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An Immobilized Microbial Heparinase for Blood Deheparinization

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Abstract

A new medical application of an immobilized microbial enzyme is described. Extracorporeal devices require systemic heparin administration to prevent thrombus formation; however, the use of heparin often leads to serious hemorrhagic complications. Heparinase isolated from Flavobacterium has been immobilized and used in a fluidized bed reactor to eliminate heparin from blood passing through an extracorporeal circuit both in vitro and in vivo. This paper discusses the stepwise development of this heparinase reactor including: (1) improvements in the fermentation resulting in an inexpensive large-scale source of heparinase without the addition of the previously required inducer, heparin; (2) the use of batch processes to adapt previous purification schemes to large-scale heparinase production and the subsequent purification of heparinase to a single SDS-PAGE banding protein; (3) the immobilization of heparinase with a 91% activity recovery and good stability, (4) the design and successful testing of a fluidized bed reactor containing immobilized heparinase in the removal of clinically used quantities of heparin from both human blood in vitro and canine blood in vivo; and (5) the initiation of animal studies focusing on the toxicology of heparinase-derived heparin degradation products and the short and long term effects of exposure to these products and to heparinase.

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Index Entries: Medical applications of enzymes; extracorporeal circulation; blood deheparinization; heparin; *Flavobacterium heparinum;* heparinase; immobilized heparinase; deheparinization.

Introduction

The potential therapeutic importance of microbial enzymes has gained increased attention with the growing availability and reduced cost of a wide variety of highly purified enzymes such as streptokinase (*1*). The main criticism of proposed medical uses of microbial enzymes has centered around the presumed antigenicity of these foreign proteins. In an effort to avoid immunological complications, most medical applications of microbial enzymes have involved immobilized enzymes. These immobilized enzymes, used to eliminate or reduce toxic substances, include aspariginase (*2*), phenyalanine ammonium-lyase (*3*), urease (*4*), catalase (*5*), and carbonic anhydrase (*6*).

In order for microbial enzyme therapy to be useful, these enzyme systems must not only be safe and effective, but they must fulfill an important need that cannot be adequately addressed by other more conventional forms of therapy. The research reported here describes an effort to solve such a problem by an approach that may serve as a model for future medical applications of microbial enzymes.

There are nearly 20 million perfusions involving extracorporeal medical machines (e.g., artrificial kidney, pump oxygenator) performed each year. The artificial kidney is employed by over 100,000 persons several days each week (*7*). Open-heart operations employing cardiopulmonary bypass on the heart–lung machine are an everyday occurrence at many major medical centers (*8*). Still other newer applications of extracorporeal devices are in either the developmental or early stages of clinical trials (*9*).

In all of these extracorporeal devices, systemic levels of heparin are required to prevent thrombus formation. The use of heparin leads to hemorrhagic complications such as bleeding as much as 8–33% of the time (*10*). Heparin has been implicated as the drug most responsible for the greatest number of deaths in otherwise healthy patients (*11*). In this paper a method of controlling blood heparin levels, using immobilized microbial heparinase that specifically degrades heparin, is discussed.

Materials and Methods

All chemicals used in the fermentations were obtained from sources described in refs. (*14*) and (*18*). Hydroxylapatite (HA) and all chemicals and equipment used in electrophoresis were obtained from Biorad Inc. Chromatofocusing supplies, Sepharose, Sephadex, and Heparin–Sepharose were obtained from Pharmacia Inc. Cellulose phosphate was supplied by Whatman Inc. Controlled-pore dialysis bags were obtained from Spectrum Scientific Co. Epoxyactivated polyacrylic was obtained from Rohm-Pharma GmbH Darmstadt. Polyacrylamide PAN 1000 was a

generous gift of Dr. G. M. Whitesides. Thrombofax aPTT reagent was obtained from Ortho Inc. Azure A was purchased from Fisher Scientific Co. Porcine mucosal heparin sodium salt (grade II) was from Sigma Chemical Co. Complete blood counts (CBC) were performed on a Coulter Counter by the hematology laboratory at Brigham and Women's Hospital, Boston. Human blood was obtained from Children's Hospital Blood Bank, Boston.

Assays for Protein Content

Protein was measured by both the Lowry (*12*) and the Coomassie blue dye (*13*) assays.

Assays for Heparinase Activity

Several procedures were used to follow heparin in heparinase assays. These assays measured: (1) the disappearance of heparin, as judged by metachromasia on Azure A dye was used for determining heparin concentrations in crude fermentation preparations (*14*) and in plasma (*15*); (2) the appearance of heparin degradation products by measuring their end group chromaphore at 232 nm (*16*) is used for following the purfication of heparinase; and (3) the loss of heparin's anticoagulant activity by activated partial thromboplastin time (aPTT) is used in the in vitro and in vivo blood deheparinization studies (*17*).

One unit of heparinase activity is defined as the amount of enzyme that can degrade 1 mg of heparin completely (cleaved at an average of nine sites) per hour (for an explanation of why the International Unit is not used see ref. *18*).

Heparinase Production

Flavobacterium heparinum, a gram-negative, nonmotile, nonspore-forming rod (*14,18*) stored on agar slants as previously described (*14*), produced heparinase (EC 4.2.2.7) when grown in either a defined medium containing glucose, amino acids, salts (including sulfate), and heparin (as an inducer) (*14*) or a defined medium containing glucose, amino acids, salts (excluding sulfate) without added inducer (*18*).

Heparinase Purification

The cell pellet obtained from the fermentation was disrupted sonically (*14*), the nucleic acids precipitated with protamine sulfate (*14*), and bound (4 g support/g protein) to hydroxylapatite (HA), and then washed in a stepwise batch process with increasing concentrations of sodium chloride and sodium phosphate at pH 7.0 [from 0.0 and 0.01*M* to 0.50 and 0.25*M*, respectively (*16*)]. The HA heparinase was obtained in the 0.125*M* NaCl, 0.07*M* sodium phosphate wash. The HA purified heparinase was used in all immobilizations, in the in vitro and in vivo studies, and in all subsequent purification steps.

HA heparinase (200 mL,0.34 mg/mL, $I < 10$ m*M*) was bound batchwise to 16.8 mL of ion-exchange resin PBE 94 adjusted to pH 9.4, then packed into a 1.5 cm × 15.0 cm column and eluted with ampholyte Polybuffer 96–acetic acid adjusted to

pH 6.0. Chromatofocusing (CF) resulted in heparinase activity between pH 8.0 and 8.4.

HA heparinase (pH 7.0, I $<$ 10 m*M*) was bound to cellulose phosphate (CP) (1 gm support/3 mg protein) batchwise, and washed in a stepwise fashion with increasing concentrations of sodium chloride (from 0.0 to 0.3*M*) in 50 m*M* sodium phosphate at pH 7.0. The resulting CP purified heparinase was obtained in the 0.24*M* NaCl, 0.05*M* sodium phosphate wash.

HA heparinase (1 mL, 0.34 mg/mL, I $<$ 10 m*M* pH 7.0) was bound to 0.8 mL Heparin–Sepharose-CL-6B and packed into a 1.5 $\times$ 15.0 cm column and eluted using stepwise washes of increasing ammonium acetate concentrations at pH 7.0 (from 0.0 to 2.0*M*). The activity elutes in the 0.75*M* wash.

HA heparinase was also subjected to affinity chromatography by binding to polyvinylsulfate–Sepharose (*16*) at pH 7.0 and was eluted at either high or low pH (11 or 4) (*16*).

HA heparinase was loaded at pH 7.0 on a prefocused polyacrylamide gel and after IEF, was recovered at pH 8.5 $\pm$ 0.5 (*16*).

CF heparinase could be further purified by either heparin–Sepharose affinity chromatography as described above or by gel permeation chromatography on a 1.5 $\times$ 50 cm Sephadex G-75 or G-100 column.

Heparinase Immobilization

HA–heparinase was immobilized on CNBr-activated Sepharose 4B by an adaptation (*14,19*) of the procedure of March et al. (*20*). HA heparinase was immobilized to Sephadex-G in the absence of heparin (*20*). HA–heparinase was also immobilized onto cellulose (*16*), carboxymethyl (CM), Sephadex, and CM-cellulose (*16*), polyacrylamide PAN 1000 (*16*), and epoxyactivated polyacrylic (*21*).

Heparinase Fluidized Bed Reactor

The fluidized bed reactor (FBR) was constructed from a Bentley transfusion filter as described in ref. (*22*) and was loaded with 100 mL of a 50% bead suspension of Sepharose 4B-heparinase (*22*).

Blood Deheparinization

In vitro deheparinization was performed using 2 U of heparinized (6 U/mL) human blood stored in a reservoir at 37°C. The blood was passed through a FBR loaded with Sepharose 4B-heparinase (or Sepharose as a control) at a flow rate of 50 mL/min. Blood was sampled from the reservoir and analyzed by Complete Blood Count (CBC) and activated partial thromboplastin time (aPTT).

In vivo deheparinization was performed on canine blood heparinized at 4 U/mL. Several dogs were anesthetized with pentabarbital and the carotid artery and internal jugular vein were cannulated with a Scribner Shunt connected to silastic tubing that was joined directly to the FBR (*22*). The blood was passed through the FBR loaded with Sepharose 4B-heparinase (or Sepharose as a control) at a flow rate of 50 mL/min. Blood was sampled from the animal and analyzed by CBC and aPTT.

Results and Discussion

Heparinase Production

The goal of improved heparinase production (Fig. 1) by *Flavobacterium heparinum* has been accomplished largely by improvements in the bacterial growth medium, by the manipulation of environmental parameters, and by developing and understanding of the regulation of heparinase production in *F. heparinum*. The results of these studies are discussed below.

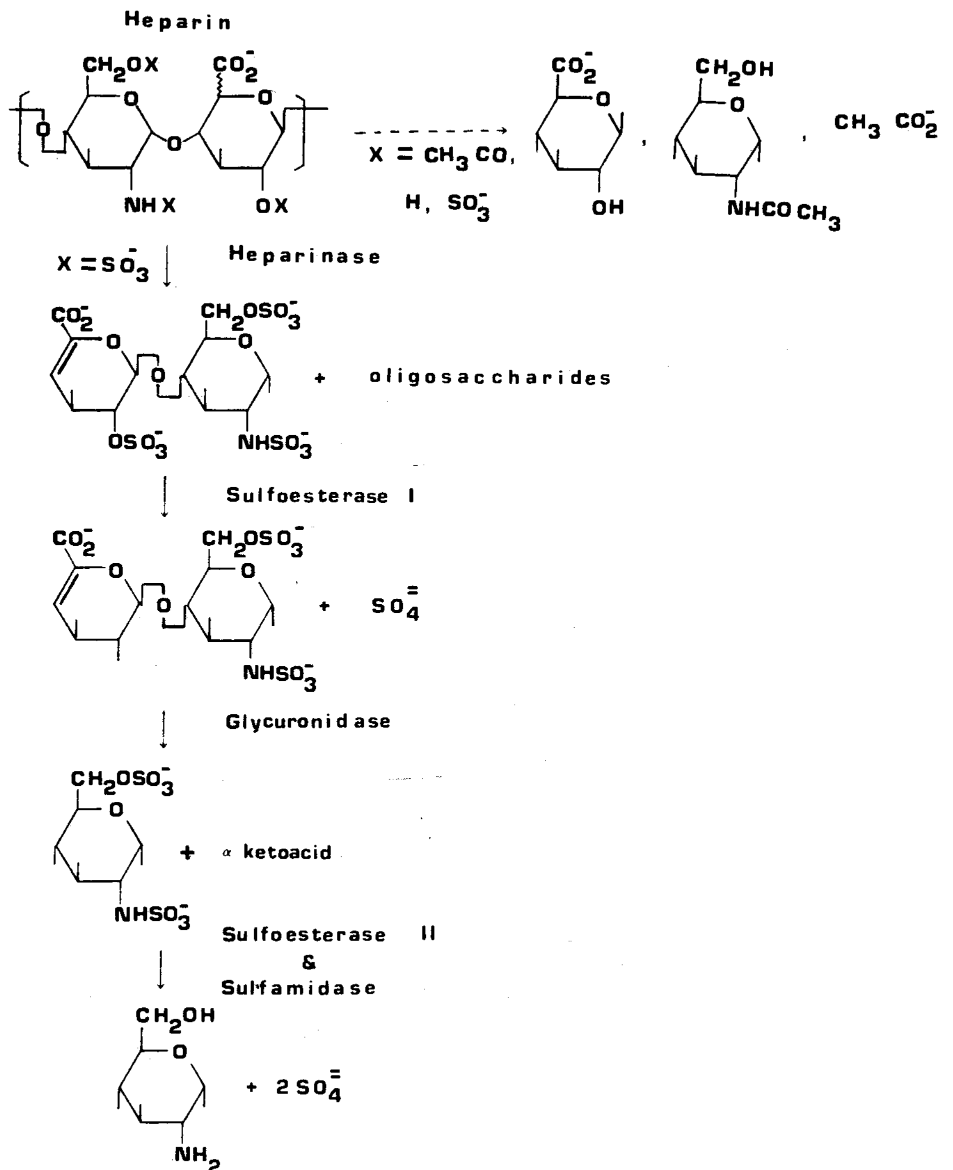

Fig. 1. Proposed sequence of heparin degradation in *F. heparinum* (29).

The production of heparinase, a cell-associated and inducible enzyme, in complex medium presented serious difficulties (*14,23*). The amount of enzyme produced is low; typically 9600 U heparinase/L fermentation broth is produced slowly with a volumetric productivity of 375 U heparinase/L-h (*14*). For the studies involving blood deheparinization, large amounts of enzyme, approximately 10,000 U HA-purified heparinase/reactor, were required. In addition, a rapid degradation of enzyme, resulting in an 86% loss of total activity within 4 h, was observed just prior to the onset of stationary phase. As a consequence, there were variations in the amount of enzyme produced and the fermentation was irreproducible (*14*).

These difficulties have been eliminated by the introduction of a defined fermentation medium consisting of glucose (as the primary carbon source), heparin (as the inducer), amino acids (methionine and histidine), and salts (including sulfate). Using this defined medium, in the fermentation (Fig. 2a) has resulted in: (1) increased microbial growth rate (30% higher than observed in complex medium); (2) increased enzyme titers reaching 96,000 U/L with volumetric productivities of 1480 U/L-h; and (3) an enzyme preparation that was stable well into the stationary phase (*14*). Although considerable effort was undertaken to find alternate (less expensive and more effective) inducers of heparinase, none was found to be better than heparin (*14*).

While studying the effect of initial ammonium sulfate concentration in the fermentation, we observed that enzyme synthesis was repressed at concentrations of 10 g/L. This repression was caused not by ammonium but by sulfate ion, a product of heparin catabolism (Fig. 1). A new defined medium (*18*), low in sulfate ($[SO_4^{2-}] \leq 10^{-4}$ *M*) was developed, enabling heparinase to be produced in the absence of the previously required inducer, heparin (Fig. 2b). In fermentations

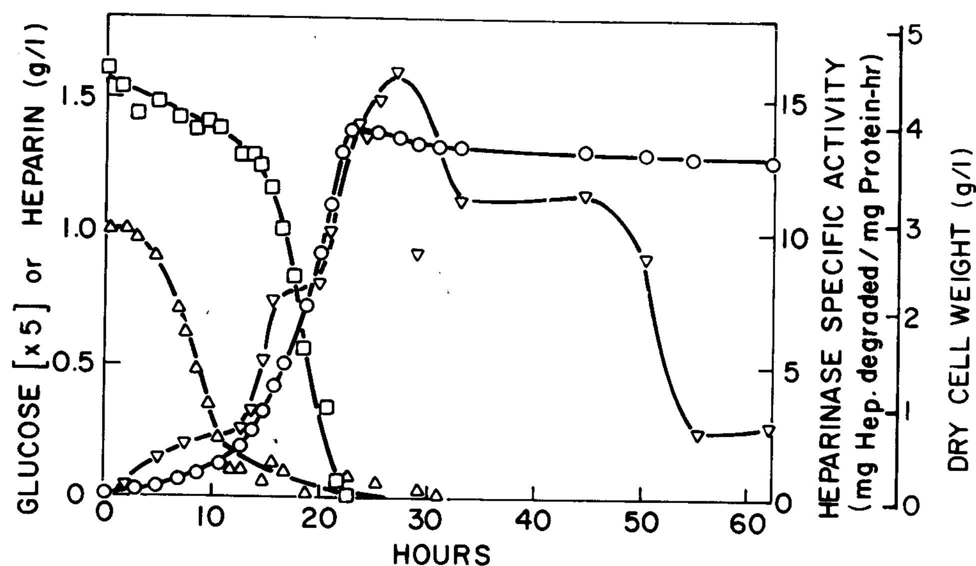

Fig. 2a. Results of a typical fermentation on defined medium showing dry cell weight (○), glucose (□), heparin (△), and heparinase specific activity (▽) as a function of time in a 2-L fermenter.

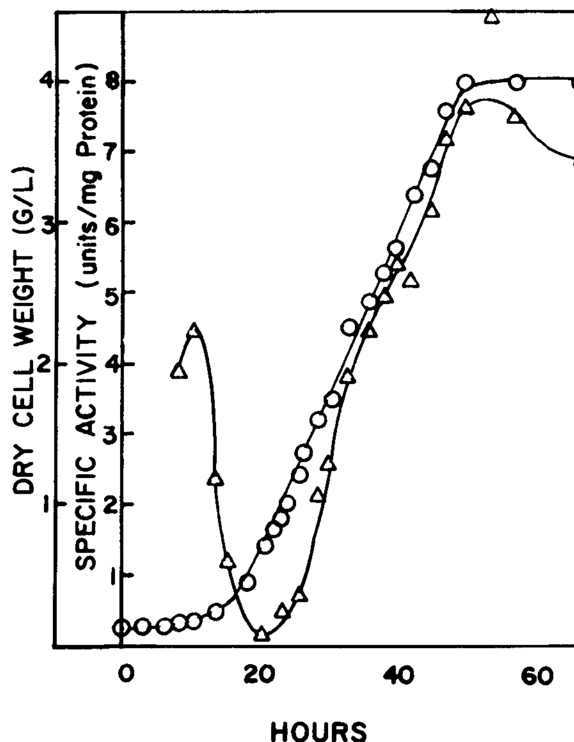

Fig. 2b. Results of a fed-batch fermentation in low sulfate defined medium showing cell concentration (○) and heparinase specific activity (△) as a function of time as determined in a 2-L fermenter.

with low sulfate, the specific activity of heparinase is 7 U/mg protein compared to the basal level of 0.01 U/mg protein in uninduced, high sulfate medium. Heparinase titers of 28,000 U/L and volumetric productivities of 620 U/L-h are typically observed. Work, currently in progress, is focused on increasing heparinase titers and volumetric productivities by better definition of nutritional requirements in the low sulfate-defined medium and exploring the metabolic regulation of heparin synthesis.

Purification of Heparinase

The objectives in the purification of heparinase were (1) to adapt previous purification schemes (*23*) to large-scale production of heparinase; and (2) to purify heparinase to homogeneity. The results of studies are presented in Table 1 and Figs 3 and 4. The first goal has been addressed by converting the first major purification step, hydroxylapatite (HA) ion-exchange chromatography, from a column to a batch process. Batch HA chromatography has been used to purify up to 100 g of crude protein compared to the milligram quantities purified on an HA column (*23*).

In our efforts to prepare a homogeneous heparinase, five routes following batch HA chromatography were examined: (1) Polyvinylsulfate affinity chromatography; (2) heparin affinity chromatography; (3) batch cellulose phosphate ion-

TABLE 1
Heparinase Purification

	Specific activity		
	mg Heparin degraded/ mg protein h, (15)	Mg protein	#Major SDS bands
Whole cells	28.5	1000	—
Sonicate	—	—	20
Protamine precipitate	82	554	11
Hydroxylapatite (HA)	136	88	8
Chromatofocused (CF)	620	8	1–2
Sephadex gel permeation	5900	0.7	1
Cellulose phosphate (CP)			
After HA	2200	0.001	2
Isoelectric focusing (IEF)			
After HA	5000	—	2
Heparin sepharose affinity			
After HA	3200	0.001	8
After CF	3800	0.0005	1

exchange chromatography; (4) isoelectric focusing; and (5) chromatofocusing chromatography. Enzyme purity was followed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in each step.

Heparinase failed to bind to a heparin–Sepharose column under the conditions initially examined. We therefore searched for a competitive and reversible heparinase inhibitor to act as an affinity ligand. Polyvinyl sulfate (PVS), a syn-

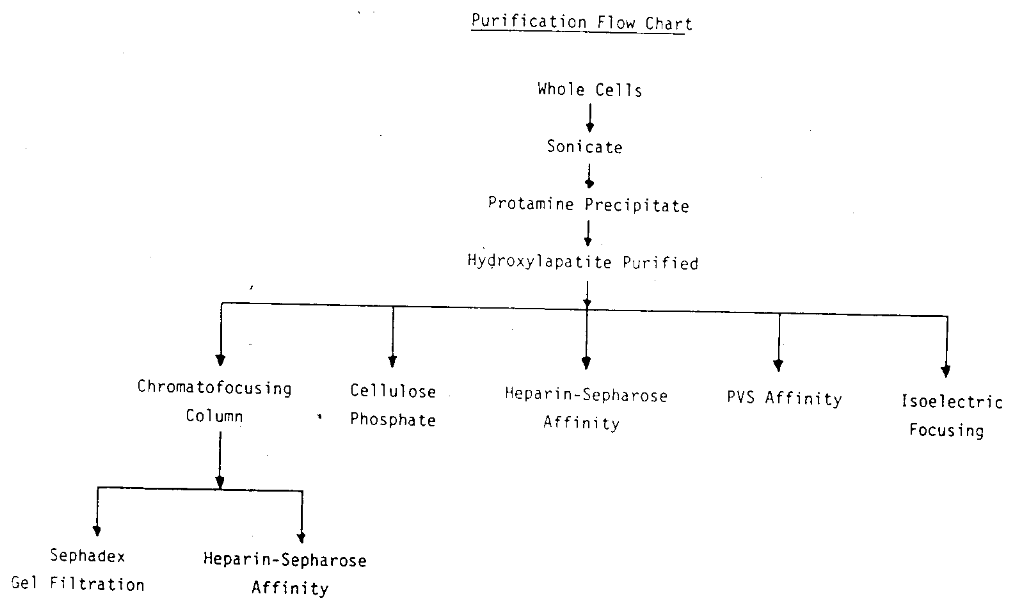

Fig. 3. Purification flow chart.

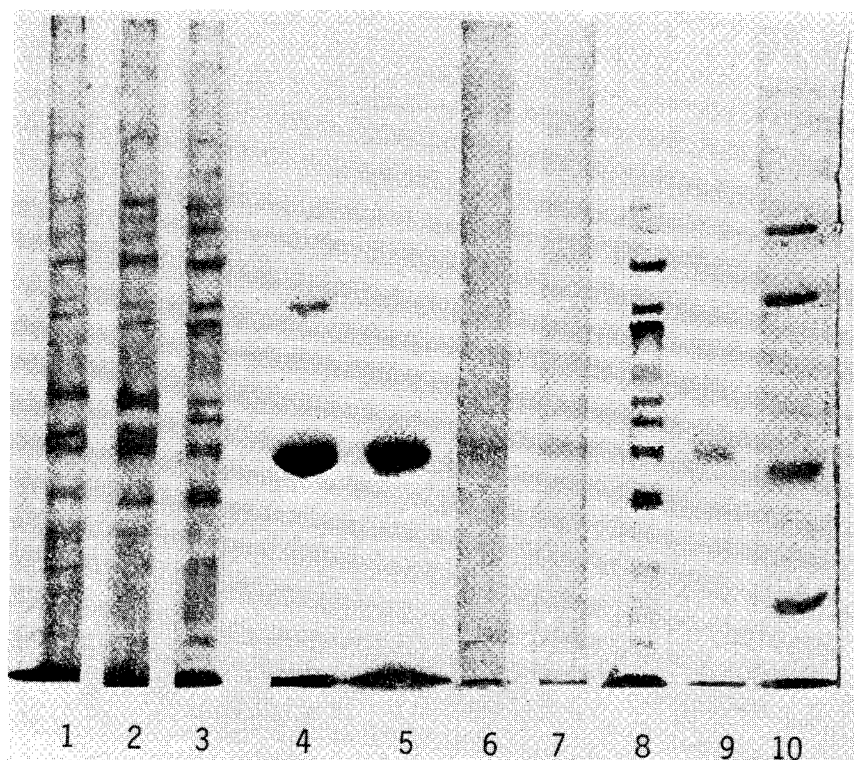

Fig. 4. Heparinase purification followed by SDS-PAGE. (1) sonicate; (2) protamine precipitate; (3) hydroxylapatite (HA), (4) dialyzed chromatofocused (CF), 50–75 mL cut (see Fig. 5); (5) dialyzed chromatofocused (CF), 60–75 mL cut (see Fig. 5); (6) Sephadex gel permeation (G-75); (7) cellulose phosphate (CP); (8) affinity chromatography (after HA); (9) affinity chromatography (after CF); (10) molecular weight markers: phosphorylase B (92,500); BSA (66,200); ovalbumin (45,000); carbonic anhydrase (31,000).

thetic heparin substitute ($K_i = 3 \times 10^{-8} M$; MW $\simeq$ 10,000), is such an inhibitor and was partially hydrolyzed and immobilized onto epoxy-activated Sepharose (*16*). Although affinity chromatography using this support resulted in a fivefold enrichment of specific activity only 5–10% of the total activity was recovered because of the harshness of the required elution conditions (pH 4 or 11). Further investigation of heparin affinity chromatography showed that HA heparinase would bind to heparin–Sepharose, but only at low ionic strengths. Mild elution conditions, increasing the ionic strength, resulted in a 2.3-fold increase in specific activity, but without a decrease in the number of major bands on SDS-PAGE.

The use of a second ion-exchanger, cellulose phosphate, in a batch process resulted in an increase in the specific activity of HA heparinase by 20-fold. However, the recovery of total activity was less than 0.02%.

The isoelectric point of heparinase has proven to be an important property in heparinase purification. Analytical isoelectric focusing (IEF) has resulted in a 50-fold increase in specific activity, but only small amounts of protein can be recovered from the polyacrylamide gel (*15*).

As a preparative technique, we have explored chromatofocusing, which uses a linear pH gradient generated by ampholytes on an ion-exchange column to separate proteins by isoelectric point (*24*). Chromatofocusing of HA heparinase from pH 9.0 to 6.0 resulted in both a broad activity peak and corresponding protein peak centered at $p_i = 8.2 \pm 0.2$ (Fig. 5). SDS-PAGE showed a single protein band corresponding to the top of the activity peak at $p_i = 8.3 \pm 0.1$.

The CF heparinase contained ampholytes that were removed by: (1) dialysis through 50,000 MW cutoff controlled dialysis tubing; (2) Sephadex gel permeation chromatography (either G-75 or G-100); and (3) heparin affinity chromatography. When CF heparinase was applied to a heparin–Sepharose column most of the protein (corresponding to heparinase as determined by SDS-PAGE) failed to bind suggesting that much of the CF heparinase was inactive. The protein which did bind to heparin-Sepharose eluted in the 0.75*M* ammonium acetate wash and showed a 17-fold enrichment with a specific activity of 3800 U/mg protein.

The removal of ampholytes using Sephadex gel permeation chromatography had the major advantage over heparin affinity chromatography of resulting in a 14,000-fold greater protein recovery, giving a specific activity of 5900 U/mg protein.

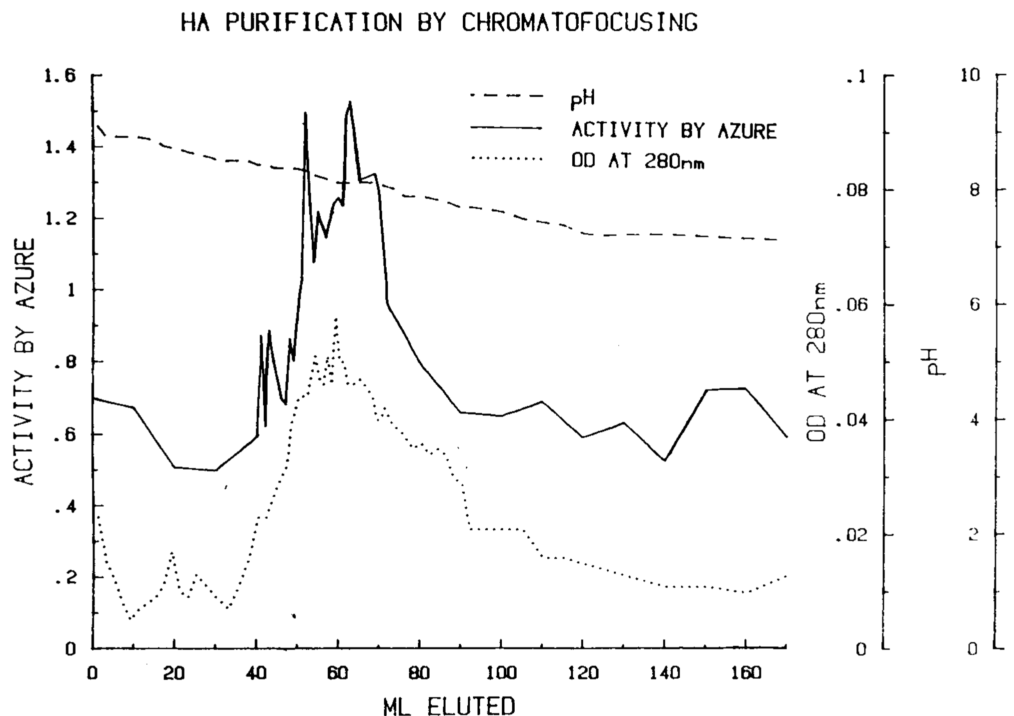

Fig. 5. Purification of HA heparinase by chromatofocusing. Heparinase activity (—), Protein concentration (· · · ·), and pH (- - -) is ploted as a function of the milliliters of column eluent.

TABLE 2
Effect of the Pore Size on the Activity of Heparinase Immobilized

Support	Relative immobilized activity	Approximate exclusion limits of globular proteins, MW (*29*)
Sephadex G15	0.06	1,500
Sephadex G50	0.11	30,000
Sephadex G75	0.42	80,000
Sephadex G100	0.51	150,000
Sephadex G150	0.60	300,000
Sephadex G200	0.62	600,000
Sepharose-4B	1.00	20,000,000

Properties of Heparinase

Heparinase has a molecular weight of 50,000 ± 2000 as determined by Sephadex G-150 gel permeation chromatography and 45,800 ± 1000 without subunits by SDS gel electrophoresis.

Heparinase is specific and acts only on heparin ($K_m = 4.2 \times 10^{-5} M$) and heparin monosulfate (at heparin-like linkages). The enzyme is an α-1–4-eliminase cleaving heparin in a random endolytic fashion (MW≃14,000) at 9–10 sites (*17,19*). The enzyme has a broad activity maximum at pH 5.8 (30°C) with a stability maximum at pH 7.0 (30°C) (*16*).

Immobilization

Heparinase (HA pure) has been covalently immobilized to a wide variety of supports with varying degrees of success (*16*). Supports giving the highest level of immobilized activity include: CNBr Activated Sepharose 4B (91% activity immobilized), Sephadex (5-56%, see Table 2); cellulose from hollow fiber kidney dialyzers (4%); carbodiimide or active ester-activated CM-Sephadex (4%), CM-Cellulose (1%), Polyacrylamide (PAN) (36%); and epoxy-activated (oxirone) acrylic beads (1%).

Several factors may be useful in predicting the success of heparinase immobilization on any given support. Macroporous supports, because of their large surface area, increase the levels of total activity that can be recovered on immobilization. Examination of the Sephadex G series of controlled pore supports (Table 2) shows that, under conditions where all the protein is immobilized, the activity of the immobilized protein increases with increased pore size. Highly (negatively) charged supports result in very low levels of immobilized activities even when a large amount of protein is immobilized. Additionally, when excess sites of activation, present in preparing Sepharose 4B-heparinase, are blocked with lysine (which imparts no charge to the support) in place of glycine (which imparts a net negative charge), both an increase in immobilized activity and a decrease in the apparent K_m value is observed. The reason for the reduced activity observed on negatively

charged supports may be electrostatic repulsion between the negatively charged substrate, heparin, and the support.

Although research continues into the search for more biocompatible supports and higher yields of immobilized activity, we have begun to examine Sepharose 4B-heparinase as a model system. Sepharose–heparinase has an enhanced thermal stability (*15*) that markedly increases its storage life at 4°C from a $t_{1/2}$ deactivation of 125 h (free native enzyme) to a $t_{1/2}$ deactivation of >3600 h (*16*). Also increased is the stability of immobilized enzyme at 37°C to a $t_{1/2}$ deactivation of 15 h in buffer (*16*). The immobilized enzyme exhibits an activity profile (*16*) broadened over a larger temperature range than the free enzyme and a shift in activity maximum from 30 to 37°C (possibly the result of the temperature dependence of the rate of substrate diffusion through the support). The apparent K_m of Sepharose 4B-heparinase (lysine-blocked) is $1.2 \times 10^{-3}M$ (*16*).

Although the linkage formed by protein binding to CNBr-activated Sepharose is not completely stable (*25*), no leaching of heparinase was detected in buffer over a 1-month period at 25°C or in blood at 37°C over a 2 h period (*22*).

In Vitro and In Vivo Blood Deheparinization

Initial experiments utilized small (1.5 mL bed volume, 1 cm diameter) packed-bed reactors containing Sepharose-heparinase to deheparinize either heparin containing buffer solutions or blood. At rates above 1 mL/min, however, the bed packed tightly, preventing flow (*22*). To avoid this problem a fluidized bed reactor (FBR) containing 100 mL of a 50% bead suspension and able to support flows up to 100 mL/min was used (*22*).

Human blood in a reservoir in vitro was deheparinized by passage through the FBR containing Sepharose–heparinase at a flow rate of 50 mL/min. At the beginning of the experiment, the anticoagulant activities in blood entering and exiting the reactor were 6 and 0 U/mL, respectively, as measured by aPTT. Blood samples taken from the reservoir at various times were also measured by aPTT and these results are shown in Fig. 6. A control FBR containing Sepharose 4B (without immobilized heparinase) caused no decrease in heparin level throughout the entire experiment (Fig. 6).

In vivo experiments performed with dogs paralleled the results of the in vitro experiments and showed that exposure of heparinized blood to the reactor caused a rapid loss of heparin's anticoagulant activity. The slow decrease of heparin's anticoagulant activity in the control experiment (where the FBR contains Sepharose 4B) corresponds exactly to the measured (not shown) in vivo decay of heparin's activity in dogs. The loss of heparin, as measured by a metachromatic dye assay corresponded to that observed by aPTT (*15,22*) in all of the experiments, eliminating the possibility that the decrease in aPTT was caused by the nonspecific effect of immobilized heparinase on coagulation factors.

At the end of the in vivo experiment, blood taken from the dogs showed no decrease in hematocrit, a 30% decrease in white blood cell count, and a 70% decrease in platelet count. These values are typical of those obtained in extracorporeal circulation in dogs (*31*). No detectable heparinase antibody formation by Ouchterlony precipitin tests as a result of this single exposure (*22,26*).

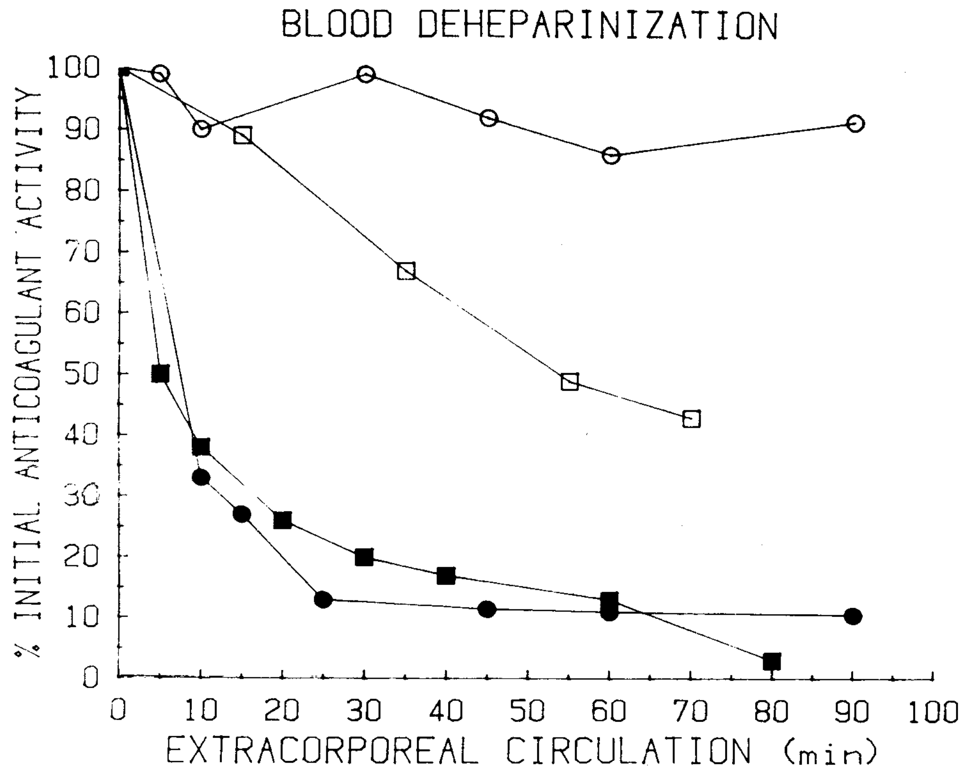

Fig. 6. Blood deheparinization. Heparinized human blood (●) and canine blood (■) was passed through a fluidized bed reactor (FBR) loaded with Sepharose 4B-heparinase. A set of control experiments was conducted in which heparinized human blood (○) and canine blood (□), was passed through a FBR loaded with Sepharose 4B. The percent of initial activity as measured by aPtt is plotted as a function of time. The total human blood and canine blood volume passed through the FBR every 20 and 30 min, respectively.

Heparin degradation products were tested using a forward mutation assay in *Salmonella typhimurium* (*28*); no cytotoxicity or mutagenicity was observed at 1000 times the expected clinical levels.

In vivo testing is currently underway in sheep to examine the effect of longterm exposure of animals to an immobilized heparinase reactor in conjunction with kidney dialysis. Additional toxicological testing of the enzymatic products of heparin degradation are also being performed in both rats and sheep.

Summary

The development of a proposed medical application for a microbial enzyme was discussed. The fermentation of *Flavobacterium heparinum* has been improved to provide an inexpensive source of large quantities of heparinase. The early steps in the enzyme purification have been converted to batch processes to permit easy scale-up; the enzyme has been further purified to a single band on an SDS gel. The

enzyme has been immobilized to a variety of supports and used to deheparinize human blood in vitro and canine blood in vivo.

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