourth heparin lyase, also with unique features, was isolated from the anaerobe mouth bacterium *Bacteroides heparinolyticus* (52). The molecular mass was determined to be 63 kDa, and the pl was 9.5. Degradation products and enzyme specificity also resembled those of heparinases I, II, and III. Furthermore, this enzyme was also inhibited by Hg²⁺. On the grounds of these unique characteristics (molecular mass and pl), it is suggested that this enzyme should be identified as heparinase IV.

Without further information about the primary, secondary, and tertiary structures of these heparinases or the nucleotide sequences of their genes, it is difficult to speculate on their phylogenetic relationship. It might be that these enzymes originated from the same ancestral gene by divergent evolution as is the case with the serine proteases trypsin, chymotrypsin, elastase, thrombin, and plasmin (53). In these proteins, a large proportion of amino acids were conserved, and the catalytic centers are similar. On the other hand, the heparinases might have originated from separate genes and developed the same functional activity by convergent evolution. The bacterial serine protease, subtilisin, for example, proteins, a large proportion of amino acids were conserved, and the catalytic centers are similar. On the other hand, the heparinases might have originated from separate genes and developed the same functional activity by convergent evolution. The bacterial serine protease, subtilisin, for example, developed the same functional activity by convergent evolution.
A Novel Heparinase: Purification and Characterization

EXPERIMENTAL PROCEDURES


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FIG. 1. Radioactivity profile of heparin III amino acid hydrolysate after digestion. (A) labelled heparinase was hydrolysed with HCl. Amino acids were separated with a Waters Amino Acid System and identified with standards. The radioactivity of 100 µl of each fraction was determined.