0273-2289/87/1613–0035$03.20

Large Scale Preparation and Characterization of Mucopolysaccharase Contamination Free Heparinase

VICTOR C. YANG,[1,2] HOWARD BERNSTEIN,[1,3] CHARLES L. COONEY,[1,3] AND ROBERT LANGER[1,4,*]

[1]Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; [4]Department of Surgery, Children's Hospital Medical Center, Boston, Massachusetts 02115; [2]College of Pharmacy, The University of Michigan, Ann Arbor, Michigan 48109-1065; [3]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

Received April 13, 1987; Accepted June 17, 1987

ABSTRACT

By a combination of hydroxylapatite chromatography and negative adsorption on QAE-Sephadex at pH 8.3, heparinase (E.C.4.2.2.7) can be successfully isolated from all the other mucopolysaccharase contaminants present in *Flavobacterium heparinum*. Hydroxylapatite isolates heparinase primarily from chondroitinases, hyaluronidase, and most glycuronidases. QAE-Sephadex chromatography at pH 8.3 further separates heparinase from heparitinases, sulfatases, and the remaining glycuronidases. The heparinase preparation thus obtained contains no statistically significant levels of other contaminating mucopolysaccharases except for heparitinases that are present at an apparent maximum level of 3.4%. Owing to the presence of a cross-reaction of heparinase on heparitin sulfate at conditions employed for the assay of heparitinase, the heparitinase level of 3.4% could be misleading because of the action of heparinase on heparitin sulfate. Characterization of this heparinase preparation shows that the enzyme has an optimum salt concentration of 0.08*M* NaCl, an optimum pH of 6.5, an activation energy of 5 kcal/mol, and a K_m of 7.95×10^{-6} M.

*Author to whom all correspondence and reprint requests should be addressed.

These parameters are almost identical to those displayed by a homogeneous heparinase preparation. The method described here is suitable for scale-up purposes using batch chromatographic procedures.

Index Entries: Heparinase (heparin lyase, E.C.4.2.2.7); mucopolysaccharases; *Flavobacterium heparinum*; batch chromatographic procedures; hydroxylapatite chromatography, negative adsorption on QAE-Sephadex; pH-Dependent Binding Analysis; mucopolysaccharase contamination free heparinase.

INTRODUCTION

Flavobacterium heparinum is recognized to contain a variety of mucopolysaccharases including heparinase (*1–3*), heparitinases (*3*), chondroitinases (A–C) (*4,5*), hyaluronidase (*4,6*), sulfatases (sulfoesterases and sulfamidase) (*1,7,8*), and glycuronidases (*2,9,10*). Heparinase, heparitinases, chondroitinases, and hyaluronidase are eliminases that act on mucopolysaccharides to yield α, β unsaturated uronic acids as the nonreducing terminals. On the other hand, sulfatases and glycuronidases are hydrolases that act further on the degradation products produced by the eliminases by a hydrolytic pathway. All these enzymes have been widely used in the identification and structural studies on mucopolysaccharides (*11–14*). Recently, our laboratory has demonstrated a potential therapeutic application of heparinase by placing an immobilized heparinase reactor at the termination of extracorporeal therapy for blood deheparinization (*15*). To test such a possibility, g quantities of heparinase free of the other mucopolysaccharase contaminants are required. Although several research groups (*16–18*) have reported the purification of heparinase from the other enzyme contaminants by successive column chromatographic procedures, such methods are time consuming and not suited for the preparation of large quantities of the enzyme.

In a previous publication (*19*), we described some preliminary observations suggesting that by a combination of two ion-exchange chromatographic procedures, g quantities of heparinase which is relatively pure (90–95% pure) from the other mucopolysaccharases can be prepared. We present here a detailed study of the methodology with regard to the development of such a purification method. We also report that by performing a QAE-Sephadex adsorption at pH 8.3, heparinase can be completely isolated from contamination by chondroitinases, hyaluronidase, glycuronidases, and sulfatases. Although a low level (3.4%) of heparitinase activity was observed in the resulting heparinase preparation, this activity is believed to be primarily a result of the crossreaction of heparinase on heparitin sulfate under the assay conditions. The heparinase thus prepared displays identical catalytic properties and kinetics to those of the electrophoretically homogeneous heparinase.

EXPERIMENTAL

Materials

Heparin (sodium salt, 150 USP U/mg from porcine intestinal mucosa) was purchased from Hepar Industries. Heparitin sulfate was generously supplied by Cifonelli of Wyler Children's Hospital, Chicago, IL. Trisulfated disaccharide was prepared by complete digestion of heparin with heparinase and purified according to the procedures of Grant et al. (*20*). Chondroitin 4- and 6-sulfates, dermatan sulfate, hyaluronic acid, *p*-nitrocatechol sulfate, QAE-Sephadex A-50, and cellulose phosphate were obtained from Sigma. Bio-Rad dye reagent, hydroxylapatite (Bio-Gel HTP), Bio-Lyte 3/10, and chemicals for polyacrylamide gel electrofocusing were purchased from Bio-Rad Laboratories. Test-mixtures (pI markers) were from Serva. All other chemicals were reagent grade, and water was twice distilled.

Methods

Assays

Protein concentrations were measured either by the method of Lowry et al. (*21*) or by the Bio-Rad protein assay (*22*). Heparinase, heparitinase, chondroitinase, and hyaluronidase activities were assayed by quenching the enzymatic reaction at various times with 0.03*N* HCl, then measuring the increase in ultraviolet absorption at 232 nm as described previously (*23*). Substrates were prepared at 7 mg/mL in "assay mixture" containing 0.25*M* sodium acetate, 0.0025*M* calcium acetate at pH 7. Glycuronidase activities were monitored by the rate of disappearance of absorption at 232 nm (*23*), using trisulfated disaccharide (5 mg/mL in "assay mixture") as the substrate. Sulfatase activities were assayed by the procedures of Ototani et al. (*18*), using *p*-nitrocatechol sulfate as the substrate. Since phosphate ions can inhibit sulfatase activity at a concentration as low as 0.03*M* (*8*), all samples for sulfatase assays were dialyzed against distilled water overnight to avoid any possible phosphate inhibition. One International U of enzymatic activity is defined as the amount of enzyme that causes one μmol of products to be formed/min, based on a molar extinction coefficient of $5.1 \times 10^3\ cm^{-1}\ M^{-1}$ at 232 nm for the degradation products, the α, β unsaturated uronic acids (*24*), and of $1.07 \times 10^4\ cm^{-1}\ M^{-1}$ at 515 nm for *p*-nitrocatechol (*25*). All enzymatic activities were measured at 37°C, with the exception of heparitinase which was measured at 43°C. This is to limit the crossreaction of heparinase on heparitin sulfate, since at 43°C heparitinase activity was maximized but the heparinase activity was minimized (*16*). The relative content of an enzyme was estimated by the amount of activity units present in each mL of the protein solution, as compared to that of heparinase

(100%). A relative content of less than 0.4 ± 0.1% is considered to be statistically insignificant, since a change in absorbance of less than 0.01 OD U/U T cannot be significantly determined spectrophotometrically.

Preparation of Crude Cell Extract

Cultures of *Flavobacterium heparinum* were grown according to the procedures of Galliher et al. (*26*). Crude cell extract was prepared by homogenization and protamine sulfate precipitation (*19*).

Batch Hydroxylapatite Chromatography

Four hundred g of hydroxylapatite preequilibrated with 10 m*M* phosphate buffer (pH 6.8) were mixed with 4 L of the crude cell extract (protein concentrations: 25 g/L; heparinase activity: about 7,000 U/L), and then washed stepwise with 5 L of buffers of increasing ionic strength. The solution employed for the sequential elutions were prepared by mixing 10 m*M* sodium phosphate buffer (pH 6.8) with 0.25*M* sodium phosphate buffer (pH 6.8) containing 0.5*M* NaCl, according to a ratio (v/v) of 8/0, 7/1, 6.5/1.5, 6/2, 5.5/2.5, 5/3, 4.5/3.5, 4/4, and 0/8, respectively (*19*).

Batch Ion Exchange Chromatography

A variety of ion-exchangers have been tested to further purify the hydroxylapatite-treated heparinase. One L of the enzyme preparation (protein concentration: 0.3–0.4 mg/mL) obtained from pooling of the 6.5/1.5 and 6/2 elutions of the hydroxylapatite chromatography was concentrated to 100 mL with a Diaflo Model 402 ultrafilter equipped with a YM 30 membrane (mol wt cutoff: 30,000 dalton). The concentrated solution then was dialyzed against 50 vol of 10 m*M* phosphate buffer (pH 6.8) overnight. Aliquots of 10 mL of the dialyzed sample were loaded onto 0.5 g of each of the ion-exchanger preequilibrated with the same buffer. For the chromatography on hydroxylapatite or on a cation-exchanger such as CM-Sephadex, SP-Sephadex and cellulose phosphate, elution was followed according to the procedures described in the previous section, using 5 mL of the eluent for each washing step. For the chromatography on an anion-exchanger such as DEAE-Sephadex and QAE-Sephadex, the protein solution was collected directly by centrifugation and no further elution was necessary. For QAE-purification performed at higher pH (pH 8.3), the above dialyzed sample was redialyzed for 2 h against 50 vol of 10 m*M* phosphate buffer at pH 8.3, then loaded onto QAE-Sephadex pretreated to pH 8.3 with the same buffer. The eluent thus obtained was assayed for protein concentration and heparinase activity.

Isoelectric Focusing

Gels containing 5% acrylamide and 2% Bio-Lyte 3/10 were prepared in the Bio-Rad CTL Casting System. The gel was placed on the Bio-Rad Model 1415 Cell connected to a refrigerated circulator precooled to 4–10°C. The anode was placed onto the wick wetted with 1*N* H_3PO_4 and

the cathode was placed onto the wick wetted with 1*N* NaOH. The gel was prefocused at 250 V for 90 min. Samples to be focused were dialyzed against distilled water for 24 h, and aliquots of 10 μL were applied in the middle of the gel after prefocusing. Isoelectric focusing was carried out at a starting voltage of 250 V for 6 h. At the end of the run, the voltage rose to 1000 V. The pH gradient was measured using a test-mixtures containing cytochrome C (pI 10.65), ribonuclease (pI 9.45), whale myoglobin (pI 8.3, 7.7), horse myoglobin (pI 7.3, 6.9), conalbumin (pI 5.9), β-lactoglobulin (pI 5.34), bovine albumin (pI 4.7), ferritin (pI 4.4), and amyloglucosidase (pI 3.5), and was also checked by the use of a Bio-Rad surface pH electrode. The gel was fixed with 12.5% trichloroacetic acid, 4% sulfosalicyclic acid for 1 h, stained with a solution of 0.04% Coomassie blue R-250 dissolved in 27% isopropanol, 10% acetic acid, and 0.5% $CuSO_4$ for 2 to 3 h, and then destained by repeated washings with 7% acetic acid in 12% isopropanol.

pI Measurements

Isoelectric points (pI) of heparinase, chondroitinases, and hyaluronidase were measured by chromatofocusing on a PBE 94 column (1 × 10 cm) with polybuffer 96 as the eluent according to procedures described earlier (*19*). Isoelectric points of heparitinases, sulfatases, and glycuronidases were measured by the pH-Dependent Binding Analysis (*27*).

RESULTS

Table 1 shows the relative contents of the contaminating enzyme activities in the crude extract of *Flavobacterium heparinum*, as compared to the heparinase activity (100%) on the basis of the units present in each of the preparations. The crude extract contained a relatively high amount of the other mucopolysaccharases, with the exception of chondroitinase B which was present at a barely detectable level (<2%). Although heparitinases were assayed at 43°C, a temperature at which the heparinase activity was minimized (*16*), a certain level of activity resulting from the crossreaction of heparinase on heparitin sulfate was still observed (data are not shown). It is also important to note that sulfatase assay measures the total sulfatase activities, including sulfoesterases and sulfamidase (*18*).

Linker and Hovingh (*16*) successfully isolated heparinase from heparitinases, chondroitinases, and hyaluronidase by hydroxylapatite column chromatography. Ototani et al. (*18*) have reported similar findings. Based on these results, hydroxylapatite chromatography was chosen as the first step for heparinase purification. An additional reason for selecting hydroxylapatite is that the purification can be easily scaled-up by converting from a column technique to a batch process. The elution patterns of the enzymes contained in the crude extract of *Flavobacterium heparinum* on batch hydroxylapatite chromatography are illustrated in Fig. 1.

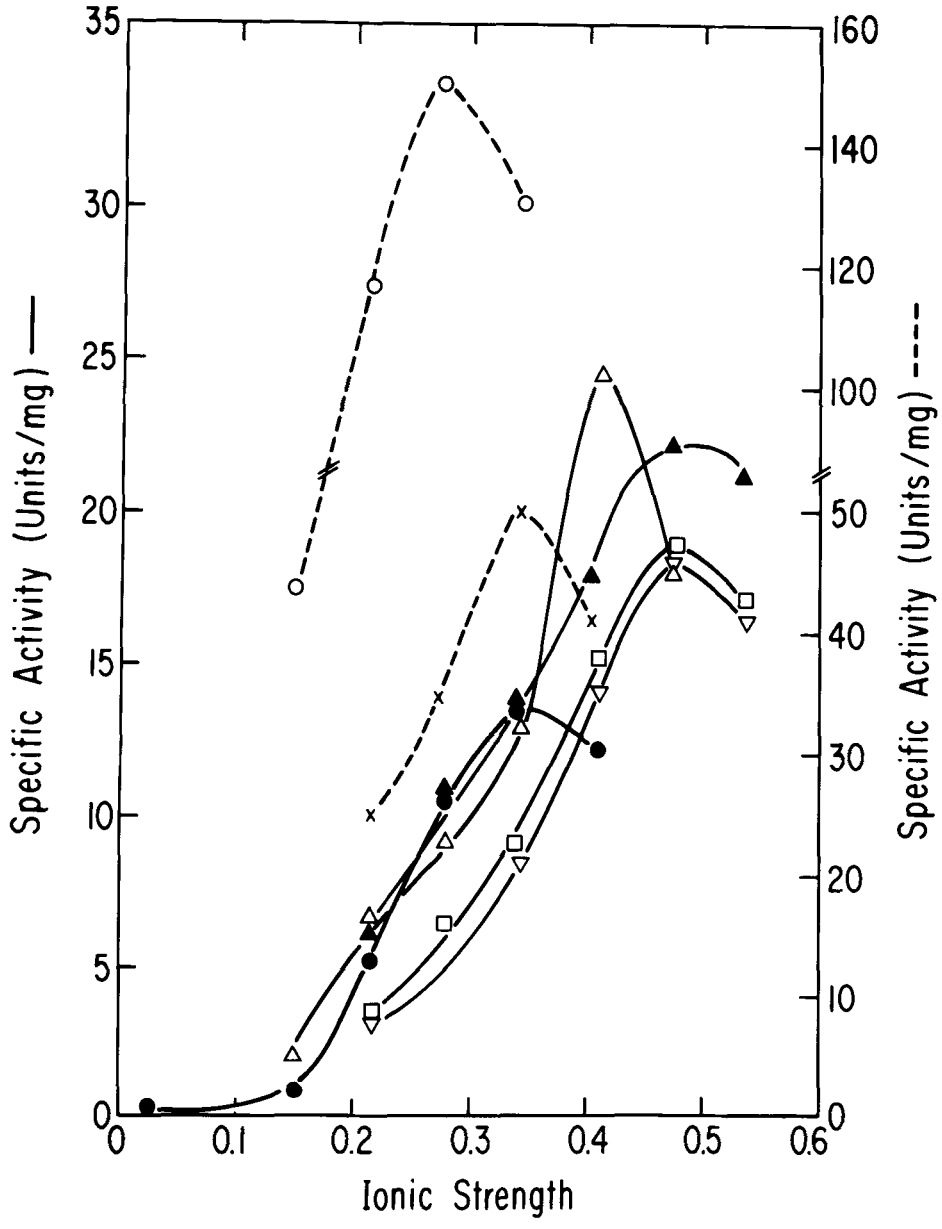

Fig. 1. Hydroxylapatite chromatography of the glycosaminoglycan-degrading enzymes present in the crude extract of *Flavobacterium heparinum*. The experimental procedures are described under "Methods": O------O, heparinase activity; X------X, heparitinase activity; △—△, glycuronidase activity; □—□, chondroitinase A activity; ▽—▽, chondroitinase C activity; ●—●, sulfatase activity; and ▲—▲, hyaluronidase activity.

As shown in this figure, heparinase was well separated from chondroitinases, hyaluronidase, and glycuronidases, but not from heparitinases and sulfatases. Table 1 shows that more than 70% of the initial relative activities attributed to chondroitinase A, chondroitinase C, hyaluronidase, and glycuronidase was removed by hydroxylapatite chromatography, whereas only minor amounts of heparitinases and sulfatases were eliminated. The resulting preparation was not free from other mucopolysaccharase contamination and further purification steps were required.

Ion-exchange chromatography based on batch procedures is a proven technique suitable for large scale protein purification and was selected as the second step for heparinase purification. A variety of cation- and anion-exchange resins were examined, and the results are shown in Table 2. Owing to the cationic nature of heparinase (pI 8.5) at neutral

Table 1
Relative Content of the Glycosaminoglycan-Degrading Enzymes in Different Preparations of *Flavobacterium heparinum*

Enzyme	Relative content[a], %	
	Crude Extract	Hydroxylapatite Preparation
Heparinase	100	100
Heparitinases	33.9	26.4
Chondroitinase A	14.1	3.5
Chrondroitinase B	1.9	0[b]
Chrondoritinase C	15.4	3.6
Hyaluronidase	26.7	8.2
Glycuronidases	15.3	6.0
Sulfatases	8.4	7.9

[a]Relative content was estimated by the U of activity present in each mL of the preparation, as compared to that of heparinase (100%).

[b]As described in the "Methods", a relative content of less than 0.4 ± 0.1% is considered to be statistically insignificant, and is therefore defined as zero content.

Table 2
Further Purification of the Hydroxylapatite-Purified Heparinase by Other Ion-Exchange Chromatography Resins

Method	Total Protein, mg	Specific Activity, U/mg	Total Activity, U	Recovery of Activity, %
Initial Sample	25	1.2	30.7	100
CM-Sephadex	7.1	1.6	12.8	39.2
SP-Sephadex	3.0	3.5	10.4	33.8
Cellulose Phosphate	2.1	4.2	8.8	28.7
Hydroxylapatite	7.1	1.7	12.8	39.2
DEAE-Sephadex	7.2	3.2	22.6	73.9
QAE-Sephadex	6.5	4.0	26.0	84.7

pH, chromatography by anion-exchangers such as QAE-Sephadex and DEAE-Sephadex at neutral pH has resulted in a "negative adsorption" (i.e., to preferentially adsorb contaminants). Contaminant removal by the anion-exchanger is superior to the previously used heparinase adsorption to cation-exchangers, because it involves a single step (i.e., no elution from exchanger is required) and provides the highest activity recovery (Table 2). Among the anion-exchangers, QAE-Sephadex is slightly better than DEAE-Sephadex with regard to activity recovery. This probably is because of the stronger ion-exchange capacity of the QAE-Sephadex. Isoelectric focusing of the hydroxylapatite preparation showed that over 70% of the proteins were acidic with their pI values below 7.0 (Fig. 2A). QAE-adsorption at pH 6.8 appears to significantly

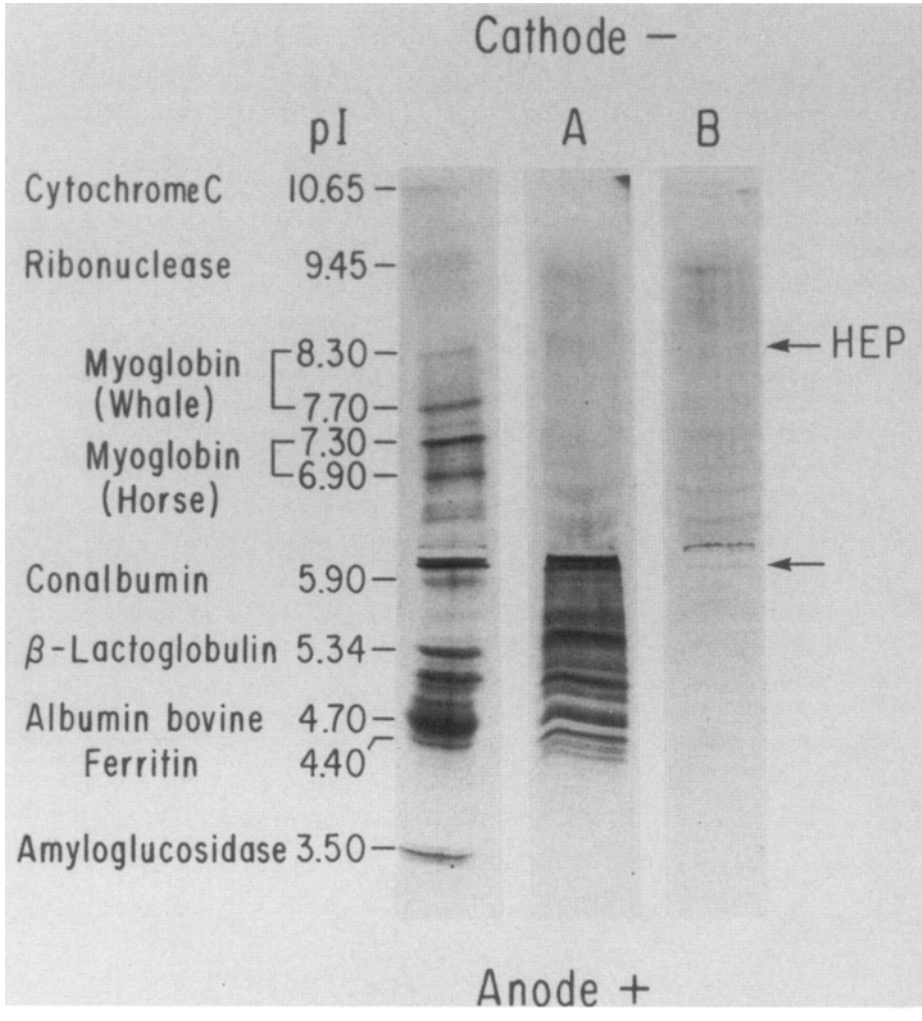

Fig. 2. Isoelectric focusing of (A) the hydroxylapatite-preparation, and (B) the QAE-preparation (performed at pH 6.8) of *Flavobacterium heparinum*. The experimental procedures are described under "Methods." Arrow indicates the position where the sample was applied.

remove all the protein bands possessing isoelectric points below 6.8 (Fig. 2B). The QAE-purification therefore removes the protein impurities on the basis of their charges and pI values. In order to determine the best conditions for QAE-purification, the pI of mucopolysaccharases present in *Flavobacterium heparinum* were measured. Table 3 lists the eluting ionic strength from hydroxylapatite chromatography and the pI for these enzymes. From a comparison of the pI, QAE-purification at neutral pH would be expected to remove most of the sulfatase and glycuronidase activities. However, isolation of heparitinase activity from the hydroxylapatite preparation requires QAE-adsorption at a pH higher than pH 7.9 which is the pI of heparitinases. The results shown in Table 4 are consistent with this expectation. The solution obtained from the QAE-adsorption step at pH 6.8 contained low amounts of glycuronidases (1.5%) and sulfatases (1.5%), and a high amount of heparitinases (25%). Adsorption by QAE-Sephadex at pH 8.3 reduced the heparitinase contamination by more than sevenfold, and completely eliminated the sulfatase and glycuronidase contamination.

Table 5 summarizes the results of a large scale heparinase purification obtained using 4 L of a solution collected from a pool of the 6.5/1.5 and 6/2 elutions from the hydroxylapatite step. The solution was ultrafiltered to 400 mL, dialyzed against 10 vol of 10 m*M* phosphate buffer (pH 8.3) 3 times over 4 h, and added to 20 g of QAE-Sephadex pre-equilibrated at pH 8.3 with the same buffer. As shown in Table 5, the heparinase was purified about 30-fold from the crude extract. The overall mass yield was 1.2%, and the recovery of the total heparinase activity was 34%. The resulting preparation contains no other mucopolysaccharase

Table 3
Eluting I from Hydroxylapatite Chromatography and pI of Glycosaminoglycan-Degrading Enzymes in *Flavobacterium heparinum*

Enzyme	Eluting I[a]/Washing Step[b]	pI
Heparinase	0.28/(6/2)	8.5[c]
Heparitinases	0.35/(5.52/2.5)	7.9[d]
Chondroitinase A	0.48/(4.5/3.5)	8.7[c]
Chondroitinase B	ND[e]	ND[e]
Chondroitinase C	0.48/(4.5/3.5)	8.7[c]
Hyaluronidase	0.48/(4.5/3.5)	8.7[c]
Glycuronidases	0.42/(5/3)	6.6, 8.3[d]
Sulfatases	0.35/(5.5/2.5)	6.3[d]

[a]Data are from Fig. 1. The eluting I represents that of the washing step where the activity peak occurs.

[b]For details of the washing steps used, see the "Batch Hydroxylapatite Chromatography" under the section of "Methods."

[c]pI was determined by chromatofocusing.

[d]pI was determined by the pH-Dependent Binding Analysis (*19*).

[e]N.D. means Not Determined.

Table 4
Relative Content of the Glycosaminoglycan-Degrading Enzymes in the QAE-Sephadex Preparation of *Flavobacterium heparinum*

Enzyme	Relative Content[a], %	
	QAE-Chromatography, at pH 6.8	QAE-Chromatography, at pH 8.3
Heparinase	100	100
Heparitinases	24.9	3.4
Chondroitinase A	0.7	0[b]
Chondroitinase B	0[b]	0[b]
Chondroitinase C	0.6	0[b]
Hyaluronidase	1.2	0[b]
Glycuronidaseses	1.5	0[b]
Sulfatases	1.5	0[b]

[a]Relative content was estimated on the basis of the amount of activity U present in each mL of the preparation, as compared to that of heparinase (100%).

[b]As described in the "Methods", a relative content of less than 0.4 ± 0.1% is considered to be statistically insignificant, and is therefore defined as zero content.

Table 5
Summary of the Large Scale Heparinase Purification

Purification Step	Total Protein g	Specific Activity, U/mg	Purification Fold/Crude Extract	Total Activity, U	Recovery of Activity, %
Crude Extract	100	0.28	—	28,000	100
Hydroxylapatite	7.8	1.33	4.75	10,370	37.1
QAE-Sephadex (at pH 8.3)	1.2	7.80	27.85	9,500	33.4

activity except for heparitinase, which is present at a maximum level of 3.1%. These results are consistent with those shown in Table 4 where the purification was carried out on a small scale.

Characterization of the QAE-purified heparinase activity showed the optimum salt concentration was 0.08*M* NaCl (Fig. 3A), and the optimum pH was 6.5 (Fig. 3B). The reaction kinetics of the QAE-purified heparinase are shown by a Lineweaver-Burk plot (Fig. 4). The K_m and V_m values, obtained at 37°C, pH 7 and a protein concentration of 1.6 μg/mL, were $7.95 \times 10^{-6} M$ and 3.82×10^{-7} *M*/min, respectively. The Arrhenius plot of the heparinase activity vs temperature over the range of 20–40°C gave an activation energy of 5 kcal/mol (Fig. 5). These results are in excellent agreement with the values of an optimum salt concentration of 0.1*M* NaCl, an optimum pH of 6.5, a K_m of 8.04×10^{-6} *M*, and an activation energy of 6 kcal/mol reported for the electrophoretically homogeneous heparinase preparation (*19*). Since the presence of trace amounts of cer-

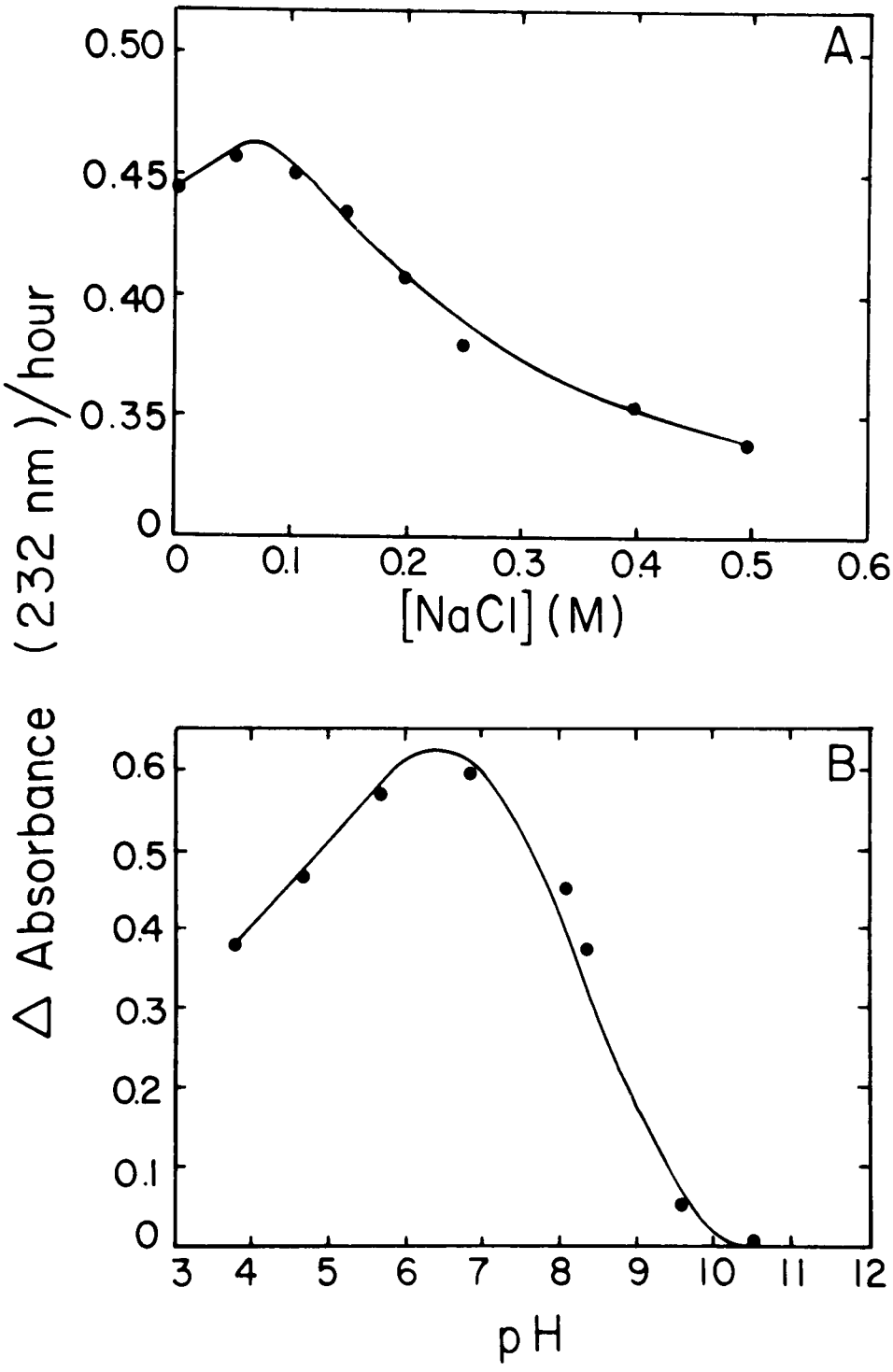

Fig. 3. Effect of (A) salt concentration, and (B) pH on the activity of the QAE-purified (at pH 8.3) heparinase. In (A), activity was determined at pH 7, a protein concentration of 15 μg/mL, and at the indicated salt concentration. In (B), activity was determined at the indicated pH and a protein concentration of 20 μg/mL. The reaction mixture also contained 10 m*M* phosphate.

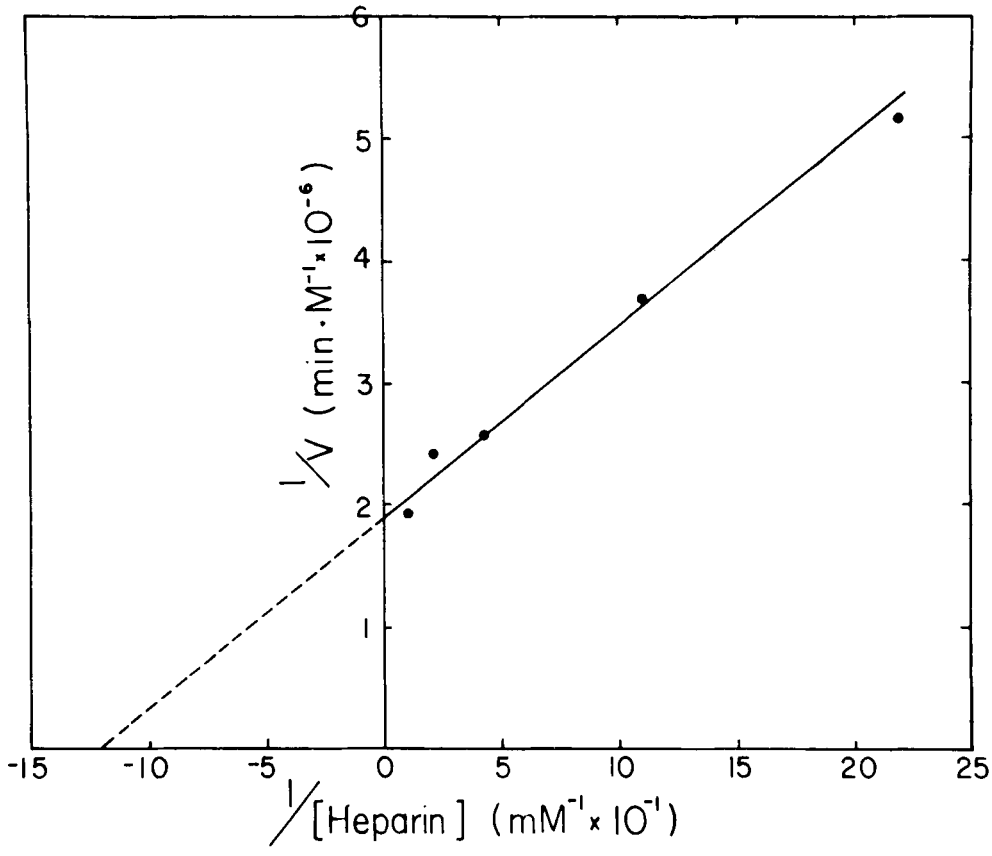

Fig. 4. Lineweaver-Burk plot of the initial rate of the reaction catalyzed by the QAE-purified (at pH 8.3) heparinase as a function of heparin concentration. Initial rates (V) were measured at the indicated heparin concentration. Protein concentration in the reaction mixture was 1.6 μg/mL. An average mol wt of 11000 daltons was used for heparin to calculate its *M* concentration.

tain contaminating enzymes such as glycuronidases would affect the kinetics of the heparinase catalyzed reaction, the agreement in kinetic data between the QAE-purified heparinase and the electrophoretically homogeneous heparinase suggests a high catalytic purity for the QAE-purified heparinase.

DISCUSSION

By a combination of hydroxylapatite chromatography and QAE-Sephadex adsorption, heparinase can be purified from the other mucopolysaccharases. Hydroxylapatite separates heparinase from chondroitinase, hyaluronidase, and glycuronidase contamination on the basis of different binding abilities. QAE-Sephadex adsorption further separates heparinase from heparitinases, sulfatases, and the remaining glycuronidases on the basis of their net charge at the adsorption pH. Purification by QAE-adsorption at pH 8.3 enhances the elimination of the foregoing

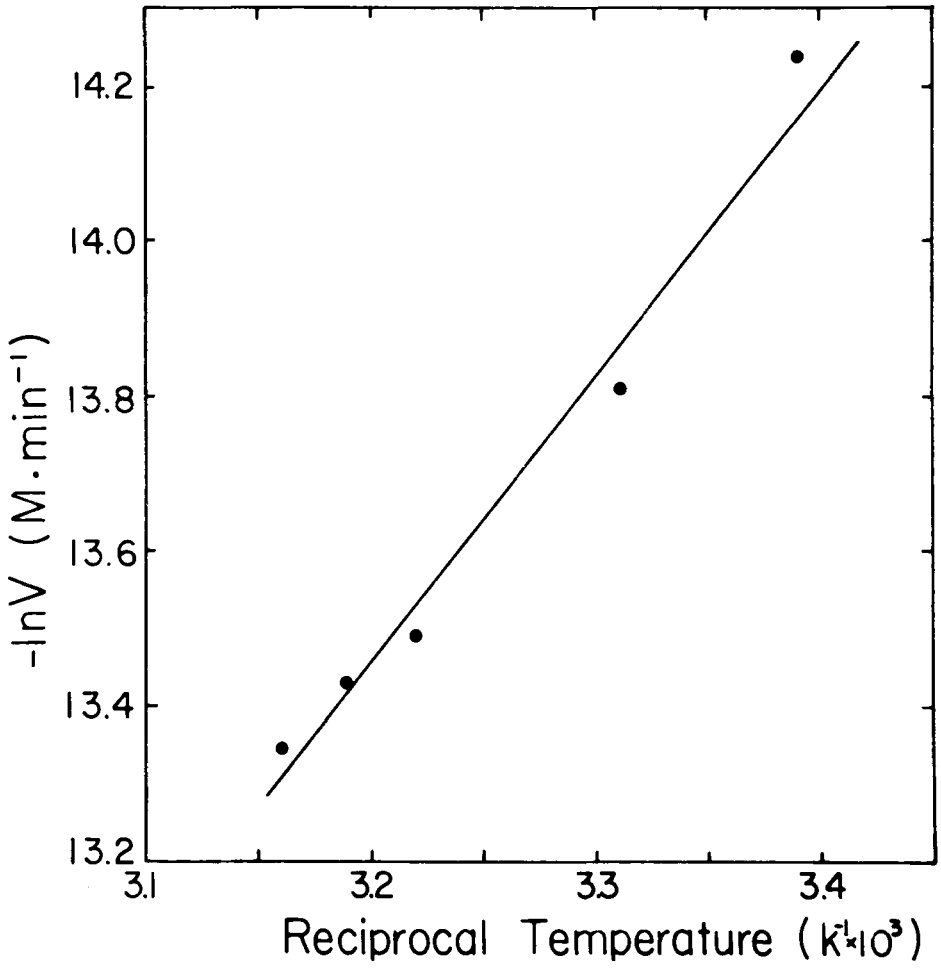

Fig. 5. Arrhenius plot of the temperature effect on the activity of the QAE-purified (at pH 8.3) heparinase. Initial rates (V) of the heparinase reaction were measured at temperatures of 20–40°C. Reactions were performed under conditions in which the enzyme (3.9 μg/mL) was saturated with substrate (heparin concentration: 11.4 mg/mL). An average mol wt of 11000 daltons was used for heparin to estimate its *M* concentration. The reaction mixture contained 10 m*M* sodium phosphate at pH 7.

enzymes, especially heparitinases. The resulting heparinase preparation contains no statistically significant levels of chondroitinases, hyaluronidase, glycuronidases, and sulfatases, and a maximum level of 3.4% of heparitinases. The heparitinase activity may primarily result from a crossreaction of heparinase on heparitin sulfate under the assay conditions.

Several authors (*4,5,18*) reported the copurification of chondroitinase A, chondroitinase C, and hyaluronidase activities throughout their preparation of these enzymes from *Flavobacterium heparinum*. It was suggested therefore that the activities might be attributed to a single protein (chondroitinase AC) acting on chondroitin 4- and 6-sulfates, as well as hyaluronic acid (*5,18*). The present study supports their findings. The ac-

tivities of chondroitinase A, chondroitinase C, and hyaluronidase were not separated by hydroxylapatite chromatography (Fig. 1). In addition, all these enzymes possess the same isoelectric point (pI 8.7). However, if indeed a single protein is responsible for all these activities, our results suggest that the protein is more specific for hyaluronic acid (a hyaluronidase?), simply because it shows a higher relative activity towards hyaluronic acid (26.7%) than towards chondroitin 4- (14.1%) or 6-sulfates (15.4%) (Table 1). Although this protein has a high isoelectric point (pI 8.7) and would presumably not bind to an anion-exchanger such as QAE-Sephadex, Table 4 showed that it was completely removed from the hydroxylapatite preparation by the QAE-Sephadex adsorption at pH 8.3. This may be accounted for in terms of the presence of a different charge form for the protein in the hydroxylapatite-purified heparinase preparation. Ototani et al. (*18*) reported that chondroitinase AC was separated into a major and minor peak in the hydroxylapatite purification of the crude cell extract from *Flavobacterium heparinum*. The minor peak was eluted at an ionic strength close to that of heparinase, whereas the major peak was eluted at a much higher ionic strength. The presence of a different eluting ionic strength for the minor peak suggests that the protein molecule in the minor peak are present in a charge form (or a pI value) differing from that in the major peak. The minor peak of chondroitinase AC was copurified with heparinase in the hydroxylapatite purification, and since it might have a more acidic pI value than 8.7, the protein was completely removed by the QAE-adsorption at pH 8.3.

The method described in this paper is suitable for production of large quantities (g) of mucopolysaccharase contamination free heparinase. Although batch procedures are sometimes less efficient in resolution than column techniques, they are adopted in order to scaleup the enzyme production from mg quantities produced by a column to g quantities. The enzyme was purified 30-fold from the crude extract and the activity recovery was 33.4%. The major loss of activity (63%) occurs with the hydroxylapatite step. The QAE-Sephadex step only results in a 3.6% activity loss. Thirty percent of the activity loss in the hydroxylapatite step is a result of the isolation of heparitinases from the heparinase fraction. Unlike the other contaminating enzymes, which differ from heparinase by a wide margin either in their eluting ionic strength (in hydroxylapatite adsorption) or in their pI, heparitinases are quite close to heparinase and can be separated only by a combination of hydroxylapatite and QAE-Sephadex chromatography. Despite a significant elution of heparinase with 5.5/2.5 (I: 0.35) washing step from hydroxylapatite chromatography (Figure 1) only the washings of 6.5/1.5 to 6/2 (I: 0.22–0.28) were collected. This is done to minimize heparitinase contamination. For preparations whose heparitinase level is not critical, the total activity recovery can be improved to as high as 50%, simply by including the 5.5/2.5 elution in the QAE-Sephadex purification.

ACKNOWLEDGMENTS

We thank Alfred Linker (Veterans Administration Hospital, Salt Lake City, UT) for generously providing the agar slants of *Flavobacterium heparinum*, Cifonelli of Wyler Children's Hospital, Chicago, IL for kindly supplying the heparitin sulfate, and Elizabeth Norton of the Massachusetts Institute of Technology for assistance in bacterial fermentation. The work was supported by Grant GM 25810 from the National Institutes of Health.

REFERENCES

1. Korn, E. D. and Payza, A. N. (1956), *J. Biol. Chem.* **223,** 859–864.
2. Dietrich, C. P. (1969a), *Biochemistry* **8,** 2089–2094.
3. Hovingh, P. M. and Linker, A. (1970), *J. Biol. Chem.* **245,** 6170–6175.
4. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968), *J. Biol. Chem.* **243,** 1523–1535.
5. Michelacci, Y. M. and Dietrich, C. P. (1974), *Biochem. Biophys. Res. Commun.* **56,** 973–980.
6. Michelacci, Y. M. and Dietrich, C. P. (1976), *J. Biol. Chem.* **251,** 1154–1158.
7. Lloyd, A. G., Law, B. A., Fowler, L. J., and Emberg, G. (1968), *Biochem. J.* **110,** 54p.
8. Dietrich, C. P. (1969b), *Biochem. J.* **111,** 91–95.
9. Linker, A. and Hovingh, P. (1965), *J. Biol. Chem.* **240,** 3724–3728.
10. Warnick, C. T. and Linker, A. (1970), *Fed. Proc.* **29,** 675.
11. Silva, M. and Dietrich, C. P. (1975), *J. Biol. Chem.* **250,** 6841–6846.
12. Linker, A. and Hovingh, P. (1979), *Heparin: Structure, Cellular Functions and Clinical Application,* McDuffie, N. M., ed., Academic, NY, pp. 3–24.
13. Oik, Y., Kimata, K., Shinomura, K., and Suzuki, S. (1980), *Biochem. J.* **191,** 193–202.
14. Toole, B. P. (1981), *Cell Biology of Extracellular Matrix,* Hay, E. D., ed., Plenum, NY, pp. 259–294.
15. Langer, R., Linhardt, R., Hoffberg, S., Larsen, A. K., Cooney, C. L., Tapper, D., and Klein, M. (1982), *Science* **217,** 261–263.
16. Linker, A. and Hovingh, P. (1972a), *Meth. Enzymol.* **28,** 902–911.
17. Dietrich, C. P., Silva, M. E., and Michelacci, Y. M. (1973), *J. Biol. Chem.* **248,** 6408–6415.
18. Ototani, N., Kikuchi, M., and Yosizawa, Z. (1981), *Carbohydr. Res.* **88,** 291–303.
19. Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L., and Langer, R. (1985a), *J. Biol. Chem.* **260,** 1849–1857.
20. Grant, A. C., Linhardt, R. J., Fitzgerald, G. L., Park, J. J., and Langer, R. (1984), *Anal. Biochem.* **137,** 25–32.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
22. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
23. Linhardt, R. J., Fitzgerald, G. L., Cooney, C. L., and Langer, R. (1982), *Biochim. Biophys. Acta* **702,** 192–203.

24. Linker A. and Hovingh, P. (1972b), *Biochemistry* **11,** 563–568.
25. Cerbelaud, E. (1983), M.S. Dissertation, Massachusetts Institute of Technology, pp. 86–88.
26. Galliher, P. M., Cooney, C. L., Langer, R. S., and Linhardt, R. J. (1981), *Appl. Environ. Micro.* **41,** 360–365.
27. Yang, V. C. and Langer, R. (1985b), *Anal. Biochem.* **147,** 148–155.