

Purification and Characterization of Heparinase from *Flavobacterium heparinum**

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Heparinase (EC 4.2.2.7) isolated from *Flavobacterium heparinum* was purified to homogeneity by a combination of hydroxylapatite chromatography, repeated gel filtration chromatography, and chromatofocusing. Homogeneity was established by the presence of a single band on both sodium dodecyl sulfate and acid-urea gel electrophoretic systems. Amino acid analysis shows that the enzyme contains relatively high amounts of lysine residues (9%) consistent with its cationic nature (pI 8.5) but contains only 4 cysteine residues/polypeptide. The molecular weight of heparinase was estimated to be $42,900 \pm 1,000$ daltons by gel filtration and $42,700 \pm 1,200$ daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme is very specific, acting only on heparin and heparan monosulfate out of 12 similar polysaccharide substrates tested. It has an activity maximum at pH 6.5 and 0.1 M NaCl and a stability maximum at pH 7.0 and 0.15 M NaCl. The Arrhenius activation energy was found to be 6.3 kcal/mol. However, the enzyme is very sensitive to thermal denaturation and loses activity very rapidly at temperatures over 40 °C. Kinetic studies of the heparinase reaction at 37 °C gave a K_m of 8.04×10^{-6} M and a V_m of 9.85×10^{-5} M/min at a protein concentration of 0.5 µg/ml. By adapting batch procedures of hydroxylapatite and QAE (quaternary aminoethyl)-Sephadex chromatography, gram quantities of heparinase that is nearly free of catalytic enzyme contaminants can be purified in 4-5 h.

Heparinase (EC 4.2.2.7) from *Flavobacterium heparinum* is an eliminase (1) which cleaves heparin at α -glycosidic linkages in heparin's major repeating unit: $\rightarrow 4$ -2-deoxy-2-sulfamino- α -D-glucopyranose 6-sulfate-(1 \rightarrow 4)- α -L-idopyranosyluronic acid 2-sulfate-(1 \rightarrow 2). Heparinase acts in a random endolytic fashion (3) to produce heparin fragments that are chain-shortened segments of heparin with minor-end group modification. The elimination reaction results in a Δ -4,5 site of unsaturation in the terminal uronic acid residue (4, 5), leaving the chemical structure of the glucosamine reducing end unaltered (6).

Heparinase has found use in numerous applications includ-

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ing structural determination of heparin (2, 6, 7), new bioassays for heparin (8, 9), investigation of the anticoagulant mechanism (10), preparation of low molecular weight heparin anticoagulants (11) and anti-tumor agents (12), and the development of immobilized enzyme filters for blood deheparinization (13). Despite its importance, heparinase has never been purified to homogeneity nor have its activity, stability, or kinetic characteristics been well characterized. In this report we present two methods for heparinase purification: (i) a method conducive to producing gram quantities of nearly catalytically pure heparinase in several hours using batch ion-exchange chromatography and (ii) a method for producing heparinase that is homogeneous as judged by single band purity on two different gel electrophoretic systems. We have also characterized the purified heparinase with respect to its reaction kinetics, stability, and activity.

EXPERIMENTAL PROCEDURES

Materials

The sodium salt of heparin from porcine intestinal mucosa (157 USP units/mg) was obtained from Hepar Industries. Chondroitin 4- and 6-sulfates, dermatan sulfate, hyaluronic acid, pectin, chitin, dextran sulfate, alginate, polyvinyl sulfate, polygalacturonic acid, *p*-nitrocatechol sulfate, and protamine sulfate were purchased from Sigma. Heparan monosulfate was generously supplied by Dr. Cifonelli of Wyler Children's Hospital, Chicago. *N*-Acetyl heparin was prepared from *N*-desulfated heparin (14), according to the procedure of Roseman and Ludowig (15). Azure A dye (A-970; total dye content, 70%) and Folin reagent were from Fisher. Sephadex G-100, QAE (quaternary aminoethyl)-Sephadex, Sephadex G-100 superfine, Polybuffer exchanger PBE 94, Polybuffer 96, and protein markers were obtained from Pharmacia Fine Chemicals. Hydroxylapatite (Bio-Gel HTP), Bio-Rad dye reagent, and chemicals for SDS¹-polyacrylamide gel electrophoresis were purchased from Bio-Rad. Other chemicals were reagent grade, and water was twice distilled.

Methods

Protein Assays

Protein concentrations were measured by the Lowry method (16) or by the Bio-Rad protein assay (17).

Enzyme Assays

Heparinase activity was measured by the increase in ultraviolet absorption at 232 nm (5), according to the procedure of Linhardt *et al.* (3). However, this UV 232-nm assay was utilized only on relatively pure preparations of heparinase because contaminating enzymes such as glucuronidase (1, 4) can act on degradation products resulting in a

¹ The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

loss of the chromophore being measured. In the less pure preparations, heparinase activity was assayed by the metachromatic shift of azure A from blue to red in the presence of heparin (18), according to the procedure of Galliher *et al.* (19). The change in absorbance was measured at 620 nm throughout the entire linear range of the assay and compared with a standard curve of 0 to 8.3 mg of heparin/ml in assay broth. One international unit of heparinase activity is defined as that amount which causes 1 μ mol of double bonds formed/min, based on a molar extinction coefficient of $5.1 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ at 232 nm for the degradation products, the α, β -unsaturated uronic acids (5). However, in the less pure preparations where the azure A assay is used, the international unit is no longer suitable. One unit of enzyme activity is, therefore, defined as the amount of enzyme which degrades 1 mg of heparin/h. Because of the cross-reaction of heparinase on heparan monosulfate, heparitinase activity was assayed at 43 °C, a temperature at which the heparitinase activity was maximized but the heparinase activity was minimized (4). Sulfatase activity was assayed by the procedure of Ototani *et al.* (20) using *p*-nitrocatechol sulfate as the substrate. Glycuronidase activity was measured by monitoring the rate of disappearance of absorption at 232 nm in the assay solution which had been previously used to measure heparinase activity, according to the method of Warnick and Linker (21).

Crude Enzyme Preparation

F. heparinum was grown in a complex protein digest medium as described previously (19), with heparin as the inducer. Approximately 100 g (dry weight) of cells were obtained in a 10-liter fermentation. The cells were harvested by centrifugation and resuspended in 3 volumes of cold 0.01 M sodium phosphate buffer, pH 6.8. The suspended microorganisms were homogenized at 5000 p.s.i. in 3–4 passages through a Gaulin model 15 M homogenizer thermostatically controlled at 4 °C by circulating ice water. After the addition of protamine sulfate to the homogenate (125 mg of protamine sulfate/g of protein) the cell debris and precipitated nucleic acids were removed by centrifugation at $10,000 \times g$ for 15 min. The supernatant fluid thus obtained (the "crude extract") was subjected to further purification. Unless otherwise stated, all the enzyme preparations and purifications were carried out at 4 °C.

Large Scale Production of Heparinase

This includes batch procedures of hydroxylapatite and QAE-Sephadex chromatography.

Hydroxylapatite—The crude extract was batch loaded onto hydroxylapatite pre-equilibrated with 0.01 M phosphate buffer (pH 6.8), using 4 g of hydroxylapatite/g of protein. The hydroxylapatite-bound protein was then washed stepwise with buffers of increasing concentrations of sodium phosphate and NaCl. The solutions used for the sequential stepwise elutions were prepared by mixing 0.01 M sodium phosphate buffer, pH 6.8, with 0.25 M sodium phosphate, 0.5 M NaCl buffer, pH 6.8, according to a ratio (v/v) of 8/0, 7/1, 6.5/1.5, 6/2, 5.5/2.5, 5/3, 4/4, 3/5, 2/6, 1/7, and 0/8, respectively. The hydroxylapatite particles were removed by centrifugation at $3000 \times g$ for 3 min, and the supernatant was assayed for protein concentration and heparinase activity.

QAE-Sephadex Adsorption—A negative adsorption on QAE-Sephadex was carried out. The resulting enzyme preparation (0.2–0.4 mg/ml), obtained from the hydroxylapatite chromatography in a 0.07 M sodium phosphate and 0.125 M NaCl wash (*i.e.* the 6/2 solution), was dialyzed for at least 4 h against 100 volumes of 0.01 M phosphate buffer at pH 6.8 and then added to QAE-Sephadex (5 mg of QAE-Sephadex/ml of protein solution). The mixture was kept at 4 °C for 10 min, with occasional and gentle vortexing, and the gel beads were removed by centrifugation at $3000 \times g$ for 3 min. The supernatant was assayed for protein concentration and heparinase activity.

Polyacrylamide Gel Electrophoresis

The enzyme preparations obtained during the successive purification stages were subjected to SDS-PAGE according to a modified procedure of Laemmli (22). The separation gel contained 10% acrylamide and 0.1% SDS in 0.375 M Tris-HCl buffer (pH 8.8). The stacking gel contained 4% acrylamide and 0.1% SDS in 0.125 M Tris-HCl buffer (pH 6.8). Protein samples were dissolved in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% 2-mercaptoethanol, and 12.5% glycerol, and then heated in a boiling water bath for 2 min prior to electrophoresis. Electrophoresis was carried out at room

temperature at a constant voltage of 40 V until the bromphenol blue marker reached the bottom of the gel. The gels were fixed in 50% trichloroacetic acid overnight, stained for 3 h at room temperature with 0.25% Coomassie Blue R-250 in 45% methanol and 9% glacial acetic acid, and then diffusion destained by repeated washing with 15% glacial acetic acid in 10% methanol. To demonstrate the homogeneity of the purified enzyme, the urea-acetic acid system of Panyim and Chalkley (23) was also employed. The slab gel contained 10% polyacrylamide and 6.25 M urea in 0.9 M acetic acid (pH 3.2). The sample buffer contained 6.25 M urea and had a pH of 2.6. The electrode buffer was 0.9 M acetic acid (pH 2.6). The gel was stained and destained by the same procedures used in the SDS-PAGE system.

Elution of Heparinase from SDS-Polyacrylamide Gels

The concentrated QAE-purified heparinase was subjected to electrophoresis on a slab SDS-polyacrylamide gel as described above. After electrophoresis, a slot containing protein markers together with a slot containing the QAE-purified sample was cut out of the slab, fixed for 20 min, stained for 20 min, and destained for 15 min while the rest of the gel was stored at 4 °C in a refrigerator. The stained and unstained gels were lined up with each other, and the regions containing polypeptide bands were excised in slices. The gel slices were incubated overnight at 4 °C in separate tubes containing 3 ml of 1% (v/v) Triton X-100 in water. The eluate was dialyzed against 100 volumes of 0.01 M phosphate buffer (pH 7) for 10 h to remove the Triton since it produced a high background in the UV 232-nm assay. The preparations were assayed for heparinase activity.

Gel Filtration Chromatography

Sephadex G-100 gels (515 ml) and Sephadex G-100 superfine gels (190 ml) were packed in glass Pharmacia columns, equilibrated, and eluted with 0.01 M sodium phosphate buffer, pH 6.8. Samples (6 ml) were injected through a three-way valve and eluted at a flow rate of 48 ml/h for the Sephadex G-100 column and of 3.6 ml/h for the Sephadex G-100 superfine column. Protein markers used were albumin ($M_r = 67,000$), ovalbumin ($M_r = 43,000$), chymotrypsinogen A ($M_r = 25,000$), and ribonuclease A ($M_r = 13,700$).

Chromatofocusing

Chromatofocusing was performed with a 0.9-cm diameter column containing 15 ml of anion exchanger PBE 94. The anion exchanger was equilibrated with 0.25 M ethanolamine- CH_2COOH buffer at pH 9.4 and eluted with Polybuffer 96 diluted 1:10 with deionized water and adjusted to pH 6.0 with acetic acid. Proteins were monitored at 280 nm since Polybuffer interferes with the Lowry assay.

Amino Acid Analysis

The amino acid composition of purified heparinase was determined with a Beckman 121 M amino acid analyzer. About 20 μ g of sample were hydrolyzed at 110 °C for 24, 48, and 72 h in 6 N HCl in an evacuated sealed tube. The sample was then dried, dissolved in 150 μ l of 0.2 N sodium citrate buffer (pH 2.2) containing 0.5% thioglycol and 0.1% phenol, and loaded onto the analyzer. Tryptophan was destroyed under the conditions for hydrolysis. Asparagine and glutamine were converted to aspartic acid and glutamic acid, respectively. Cysteic acid was determined by hydrolysis of protein oxidized with performic acid (24).

Kinetics and Substrate Specificity

The kinetic constants of the heparinase reaction were determined at 37 °C by measuring the initial rates of heparin degradation as a function of heparin concentrations ranging from 9.1 μ M to 2.27 mM. The substrate specificity of the purified heparinase on various polysaccharides including heparin, *N*-acetyl heparin, heparan monosulfate, chondroitin 4- and 6-sulfates, hyaluronic acid, dermatan sulfate, pectin, chitin, dextran sulfate, polygalacturonic acid, and alginic acid were measured at 30 °C, pH 7, by the UV 232-nm assay. The reaction mixture contained 10 mM phosphate, a protein concentration of 28 μ g/ml, and a substrate concentration of 8.3 mg/ml. All substrates above were tested for their inhibitory effect on heparinase at a concentration of 8.3 mg/ml and in the presence of a heparin concentration of 8.3 mg/ml.

RESULTS

Large Scale Production of Heparinase—A high demand for heparinase in blood deheparinization studies (13) led us to seek methods suitable for production of large quantities of highly purified enzyme with a minimum of expense and inconvenience. This has been achieved by converting two successive ion-exchange chromatographic steps from a column technique to a batch process. The first step was a batch hydroxylapatite separation, the results of which are shown in Fig. 1. Heparinase activity was eluted over a broad range of ionic strength ($I = 0.22-0.42$); however, the maximum activity was obtained in a $I = 0.29$ wash. These results are consistent with those reported by other authors using hydroxylapatite column chromatography (20, 25). Although batch procedures are less efficient in resolution than column techniques, they allow enzyme production to be scaled up from the milligram quantities produced by a hydroxylapatite column to hundreds of grams. Polyacrylamide gel electrophoresis showed the presence of about 15 major bands in this preparation (Fig. 1). Enzymatic assays indicated that this preparation was not "catalytically" pure and contained low amounts of activities attributed to enzymes such as chondroitinases (26, 27), heparitinase (4, 20), and hyaluronidase (20, 27) which act on heparin-like mucopolysaccharides as well as other enzymes, sulfatases (4, 28) and glycuronidase (4, 20, 29), that act on heparin degradation products.

Further purification was achieved through anion-exchange chromatography on QAE-Sephadex. This is a convenient and efficient method since over 70% of the proteins in the hydrox-

ylapatite preparation are acidic (as judged by isoelectric focusing) while heparinase is a cationic molecule. After QAE-Sephadex purification, 85% of the total activity was recovered, and the specific activity was enriched 3- to 4-fold over that obtained from the hydroxylapatite purification. In addition, the resulting enzyme preparation was nearly free of the catalytic enzyme contaminants. The relative activities of these contaminating enzymes, as compared to the heparinase activity (100%) at 30 °C on the basis of absorbance change at 232 nm/h were: chondroitinase B, 0%; heparitinase (measured at 43 °C as described under "Methods"), <4.5%; chondroitinase AC, <4.0%; hyaluronidase, <4.2%. Only minor sulfatase and glycuronidase activities were detected. Over a period of 1 h, less than 2.5% of the sulfate groups were desulfated and 1-2.8% of the α,β -unsaturated uronide chromophores were degraded.

Elution of Heparinase after SDS-PAGE—When QAE-purified heparinase was subjected to SDS-PAGE and eluted from the gel, no heparinase activity was found in the eluate as the gel slices were eluted with 1% SDS and dialyzed thereafter. However, about 20% of the enzyme activity was recovered when the SDS gel slices were eluted with 1% Triton X-100. It appears that heparinase is denatured faster in SDS than in Triton X-100. This may be accounted for by the non-ionic nature of the Triton X-100, such that conformation change of the highly charged heparinase is much slower in Triton X-100 than in SDS. Fig. 2 shows that heparinase activity was only found in the region corresponding to a molecular weight between 38,000 to 50,100 daltons.

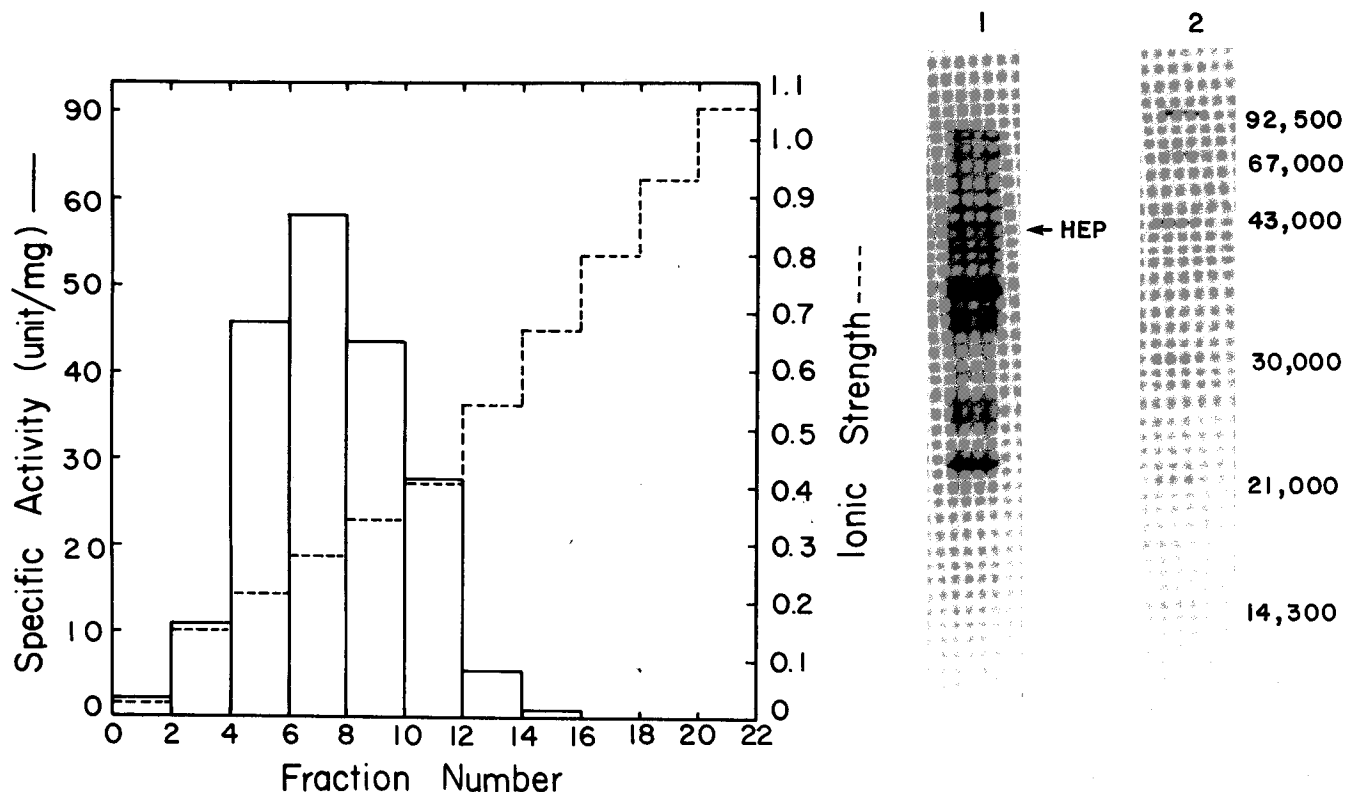
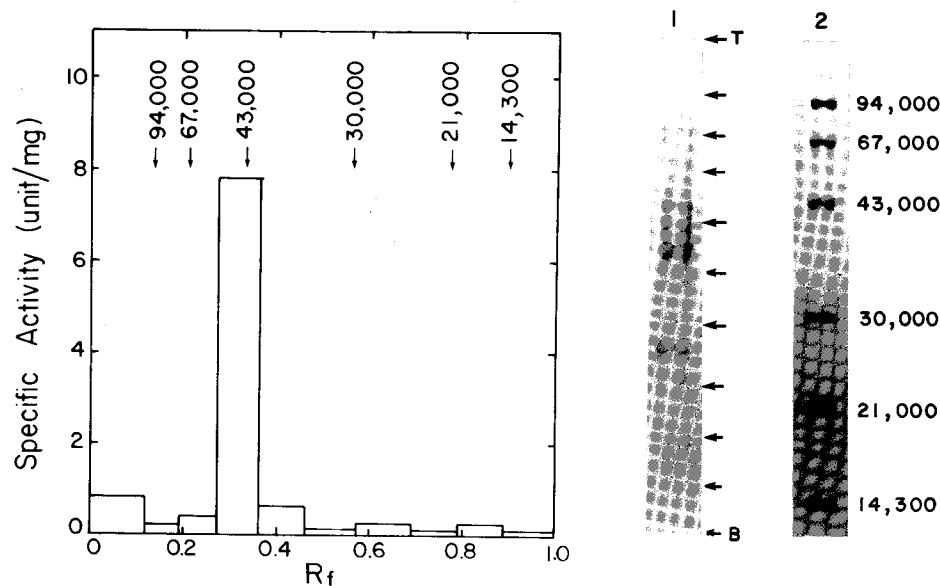


FIG. 1. Elution of heparinase from batch hydroxylapatite chromatography. One liter of the crude enzyme extract (30 mg/ml) was batch loaded onto 120 g of hydroxylapatite and then washed stepwise with buffers of increasing ionic strength as described under "Methods." Specific activity was measured by the azure A assay. One unit of heparinase activity is defined as the amount of enzyme which degrades 1 mg of heparin/h. Each fraction represents a volume of 1 liter of the eluate. Right, the active fractions resulting from an $I = 0.29$ wash were analyzed by SDS-polyacrylamide gel electrophoresis (slot 1). HEP designates heparinase. Slot 2, markers are phospholipase ($M_r = 94,000$), albumin ($M_r = 67,000$), ovalbumin ($M_r = 43,000$), carbonic anhydrase ($M_r = 30,000$), soybean trypsin inhibitor ($M_r = 21,000$), and lysozyme ($M_r = 14,300$).

FIG. 2. Elution of heparinase after SDS-polyacrylamide gel electrophoresis. The experimental procedures are described under "Methods." R_f represents the distance from the top of the gel to the place where the gel is cut, as normalized with respect to the gel length. One unit of heparinase activity used here is defined as the amount of enzyme which can completely degrade 1 mg of heparin/h. Right, the QAE preparation was analyzed by SDS-polyacrylamide gel electrophoresis (slot 1). The arrows indicate the positions where the gel is cut. Slot 2, markers (see Fig. 1).



Purification of Heparinase to Homogeneity—Purification of heparinase to homogeneity was accomplished by additional column chromatography of the hydroxylapatite-purified heparinase through Sephadex G-100 gel filtration, chromatofocusing, and Sephadex G-100 superfine gel filtration.

Sephadex G-100 Gel Filtration Chromatography—Prior to gel filtration, the hydroxylapatite-purified heparinase was concentrated about 30-fold by ultrafiltration under a pressure of 40 p.s.i., using a Diaflo model 52 ultrafilter equipped with a hydrophilic and low nonspecific binding YM 30 membrane. More than 96% of activity was recovered in the retentate after the concentration. The concentrated enzyme solution (6 ml) was loaded onto a Sephadex G-100 column (2.6 × 97 cm) and eluted with 0.01 M sodium phosphate buffer (pH 6.8). Fig. 3 shows the elution pattern. The activity peak was found at the elution volume corresponding to a molecular weight of $42,900 \pm 1,000$ daltons. A significant fraction (about 20%) of the protein, possibly high molecular weight protein contaminants together with some protein aggregates formed from the effect of concentration polarization during the ultrafiltration process, was eluted at the void volume. However, no heparinase activity was found in this region. The resulting enzyme preparation showed no glycuronidase activity and had a specific activity of 1.4 IU/mg. SDS-PAGE showed 9 major bands in this preparation. As indicated in Fig. 2, the heparinase activity was eluted from a SDS-polyacrylamide gel in the region corresponding to a molecular weight between 38,000 to 51,000 daltons; the band with a molecular weight of 43,000 daltons, therefore, may be heparinase (Fig. 3).

Chromatofocusing—Active fractions indicated in Fig. 3 by cross-hatching were combined, concentrated to a volume of 6 ml by ultrafiltration, and then applied to a chromatofocusing column. As shown in Fig. 4, the heparinase activity was eluted as a broad symmetrical peak centered at a pI of 8.5. SDS-PAGE of the chromatofocused material showed the presence of two major bands with molecular weights of 42,700 and 25,000 daltons, respectively, as well as three very minor bands. Apparently the M_r 42,700 band is heparinase, because it is the only band lying in the region where heparinase was recovered after SDS-PAGE (Fig. 2).

Sephadex G-100 Superfine Gel Filtration Chromatography—Active fractions 25 to 40 from the chromatofocusing column (see Fig. 4) were combined, concentrated to a volume of 6 ml

by ultrafiltration (as described earlier), and applied to a Sephadex G-100 superfine column (1.6 × 95 cm) pre-equilibrated with 0.01 M phosphate buffer at pH 6.8. The fractions eluting from the column were analyzed for both protein concentration and heparinase activity (Fig. 5). The activity peak coincided with the first protein peak in fraction 47. The second protein peak, which emerged at fraction 57, was presumably the M_r 25,000 contaminant seen in Fig. 4. The two shoulders appearing before the first and the second protein peak may be proteins attributed to the three minor bands seen in the gel of Fig. 4. The homogeneity of this preparation was demonstrated as a single band shown on both the SDS-PAGE system which separated polypeptides on the basis of molecular weight and the acid-urea gel system which separated polypeptides mainly on the basis of charge (Fig. 6). Densitometer traces of both gels also indicated the presence of a single polypeptide (>99% pure) in the preparation. The molecular weight of the purified heparinase was estimated to be $42,700 \pm 1,200$ daltons by the SDS-PAGE system.

Recovery and Yield—Table I summarizes the results of the heparinase purification. The enzyme was purified 280-fold over the cell homogenate. The specific activity of the purified enzyme was 26.7 IU/mg of protein. The overall mass yield of the purified protein was only 0.003% starting with 1 g of protein in the cell homogenate. The recovery of heparinase activity was about 1%.

Amino Acid Analysis—The amino acid composition of completely purified heparinase is shown in Table II. The enzyme is characterized by a low content of sulfur-containing amino acids (<2%) and a relatively high content of basic amino acids (14%) such as lysine, arginine, and histidine. The relatively high amount of basic amino acids is consistent with the cationic nature of the heparinase (pI 8.5).

Kinetics and Substrate Specificity—The kinetics of the heparinase reaction are shown in a Lineweaver-Burk plot (Fig. 7). The values of K_m and V_m , as obtained at 37 °C, pH 7, a protein concentration of 1 μ g/ml, and from the average of three separate experiments, were 8.04×10^{-6} M and 9.85×10^{-5} M/min, respectively.

Substrate specificity studies of the purified heparinase show that the enzyme is very specific, acting only on heparin and heparan monosulfate out of 12 polysaccharides tested (see "Methods."). There was 72% less activity on heparan mono-

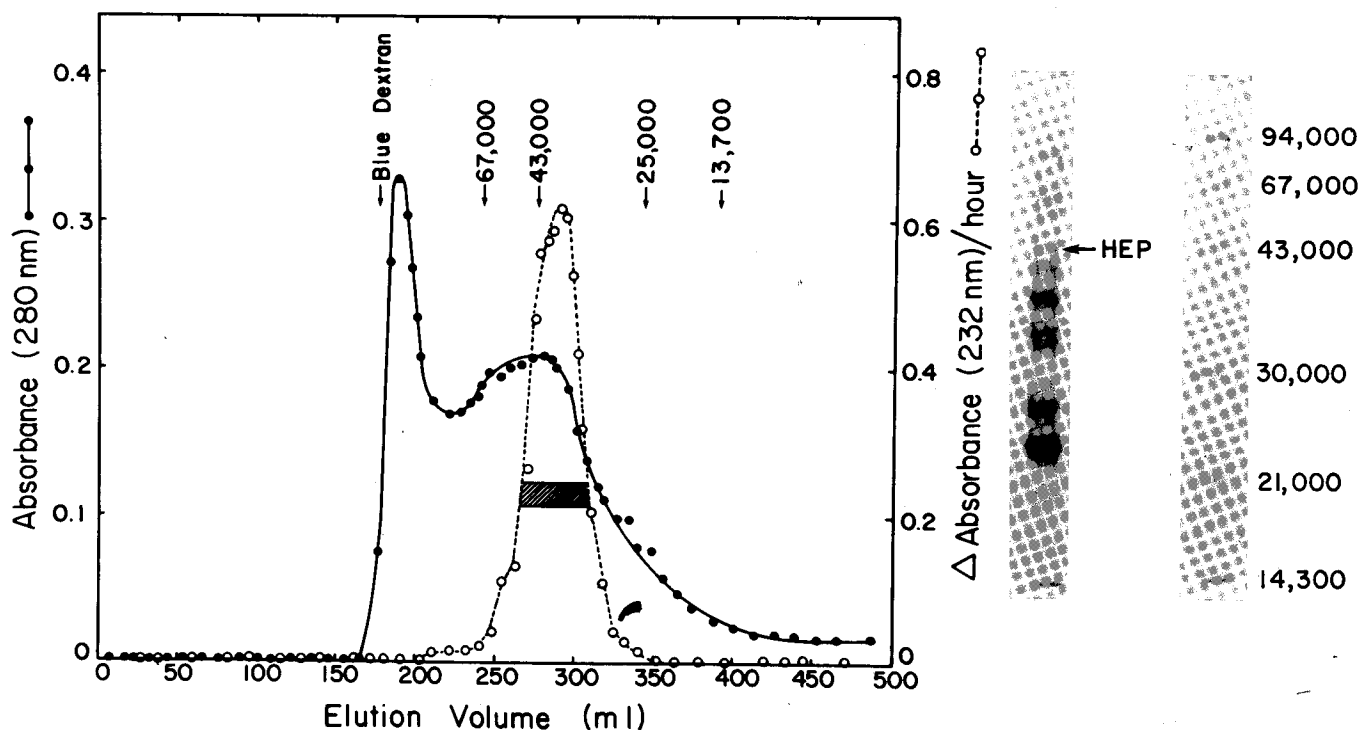


FIG. 3. Gel filtration of heparinase on Sephadex G-100. Two hundred milliliters of the hydroxylapatite-purified heparinase obtained in an $I = 0.29$ wash in Fig. 1 were concentrated to 6 ml by ultrafiltration and applied onto a Sephadex G-100 column (2.6 \times 97 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The column was then eluted with the same buffer. The molecular weight markers are blue dextran ($M_r = 2 \times 10^6$), bovine serum albumin ($M_r = 67,000$), ovalbumin ($M_r = 43,000$), chymotrypsinogen A ($M_r = 25,000$), and ribonuclease A ($M_r = 13,700$). The flow rate was 48 ml/h, and the sample size was 6 ml of the protein solution with a concentration of 10 mg/ml. Right, the active fractions indicated by cross-hatching were pooled and analyzed by SDS-polyacrylamide gel electrophoresis (slot 1). HEP designates heparinase. Slot 2, markers (see Fig. 1).

sulfate compared with heparin. The lack of activity on *N*-acetyl heparin suggests that the purified heparinase contains no heparitinase activity. The activity on heparan monosulfate, therefore, is due to the cross-reaction of heparinase on heparan monosulfate rather than that there is heparitinase contaminant present. The other compounds were not active as substrates nor did they inhibit heparin degradation when present at concentrations as high as 8.3 mg/ml.

Effects of Salt, pH, and Temperature on Activity and Stability—The effects of salt concentration and pH on heparinase activity are shown in Fig. 8. The optimum salt concentration was around 0.1 M NaCl (Fig. 8A), and the optimum pH occurred at pH 6.5 (Fig. 8B). As shown in Fig. 9A, the stable salt concentration was 0.15 M NaCl where 93% of the enzyme activity remained after 96 h of incubation at 30 °C. The stable pH, after 72 h of incubation at 30 °C, was found to be at pH 7.0 (Fig. 9B).

The effect of temperature on enzyme activity was illustrated by an Arrhenius plot (Fig. 10). Initial rates were measured at pH 7 under conditions in which the enzyme was saturated with substrate. As is true for most enzymatic reactions, the reaction rate increases with temperature. The initial rates of degradation of heparin at 17, 31, and 41 °C were 0.17, 0.29, and 0.38 mm/min, respectively. The temperature coefficient (Q_{10}) was estimated to be 1.45, i.e. the reaction rate increased 1.45-fold for each 10 °C rise in temperature. The Arrhenius activation energy was calculated to be 6.3 kcal/mol. Although heparinase was very stable at 30 °C, it was sensitive to thermal denaturation above 38 °C. While over 90% of activity remained after 72 h of incubation at 30 °C, a loss of more than 25% of activity was observed after 20 min of incubation at 41 °C.

Storage Conditions—The hydroxylapatite-purified heparinase was stable when stored either frozen or lyophilized at -20 °C with a protein concentration of 0.2–0.4 mg/ml. More than 90% of activity was recovered after storage in the freezer for 6 months. Chromatofocused heparinase was also stable on storage and maintained 80% of the activity after 2 months of storage in the Polybuffer at -20 °C and a protein concentration of 50–100 μ g/ml. However, in the absence of high salt concentration (0.1–0.2 M NaCl) or ampholytes, the enzyme is more stable when stored at -20 °C in the presence of 7.5% glycerol or 5% sucrose. An approximate 40% increase in stability was found for a preparation containing low ionic strength (10 mM sodium phosphate) and no ampholytes, when the sample was stored frozen for 2 months in the presence of 5% sucrose.

DISCUSSION

Heparin-induced *F. heparinum* contains a variety of enzymes acting on glycosaminoglycans by an eliminase mechanism (1, 28), as well as several enzymes acting on heparin degradation products by a hydrolysis mechanism (1, 29). The eliminases are heparinase (25, 29, 30), heparitinase (25), chondroitinases (26, 31), and hyaluronidase (26, 32). The hydrolases are sulfatases (sulfamidase and sulfoesterase) (30, 33, 34) and glycuronidase (1, 29, 35). Among these enzymes heparinase has gained increased attention with its potential therapeutic application for blood deheparinization in an immobilized enzyme reactor (13), as well as its importance in the elucidation of heparin structure (2, 6, 7). For the former studies it is essential to develop a method conducive to rapidly producing large quantities (grams) of catalytically pure hep-

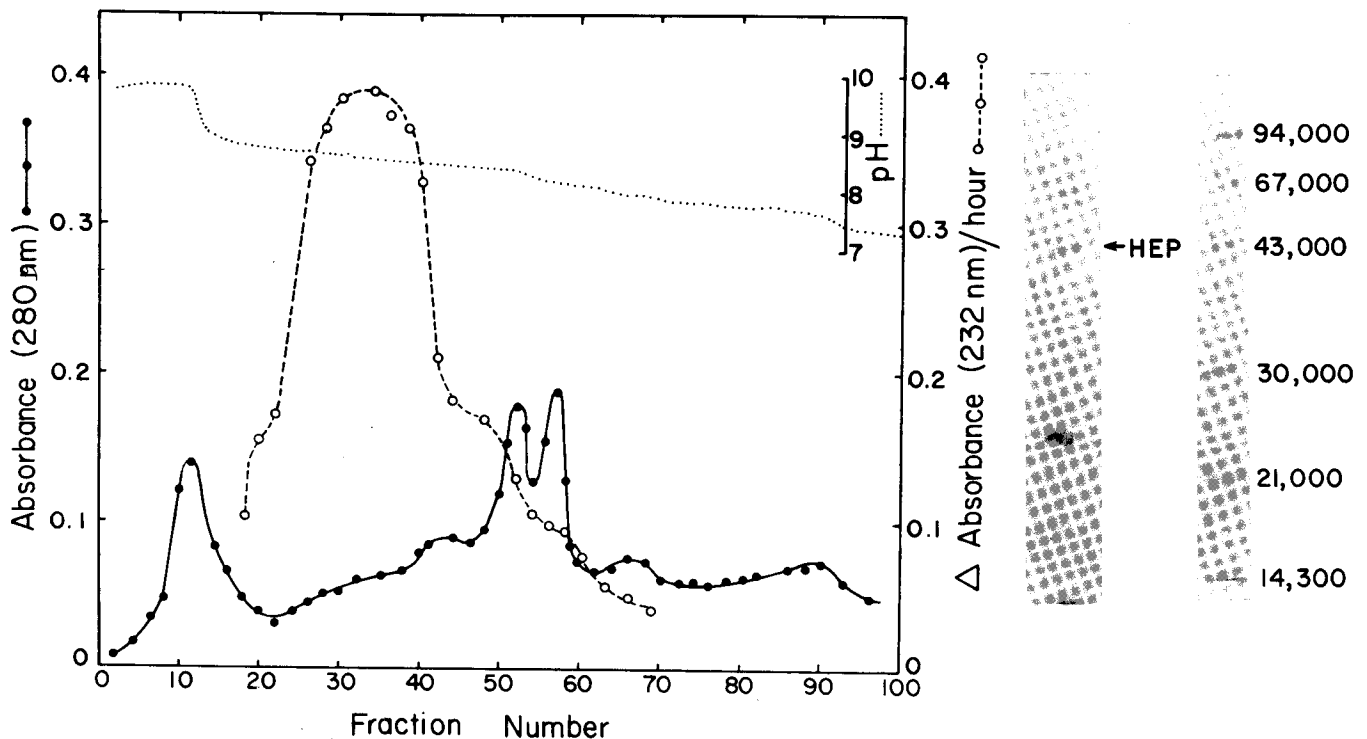


FIG. 4. Chromatofocusing of heparinase on anion exchanger PBE 94. Active fractions indicated in Fig. 3 by cross-hatching were concentrated to 6 ml by ultrafiltration, applied to the PBE 94 chromatofocusing column (0.9×24 cm), and eluted with Polybuffer 96- CH_3COOH (pH 6.0) as described under "Methods." Fractions of 1 ml are collected. The flow rate was 20 ml/h, and the sample size was 6 ml of the protein solution with a concentration of 1 mg/ml. Right, active fractions (25–40) were combined and analyzed by the SDS-polyacrylamide gel electrophoresis (slot 1). HEP designates heparinase. Slot 2, markers (see Fig. 1).

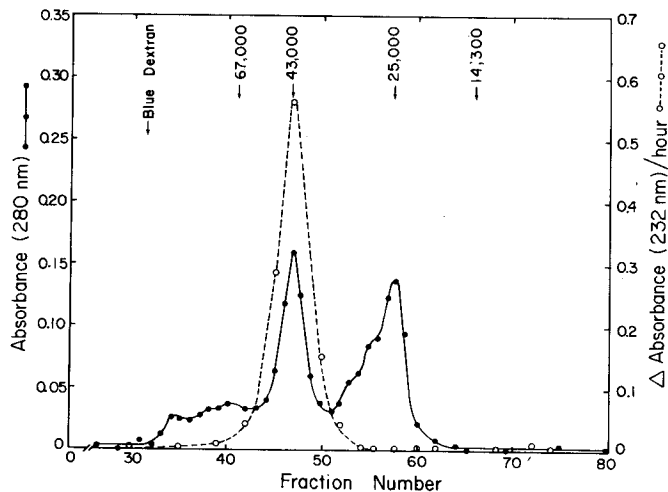


FIG. 5. Gel filtration of heparinase on Sephadex G-100 superfine. Active fractions (25–40) in Fig. 4 were pooled, concentrated to 6 ml by ultrafiltration, and applied to the Sephadex G-100 superfine column (1.6×95 cm) equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The column was then eluted with the same buffer. The flow rate was 3.6 ml/h, and the sample size was 6 ml of the protein solution with a concentration of 50 $\mu\text{g}/\text{ml}$.

a completely purified heparinase and to characterize its properties with regard to reaction kinetics, activity, and stability. Moreover, a homogeneous heparinase might eventually be used to produce antibodies which could subsequently be useful to facilitate heparinase purification through immunoaffinity chromatography.

Linker and Hovingh isolated heparinase from the other

eliminases by a combination of hydroxylapatite and cellulose phosphate column chromatography (4). Dietrich and co-workers separated heparinase from heparitinase and sulfatases by preparative electrophoresis on agarose (7). Ototani *et al.* reported that by using repeated column chromatographic steps with hydroxylapatite, cellulose phosphate, and AH (amino-hexyl)-Sephadex 4B coated with dermatan sulfate, heparinase can be purified to a point where all the contaminating activities are no longer present (20). However, these foregoing methods are time consuming and are not well suited for the preparation of large quantities of the enzyme. We have shown that by adapting a batch procedure of ion-exchange chromatography including hydroxylapatite and QAE-Sephadex, a facile large scale purification can be accomplished within a few hours. The preparation obtained after the QAE-purification is nearly free of sulfatases and glycuronidase and contains less than 5% of each of the other catalytic enzyme contaminants such as chondroitinase AC, hyaluronidase, and heparitinase. It is possible that the chondroitinase AC and hyaluronidase activities may be attributed to a single protein acting on both chondroitin sulfate and hyaluronic acid, since several authors (20, 26, 32) have reported the presence of co-purification of both activities throughout all their preparations of these enzymes from *F. heparinum*.

Heparinase has been purified to homogeneity by several successive chromatographic procedures including hydroxylapatite ion exchange, Sephadex G-100 gel filtration, chromatofocusing with PBE 94 and Polybuffer 96- CH_3COOH , and Sephadex G-100 superfine gel filtration. The homogeneity of the purified heparinase is demonstrated by the presence of a single polypeptide band in both the SDS and the acid-urea polyacrylamide gel electrophoretic systems. The molecular weight was estimated to be $42,900 \pm 1,000$ daltons by gel

FIG. 6. Homogeneity of the purified heparinase. Active fractions (46-48) in Fig. 5 were combined and analyzed by the SDS-polyacrylamide gel electrophoresis and acid-urea polyacrylamide gel electrophoresis. A, densitometry trace of the SDS-polyacrylamide gel (slot 1 on the right); slot 2, markers (see Fig. 1). B, densitometry trace of the acid-urea polyacrylamide gel (right). T, top of the gel; B, bottom of the gel. R_f represents the mobility of the polypeptide band.

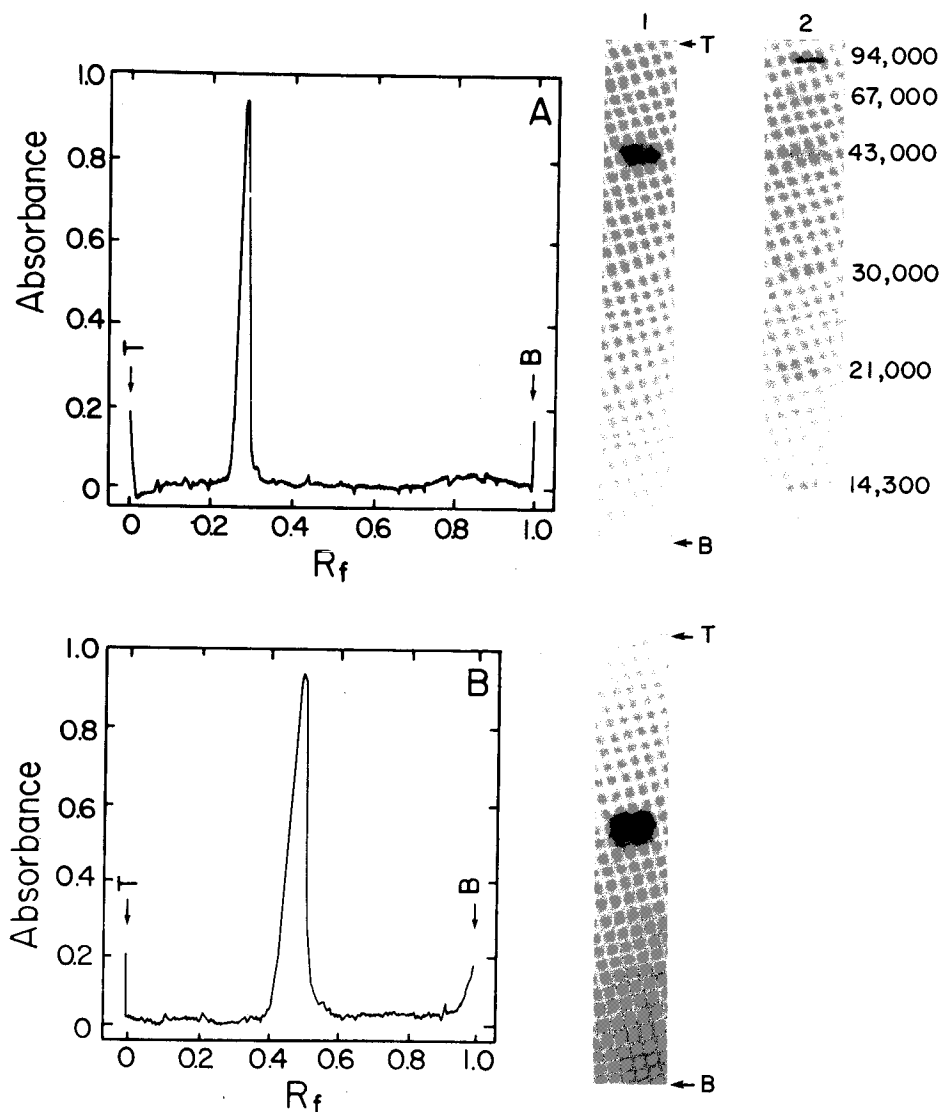


TABLE I

Summary of purification of heparinase

Except in the less pure preparations where the glucuronidase is present (i.e. azure A assay is used), the international unit is used throughout the entire work.

Purification step	Total protein	Specific activity	Specific activity	Total activity	Recovery of activity
	mg	units ^a /mg	IU ^b /mg	units ^a	% ^a
Cell homogenate	1000	7.4		7400	
Protamine sulfate precipitation	670	10.0		6700	90
Hydroxylapatite	50	35.0		1750	23.8
Sephadex G-100	5.31	104.3	1.4	553.8	7.5
Chromatofocusing	0.23	619.3	8.1	142.4	1.9
Sephadex G-100 superfine	0.03	2045.8	26.6	61.4	0.8

^a One unit of heparinase activity is defined as the amount of enzyme which degrades 1 mg of heparin/h.

^b One international unit of activity is defined as the amount of enzyme which causes 1 μ mol of double bonds formed/min.

filtration chromatography and $42,700 \pm 1,200$ daltons by SDS-PAGE. Furthermore, enzymatic assay of the polypeptides eluted from the polyacrylamide gel after SDS-PAGE showed that heparinase activity was present only in the region corresponding to a molecular weight between 38,000 to 50,100 daltons. These results suggest that heparinase is a single polypeptide chain with a molecular weight around 43,000 daltons.

Amino acid analysis of the purified heparinase indicates

that the enzyme contains only 4 cysteine residues/polypeptide. In contrast, it contains relatively high amounts of lysine residues (9%). Preliminary results (data are not included) showed that heparinase was not inactivated by 2-mercaptoethanol. This resistance to the sulfhydryl-reducing reagent may be rationalized in terms of the low content of cysteine residues. The high level of lysine residues is consistent with the cationic nature of heparinase (pI 8.5). The lysine residues may be responsible for the coupling in heparinase immobili-

TABLE II
Amino acid composition of heparinase

Amino acid	Moles	Nearest integer ^a
	%	
Half-cystine ^b	0.89	4
Aspartic acid ^c	12.82	52
Threonine ^d	5.59	23
Serine ^d	6.17	25
Glutamic acid ^c	10.12	41
Proline	5.23	21
Glycine	8.62	35
Alanine	12.21	50
Valine	3.68	15
Methionine	0.74	3
Isoleucine	3.87	16
Leucine	6.93	28
Tyrosine	3.93	16
Phenylalanine	5.01	20
Lysine	9.04	37
Histidine	2.65	11
Arginine	2.50	10
Tryptophan	ND ^e	ND
Total		407

^a The values are calculated assuming that the molecular weight of heparinase is 43,000 daltons.

^b Represents moles (%) of cysteic acid generated by performic acid oxidation.

^c Asparagine and glutamine were converted to aspartic acid and glutamic acid, respectively.

^d Based on extrapolation of data obtained by hydrolysis of 24, 48, and 72 h.

^e ND, not determined.

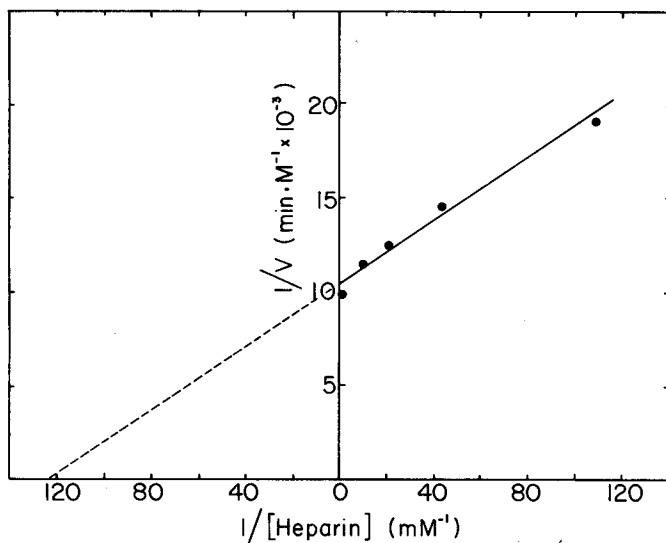


FIG. 7. Lineweaver-Burk plot of initial rates of the heparinase reaction as a function of heparin concentration. Initial rates (V) were measured by the UV 232-nm method at 37 °C, pH 7, and the indicated concentrations of heparin. Protein concentration in the reaction mixture was 0.5 $\mu\text{g}/\text{ml}$. An average molecular weight of 11,000 daltons was used for heparin to calculate its molar concentration.

zation to CNBr-activated Sepharose 4B (3) through its primary amino groups. However, if the active site is not protected with heparin during immobilization the enzyme loses activity (3). This suggests that certain lysine residues may be located either at or near the active site of heparinase.

The activity maximum of purified heparinase occurs at pH 6.5 and 0.1 M NaCl, while the stability maximum occurs at slightly higher pH (pH 7) and salt concentration (0.15 M NaCl). In order to maintain the stability of the heparinase, it

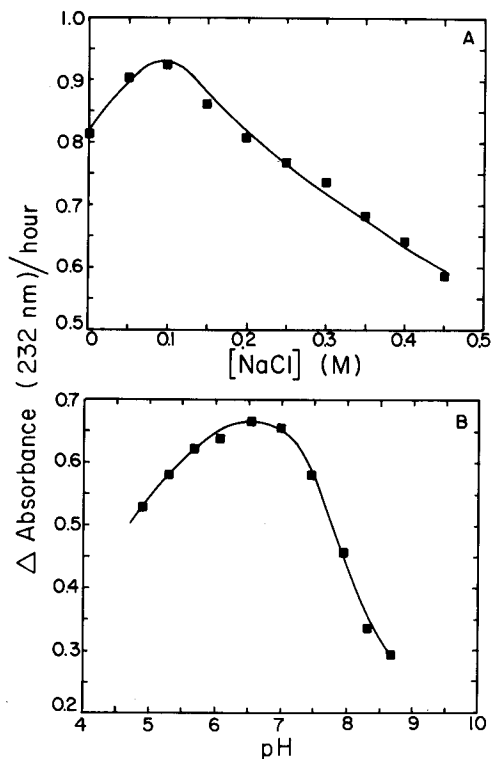


FIG. 8. Effects of (A) salt concentration and (B) pH on heparinase activity. Heparinase activity was assayed by the UV 232-nm method. In A, activity was determined at 30 °C, pH 7, a protein concentration of 12.8 $\mu\text{g}/\text{ml}$, and at the indicated salt concentrations. In B, activity was determined at 30 °C, a protein concentration of 11.2 $\mu\text{g}/\text{ml}$, and at the indicated pH. Reaction mixtures also contained 10 mM sodium phosphate.

is suggested to store the enzyme at -20 °C, pH 7, and in the presence of 0.15 M NaCl. The Arrhenius plot shows that the heparinase reaction has an activation energy of 6.3 kcal/mol, indicating that the degradation of heparin with heparinase proceeds faster at higher temperatures. However, the rate of thermal denaturation of heparinase also increased with temperature. The apparent temperature optimum is thus dependent upon the processing time of the activity assay. During an assay period of 40 min or less, the temperature optimum was found to be 37 °C. Prolonged incubation shifted the temperature optimum to a lower temperature. Dietrich (29) reported that the temperature optimum for heparinase was 30 °C. However, their assays included a 3-h incubation at each temperature.

Heparinase is very specific, acting only on heparin and heparan monosulfate. The activity toward heparan monosulfate may be rationalized in terms of the presence of heparin-like regions in the heparan monosulfate polymer (20, 25). Heparan monosulfate was a relatively poor substrate when compared to heparin, and the extent of reaction was only 28% that of heparin. This value is consistent with that reported by Hovingh and Linker on a relatively pure enzyme preparation (25) but is lower than that reported by Dietrich *et al.* (7). The difference may be attributed to the difference in sulfate content in the heparan monosulfate preparations (7, 25, 36). Sulfate groups appear to be an important structural feature required for recognition by the active site of the heparinase molecule. Preliminary results (data are not included) show that polyvinyl sulfate, a synthetic heparin analogue (37) which bears no obvious structural resemblance to heparin except the high sulfate content, is a competitive inhibitor of heparinase with a K_i of 3.0×10^{-8} M. The inhibitory effect declines as

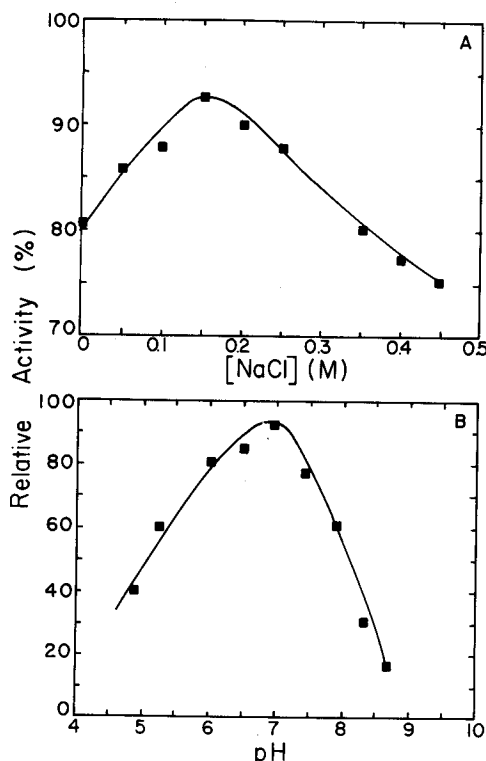


FIG. 9. Effect of (A) salt concentration and (B) pH on heparinase stability. In A, aliquots of the enzyme solution were incubated at 30 °C, pH 7, and a protein concentration of 12.8 $\mu\text{g}/\text{ml}$ for 96 h. In B, aliquots were incubated at 30 °C and a protein concentration of 11.2 $\mu\text{g}/\text{ml}$ for 72 h. The residual activity after incubation was assayed at 30 °C by the UV 232-nm method and was plotted as the relative activity. Reaction mixtures also contained 10 mM sodium phosphate.

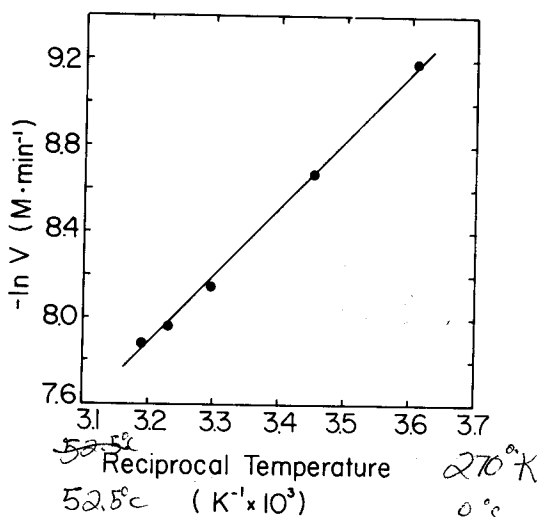


FIG. 10. Arrhenius plot of the temperature effect on heparinase activity. Initial rates (V) of the heparinase reaction were measured by the UV 232-nm method at temperatures of 4–41 °C. Reactions were conducted under conditions in which the enzyme (5.2 $\mu\text{g}/\text{ml}$) was saturated with substrate (heparin concentration, 24 mg/ml). An average molecular weight of 11,000 daltons was used for heparin to calculate its molar concentration. Reaction mixtures contained 10 mM sodium phosphate at pH 7.

the compound is desulfated by hydrolysis. The necessity of the sulfate group for either binding or reactivity becomes more evident as studies by several research groups show that heparinase cleaves the α -glycosidic linkages between a trisulfated disaccharide (2, 6). It is also interesting to note that heparinase possesses some properties resembling those discovered in a *Salmonella typhimurium* sulfate-binding protein (38). First, synthesis of both proteins is repressed by sulfate in the fermentation medium (38, 39). Second, the heparinase has a very low level of sulfur-containing amino acids (<2%), and the sulfate-binding protein has no such amino acids at all. It is not yet known whether other proteins acting upon sulfated substrates have characteristics and properties comparable to those of heparinase.

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