

## A System for Heparin Removal

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*Extracorporeal medical machines rely on systemic heparinization to improve blood compatibility. However, heparin can lead to serious complications such as hemorrhaging. We propose a new approach to control heparin levels by employing a blood filter containing immobilized heparinase. Such a filter could potentially enable heparinization of an extracorporeal circuit without simultaneous heparinization of the patient. The principal findings of our work thus far include (1) increasing volumetric enzyme production over 1000-fold from previously published procedures; (2) purifying heparinase by over 1000-fold from the crude cell extracts; (3) characterizing the biochemical properties of heparinase; (4) isolating the first heparinase inhibitors; (5) immobilizing heparinase with 91% activity recovery and excellent stability; and (6) demonstrating that columns as small as 1.5 mL can remove clinically used quantities of heparin in aqueous medium and in blood.*

**E**xtracorporeal medical machines (e.g., artificial kidney, pump-oxygenator) perfused with blood have been an effective part of the therapeutic armamentarium for many years. These devices all rely on systemic heparinization to provide blood compatibility. Despite continuous efforts to improve anticoagulation techniques, many patients still develop coagulation abnormalities with the use of these devices (1-3). Even longer perfusion times may occur with machines such as the membrane oxygenator. In such cases, the drawbacks of systemic heparinization are multiplied (4). A number of ap-

proaches have been attempted to solve this problem. These include: (1) administration of compounds to neutralize heparin (5); (2) development of heparin substitutes (6); (3) bonding of heparin (7-12) or other substances (13) to the extracorporeal device; and (4) development of new blood-compatible materials for construction of the extracorporeal device (14). In spite of these efforts, heparinization continues to be used extensively in all extracorporeal treatments, and control of blood heparin levels remains a serious problem.

We propose a new approach that would allow the full heparinization of the extracorporeal device, yet could enable, on-demand, elimination of heparin in the patient's bloodstream. This approach consists of a blood filter containing immobilized heparinase, which could be placed at the effluent of any extracorporeal device (Figure 1). Such a filter could theoretically be used to eliminate heparin after it had served its purpose in the extracorporeal device and before it returned to the patient. In this chapter we discuss our efforts to develop such a filter. Our work has focused on several areas: (1) enzyme production; (2) enzyme purification; (3) characterization of heparinase; (4) immobilization of heparinase; and (5) in vitro testing of immobilized heparinase.

### *Experimental*

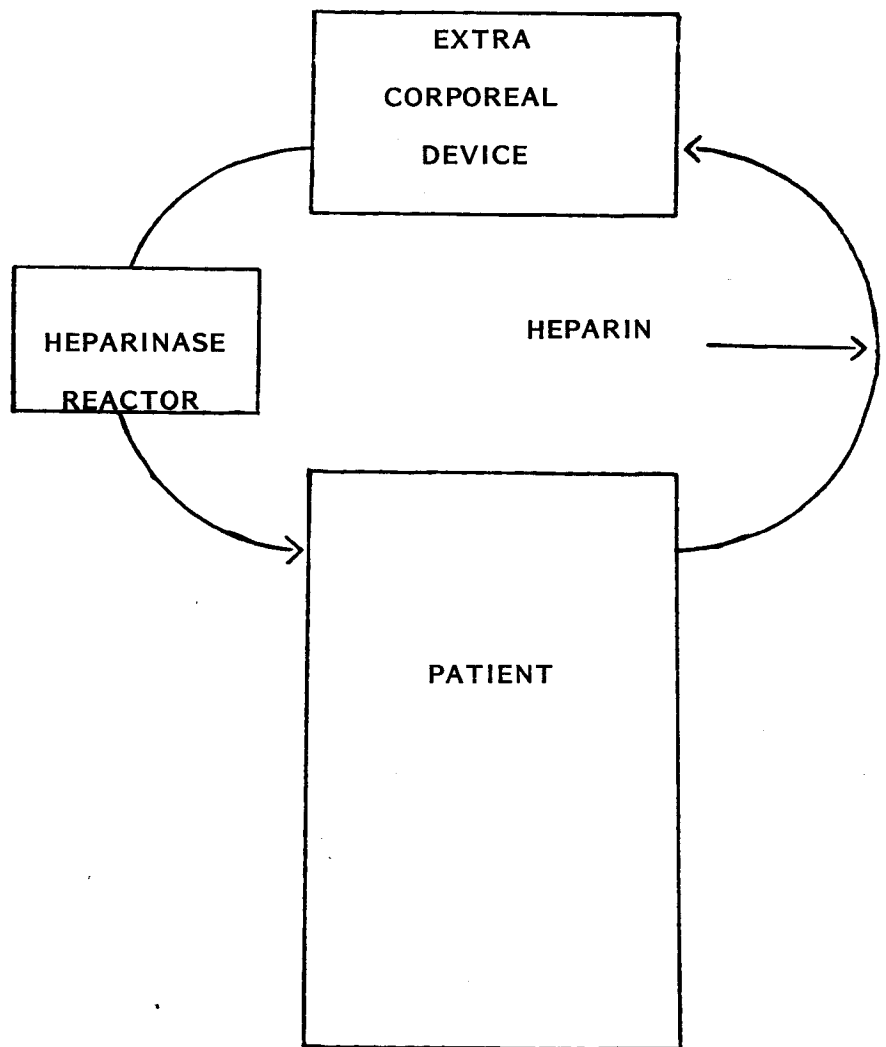
**Materials.** Heparin, as the sodium salt, from porcine intestinal mucosa, was purchased from Sigma Chemical Co. (Grade II, 153 USP k units). Azure A dye was purchased from Fisher Scientific Co. (A-970, certified biological stain, total dye content 70%).

The following polymer supports used in the immobilization were obtained pre-activated: (1) Sepharose-CNBr 4B from Pharmacia; (2) polyacrylamide polyacetyl and enzacyl AH from Aldrich Chemical Co.; and (3) polyacrylamide NHS active ester (PAN 1000) as a gift from George M. Whitesides, MIT, Department of Chemistry.

The unactivated polymer supports were obtained from the following sources: (1) poly(2-hydroxyethyl methacrylate) (PHEMA) from Polysciences Inc. and PHEMA (Spheron) from Hydron Inc.; (2) Dacron (poly(ethyleneterephthalate)) from Aldrich Chemical Company; (3) poly(methyl methacrylate) (PMA) 7% divinyl benzene cross-linked from Rohm and Haas Company; and (4) silicone (Masterflex silicone tubing) from Cole-Parmer. The activating agents used were: (1) cyanogen bromide (CNBr), hexamethylene diisocyanate, and Woodward's K reagent from Aldrich Chemical Company; (2) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) from Calbiochem-Behring Corporation; (3) glutaraldehyde (EM grade 25%) from Polysciences, Inc.; and (4) organosilane ester (A-1100) from Union Carbide Corporation, Silicones division.

The heparinase inhibitors were purchased from the following sources: (1) poly(vinyl sulfate) from Sigma Chemical Company; (2) polyanethole sulfonate from Calbiochem Corporation; and (3) polystyrene sulfonate (aqueous solution MW 70,000) from Polysciences Inc.

The purification used hydroxylapatite (HTP) from Biorad Inc., protamine sulfate, bovine serum albumin (BSA), and polylysine (Type VI) from Sigma Chemical Company, and epoxy-activated Sepharose from Pharmacia Inc. Sodium dodecyl sulfate (SDS) gel electrophoresis and isoelectric focusing (IEF) were performed using chemicals and equipment from Biorad Inc.



*Figure 1. Proposed heparin circuit. The extracorporeal device could be a renal dialysis unit or a pump-oxygenator. The heparinase reactor could be part of a blood filter to be used either continuously (in which case heparin would be added continuously at the start of the circuit) or at the end of an operation. Heparin could thus be confined to the extracorporeal circuit.*

Chondroitinase ABC from *Proteus vulgaris* was purchased from Sigma Chemical Company. All inorganic chemicals were reagent grade.

All chemicals used in heparinase production are as described previously (15).

**Analytical Determinations. PROTEIN CONTENT.** Protein was measured by the method of Lowry (16).

**HEPARINASE ACTIVITY.** Several assays were used to follow heparinase activity. These assays followed (1) the disappearance of heparin, (2) the appearance of heparin degradation products, or (3) the loss of the physiological function of heparin in anticoagulation. The basis of these assays and explanations as to when they are routinely used are listed below.

1. *Metachromasia in Azure A.* This assay measures heparinase activity by following the disappearance of heparin. Jacques (17) proposed that Azure A dye molecules dimerize in the presence of heparin, resulting in a decrease in the  $\pi$ -delocalization. This effect is observed as a shift in the absorption maximum from  $\lambda_{\text{max}} = 620 \text{ nm}$  to  $\lambda_{\text{max}} = 520 \text{ nm}$ . Since heparinase cleaves the  $\alpha$ -linkage of heparin, its action causes chain shortening, resulting in less metachromasia. The presence of heparin or heparin-derived polysaccharide chains of hexasaccharide or larger (18) can be measured easily and reproducibly at levels of 1–10  $\mu\text{g/mL}$  in crude (i.e., fermentation broths and cell sonicates) and purified preparations. Experimental details for utilizing this assay were reported in a previous publication (15).

2. a. *Reducing Sugar Assay.* With each cleavage of the heparin chain by heparinase (which is an  $\alpha$ -eliminase), one reducing end group is formed and one  $\alpha$ - $\beta$ -unsaturated end group. The increase in reducing capacity, therefore, gives a measure of enzyme activity and product formation. This assay has been used routinely in assaying crude preparations of heparinase. The reducing capacity of the products produced from heparin at different stages in the purification of heparinase is variable. For example, the reducing capacity of products produced by the action of purified heparinase is lower than that obtained by the action of crude heparinase preparations. This result is apparently due to enzymes in crude preparations that cause further degradation of the products formed by heparinase. The Park and Johnson method (19) of measuring reducing sugars was used throughout these studies.

b. *Ultraviolet Assay.* The  $\alpha$ - $\beta$ -unsaturated acid end group resulting from heparin cleavage is a chromophore with a  $\lambda_{\text{max}}$  at approximately 232 nm and a molar absorptivity of about  $5 \times 10^5 \text{ M}^{-1}$  (20). The action of heparinase can be measured by sampling the reaction mixture, quenching it in 0.03N hydrochloric acid, and measuring the absorbance. This assay can only be used to measure activity in relatively pure preparations due to two factors: (1) high concentration of protein interferes with the measurement of product, and (2) contaminating enzymes are capable of acting on the heparinase-derived product, resulting in the loss of the chromophore being measured. Because of its accuracy and ease of performing, this assay was the method of choice for measuring heparinase activity in the more purified heparinase preparations.

3. *Assays Measuring the Loss of Heparin's Biological Activity.* The loss of heparin's biological activity through the action of heparinase can be tested by a number of available assays. Three assays were chosen for their ease of use and the range of activity they measure. Whole blood recalcification time (21) involves the measurement of heparin in citrated whole blood by recalcifying the blood and measuring the clotting time. Factor  $X_a$  Assay (22) involves measuring the heparin in citrated plasma which has been enriched in Factor  $X_a$  by recalcifying and measuring the clotting time. Thrombin-antithrombin time (23) determines the action of heparin on the thrombin-antithrombin interaction by measuring the appearance of the chromophore released by thrombin from a synthetic polypeptide substrate. In each of these assays a standard curve was constructed with each determination.

### Results

**Heparinase Production.** The objective of these studies was to develop an understanding of what factors influence heparinase production by *Flavobacterium heparinum* (15). This was done by studying the kinetics of microbial growth and heparinase production, and by developing a simple, defined medium to support this growth and production. A 1000-fold increase in volumetric heparinase production over previously reported results was obtained by implementing improved techniques of microbial cultivation and environmental and genetic manipulations. These improvements and our findings are summarized below.

The wild type strain of *Flavobacterium heparinum* produces a non-extracellular heparinase in the growth stage only when heparin is supplied to the growth medium as an inducer (15). Enzyme production occurs during growth, so factors affecting growth can directly affect enzyme production. A reliable heparinase production scheme was first worked out by growing the bacteria in a complex protein digest medium. Inducer was provided at the time of inoculation of the sterile medium. Growth was initially exponential, and heparin was rapidly taken up by the cell at a rate of 1.1 g/g cell-h (Figure 2). Enzyme specific activity began to increase just as heparin uptake was finishing, and increased at a volumetric rate of 375 units/L-h. At the onset of the stationary cell growth phase, enzyme production stopped, and a deactivation was observed, resulting in an 86% loss of total activity within 4 h. To avoid this deactivation, the kinetics of enzyme production had to be understood. Timely harvest was thus important to obtain highly active heparinase. Fifteen fermentations were performed, all yielding a total enzyme level on the average of 9600 units of heparinase/L of fermentor broth, demonstrating the reliability of this method.

To understand better the environmental factors governing enzyme production, a defined growth medium was developed. This medium was the result of nutritional requirement experiments performed to elucidate the growth factors required by this bacterium. The bacterium was a histidine auxotroph with an additional (though not obligate) requirement for methionine. No vitamin requirement was observed. This result permitted the use of the following defined growth medium: glucose (main carbon source), heparin (inducer),  $(\text{NH}_4)_2\text{SO}_4$  (nitrogen source),  $\text{K}_2\text{HPO}_4$ ,  $\text{Na}_2\text{HPO}_4$ , L-histidine, L-methionine, trace salts, and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . A 30% increase in growth rate was observed using this medium. Volumetric heparinase production was increased fourfold over the complex medium production to 1480 units/L-h. Additionally, higher cell densities were obtained routinely in this defined medium. A typical production run using 20 g/L of glucose results in a tenfold increase in total enzyme obtained to 96,000 units/L fermentor broth. This fermentation has been repeated eight times to date, demonstrating the reliability of this method. In addition, the product heparinase is

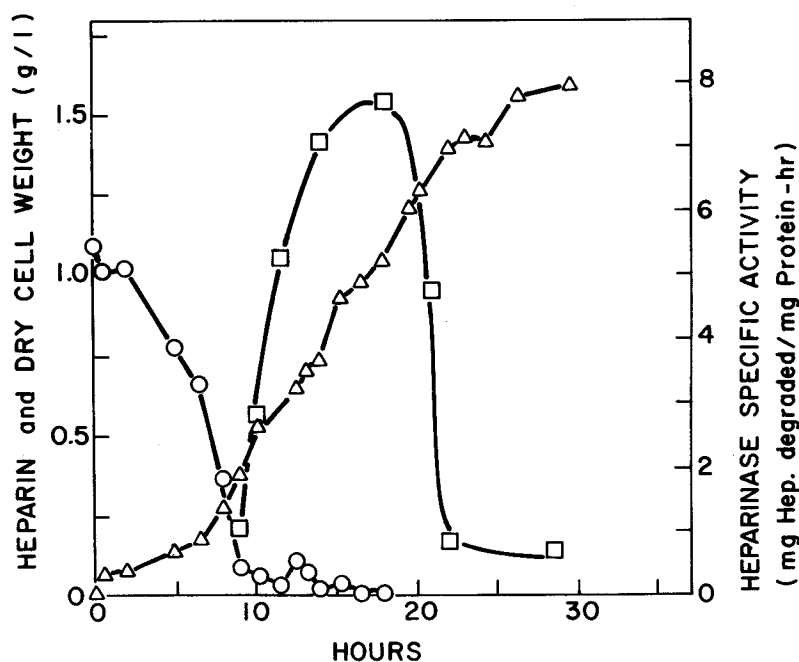


Figure 2. Results of a typical fermentation on complex medium showing heparin (○), heparinase specific activity (□), and dry cell weight (△) as a function of time as determined in a 2-L fermentor (15).

more stable, since no rapid loss of the enzyme occurs in this medium (Figure 3), and allows more flexibility and reliability for product recovery. The use of this defined medium also permitted tests concerning the effect of medium components and environmental factors on enzyme production. Optimum initial glucose and  $(\text{NH}_4)_2\text{SO}_4$  concentrations were 8 g/L and 0.5 g/L, respectively. The effect of temperature on growth rate and enzyme production was studied. Optimum growth temperature was 29°C, whereas optimum temperature for enzyme production was 24°C. The maximum phosphate concentration not deleterious to growth was 20mM.

Other methods of increasing the specific heparinase production of *Flavobacteria* currently are under study. A strain improvement program of mutation and selection has been implemented using ultraviolet and  $\gamma$ -irradiation, followed by growth selection methods. Many mutant cultures have been obtained using these methods. Of those currently under investigation, one particular mutant has provided a twofold increase in specific productivity of heparinase over the wild type in defined medium. Genetic manipulation studies will be the main focus of continuing work, with the ultimate objective of obtaining a constitutive mutant capable of producing heparinase at high levels.

**Purification of Heparinase.** The objectives of our work on the purification of heparinase were twofold: (1) to adapt previous purification schemes of Hovingh and Linker (24) to large-scale production of heparinase; and (2) to purify heparinase to homogeneity. The first goal has been met largely by moving from a column to a batch purification. The cell pellet produced from centrifugation of the fermentation broth at  $10,000 \times g$  was resuspended at 100 mg/mL protein in 0.01M phosphate buffer at pH 7.0, and disrupted sonically; the nucleic acids were precipitated with 12.5 mg/mL of protamine sulfate; and the protein solution was added to 4 g of hydroxylapatite per g of protein. The hydroxylapatite-bound protein was then washed stepwise with increasing concentrations of NaCl and sodium phosphate (from 0.0M and 0.01M to 0.50M and 0.25M, respectively). The resulting enzyme preparation (HA), obtained in a 0.125M NaCl and 0.07M sodium phosphate wash, was of sufficient purity to have its activity determined by any of the available assays.

As a further purification technique, affinity chromatography was explored. In preliminary experiments, a heparin-Sepharose column failed to bind heparinase. We therefore searched for a competitive and reversible heparinase inhibitor to act as a ligand. Three synthetic heparin substitutes—poly(vinyl sulfate), polyanethole sulfonate, and polystyrene sulfonate—met these requirements. The inhibitory effect of poly(vinyl sulfate) ( $K_i = 3.0 \times 10^{-8}M$ ; MW  $\sim 10,000$ ) appeared to be linked to the presence of sulfate groups because inhibition was lost when poly(vinyl sulfate) was hydrolyzed.

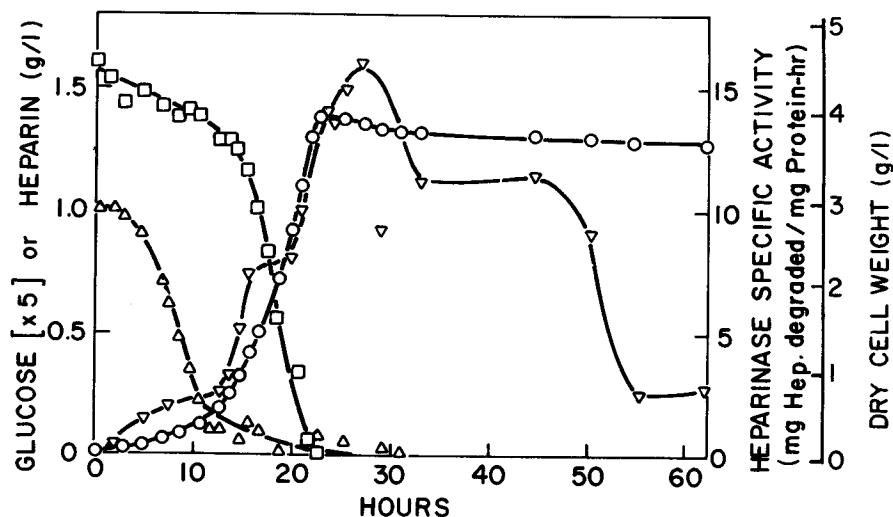


Figure 3. Results of a typical fermentation on defined medium showing dry cell weight (○), glucose (□), heparin (△), and heparinase specific activity (▽) as a function of time in a 2-L fermentor (15).

An affinity column was prepared by immobilizing partially hydrolyzed poly(vinyl sulfate) on epoxy-activated Sepharose (25). Heparinase (HA purified) was bound to this column, and was released at either high or low pH (11 or 4) with 5–10% total activity recovery and up to 500% enrichment (21).

IEF also was applied towards the HA-purified enzyme to obtain highly pure heparinase. The enzyme was loaded onto a prefocused acrylamide gel at pH 7.0. After IEF, the enzymatic activity was recovered at pH  $8.5 \pm 0.5$ . The resulting enzyme had a specific activity of about 5000 units/mg protein, having undergone an enrichment of 50-fold (21).

The purification of heparinase has been followed by SDS gel electrophoresis. The crude sonicate gave more than 20 major bands; the HA purified enzyme, 3 major bands; and the IEF purified enzyme, 2 major bands. A summary of the specific activities, protein recoveries, and enzyme purity obtained using our purification procedures is listed in Table I.

**Properties of Heparinase.** Our studies of the structure of heparinase show it to have a molecular weight of  $51,000 \pm 6,000$  by Sephadex G-200 gel exclusion chromatography, and  $45,700 \pm 1,600$  without subunits by SDS gel electrophoresis.

The enzyme is very specific, acting only on heparin ( $K_m = 4.2 \times 10^{-5}M$ ) and heparin monosulfate. Heparinase acts endolytically as an  $\alpha$ -1, 4-eliminase cleaving heparin ( $MW \cong 10,000$ ) at 7 to 8 sites (42).

Detailed studies have been performed on the activity and stability of heparinase. Hydroxylapatite-purified heparinase (HA) is stable to freeze-thawing and freeze-drying, with 90 and 87% recovered activity, respectively. Highly purified heparinase requires the addition of BSA or polylysine (0.05%) and glycerol (7.5%) to permit 100% activity recovery on freeze-thawing.

The effect of salts on heparinase activity was examined. An activity maximum was obtained in 0.162M sodium chloride; however, the maximum enzyme stability occurred at a somewhat higher concentration. The effect of the cations  $Ca^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Mo^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ ,  $Sn^{2+}$ ,  $Cd^{2+}$ ,  $Pb^{2+}$ ,  $Li^+$ ,  $K^+$ ,  $Hg^{2+}$ ,  $Mg^{2+}$ ,  $NH_4^+$ ,  $Al^{3+}$ ,  $Ba^{2+}$ , was tested using the HA-purified heparinase. Slight inhibition was shown by  $Ba^{2+}$ ,  $NH_4^+$ , and  $Pb^+$ , but total loss of activity occurred for  $Hg^{2+}$  at  $10^{-5}M$ .

**Table I. Heparinase Purification**

<i>Procedure</i>	<i>Specific Activity<sup>a</sup></i>	<i>Protein (mg)</i>	<i>Major SDS Bands</i>
Whole cells	4.3	1000	—
Sonicate	7.9	730	20
Protamine precipitate	12.5	480	—
Hydroxylapatite purified	88	45	3
Affinity chromatography	2,000	—	—
Isoelectric focusing	5,000	—	2

<sup>a</sup>Milligrams of heparin degraded per milligram of protein per hour (15).



Studies of the effect of pH on HA heparinase activity and stability determined that the activity maximum occurs at pH 5.8, while the stability maximum occurs at pH 7.0.

HA heparinase has an activity maximum at 30°C (Figure 4), but greater stability at lower temperature;  $t_{1/2}$  denaturation at 4°C was 125 h and  $t_{1/2}$  denaturation at 30°C was 25 h (Figure 5).

**Heparinase Immobilization.** Heparinase has been immobilized on a variety of supports, with a widely differing degree of success. The best results have been obtained on Sepharose and polyacrylamide. Low levels of activity recovery occurred on PHEMA. The other supports tested gave either no activity recovery or only barely detectable levels of activity (Table II).

To check several of the immobilization methods, chondroitinase ABC (from *Proteus vulgaris*) was used as a control. A summary of the activity recoveries of immobilized heparinase and chondroitinase is listed in Table II.

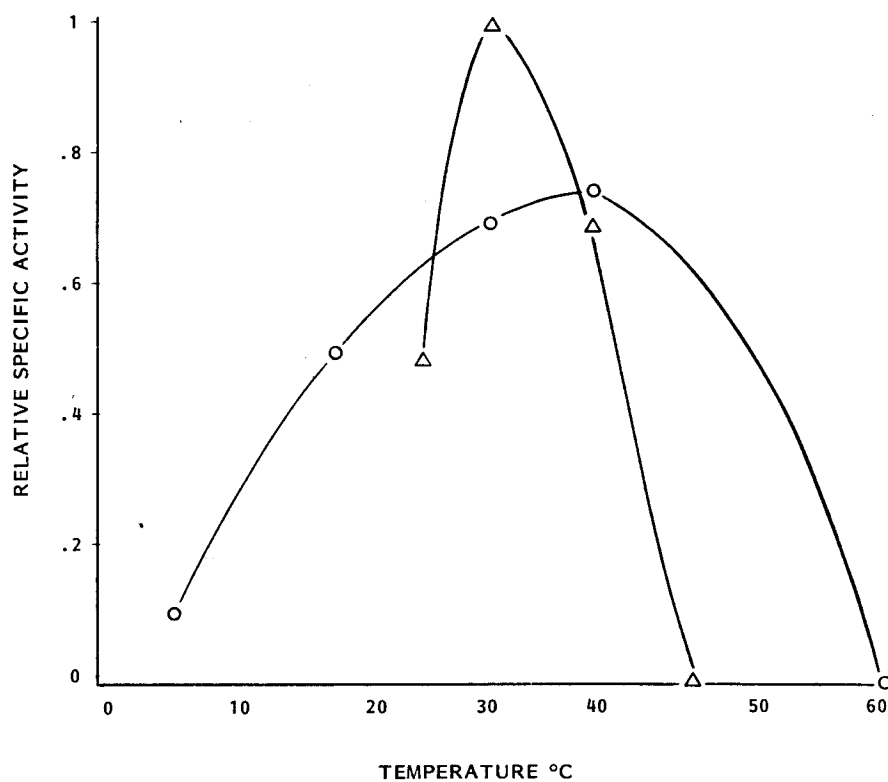


Figure 4. Activity profile of heparinase. Key:  $\Delta$ , specific activity of native enzyme, and  $\circ$ , specific activity of the Sepharose-immobilized enzyme.

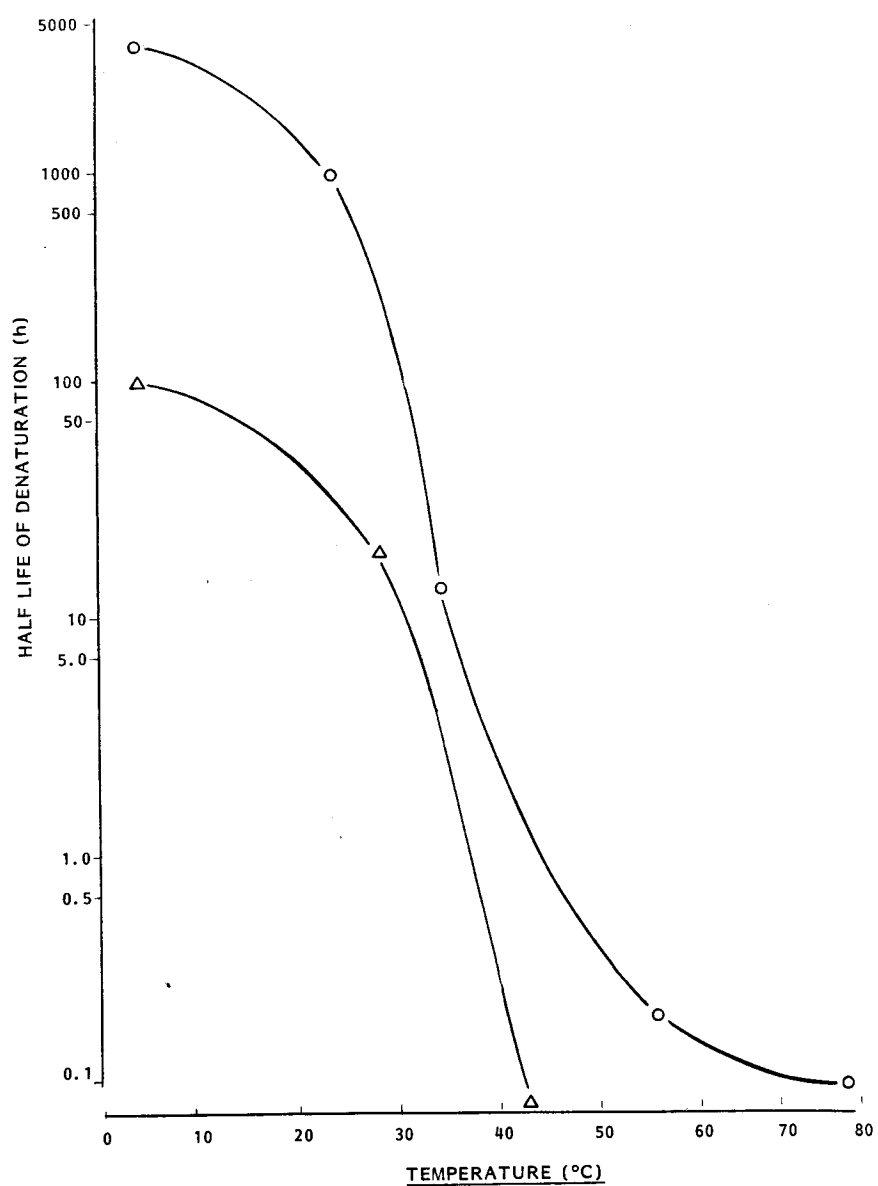


Figure 5. Stability of heparinase. Key:  $\Delta$ , half life of denaturation of native enzyme, and  $\circ$ , half life of denaturation of Sepharose-immobilized enzyme.

**Table II. Enzyme Immobilization on Various Polymer Supports**

<i>Carrier</i>	<i>Coupled By</i>	<i>Ref.</i>	<i>% Activity Heparinase</i>	<i>Immobilized Chondroitinase</i>
Sepharose	CNBr	(26)	91	0.8
Polyacrylamide	NHS active ester	(27)	56	—
	Polyacetal(aldehyde)	(28)	1.2	—
	Acyl hydrazide	(28)	0.7	—
PHEMA	CNBr	(26, 29)	1.6	—
PHEMA(Spheron)	CNBr	(26, 29)	0.03	2.2
PMA-CO <sub>2</sub> H	EDC	(30)	0.01	0.4
PMA-CONH(CH <sub>2</sub> ) <sub>2</sub> OH	CNBr	(26, 30)	0.0	—
PMA-BSA	(CH <sub>2</sub> ) <sub>6</sub> (CHO) <sub>2</sub>	(30)	0.0	—
PMA-(CH <sub>2</sub> ) <sub>2</sub> NH <sub>2</sub>	(CH <sub>2</sub> ) <sub>6</sub> (CHO) <sub>2</sub>	(30)	0.0	—
PMA-(CH <sub>2</sub> ) <sub>6</sub> NH <sub>2</sub>	(CH <sub>2</sub> ) <sub>6</sub> (CHO) <sub>2</sub>	(30)	0.0	—
Dacron	(EtO) <sub>3</sub> Si(CH <sub>2</sub> ) <sub>3</sub> N = CH(CH <sub>2</sub> ) <sub>3</sub> CHO	(31)	0.2	10.0
Dacron-NH <sub>2</sub>	(CH <sub>2</sub> ) <sub>6</sub> (CNO) <sub>2</sub>	(32)	0.0	—
	(CH <sub>2</sub> ) <sub>6</sub> (CHO) <sub>2</sub>	(33)	1.0	—
Dacron-CO <sub>2</sub> H	Woodwards K reagent	(34)	0.2	—
Silicone-NH <sub>2</sub>	(CH <sub>2</sub> ) <sub>6</sub> (CHO) <sub>2</sub>	(35)	0.0	0.4

The CNBr-activated Sepharose 4B support (1 g dry weight) was swelled in 25 mL of hydrochloric acid (0.001M), and then washed with 100 mL of 0.5M NaCl, 0.1M NaHCO<sub>3</sub> buffer at pH 8.3. To this support 5.5 mL of hydroxylapatite-purified heparinase (0.2 mg/mL protein with an activity of 88 units/mg protein in 0.2M phosphate buffer at pH 7.0) and 60 mg of heparin were added. The mixture was shaken overnight at 4°C, after which the beads were washed and blocked overnight at 4°C with a solution of lysine at pH 8.2 in 0.5M NaCl, 0.1M NaHCO<sub>3</sub> buffer solution. This support showed an uptake of 87% of the protein and an immobilization of 91% of the heparinase activity.

At present, we are continuing our efforts to immobilize heparinase to support materials in order to achieve higher yields. While this work is in progress, we have begun to explore the properties of immobilized heparinase using heparinase-Sepharose as a model.

Heparinase, immobilized on Sepharose, has enhanced thermal stability. This effect is especially noticeable in the low-temperature storage of this enzyme. At 4°C the immobilized enzyme has a half life of denaturation of > 3600 h, compared with a 125-h half life of the native enzyme at the same temperature (Figure 5). The greater stability of the immobilized enzyme is also seen at higher temperatures: 25°C,  $t_{1/2}$  = 1,000 h; 37°C,  $t_{1/2}$  = 15 h; and 60°C,  $t_{1/2}$  = 0.2 h.

Along with enhanced stability, the activity profile of the enzyme is broadened over a larger temperature range as a result of the immobilization. The activity maximum is shifted to a slightly higher temperature, from  $T$  = 30°C for the free enzyme to  $T$  = 37°C for the Sepharose-immobilized enzyme (Figure 4). This result may reflect the temperature dependence on

the rate of substrate diffusion into the support. The pH maximum of both the native and immobilized (on Sepharose) enzyme are identical (data not shown).

The apparent  $K_m$  of the immobilized enzyme is  $1.2 \times 10^{-3}M$  (this can be determined by replotting the data in Figure 6 on a Lineweaver-Burk plot). This  $K_m$  is considerably higher than the  $K_m$  determined for the free enzyme ( $K_m = 4.2 \times 10^{-5}M$ ).

**In Vitro Studies on Immobilized Heparinase.** Initial experiments have been conducted to test the effectiveness of immobilized heparinase in removing heparin in vitro. Controls consisted of Sepharose-heparinase that was denatured by heating at  $100^\circ C$  for 30 min. In one set of experiments, both active and denatured immobilized heparinase were loaded into two columns, both with a 1.5-mL bed volume. Solutions of heparin, BSA (60

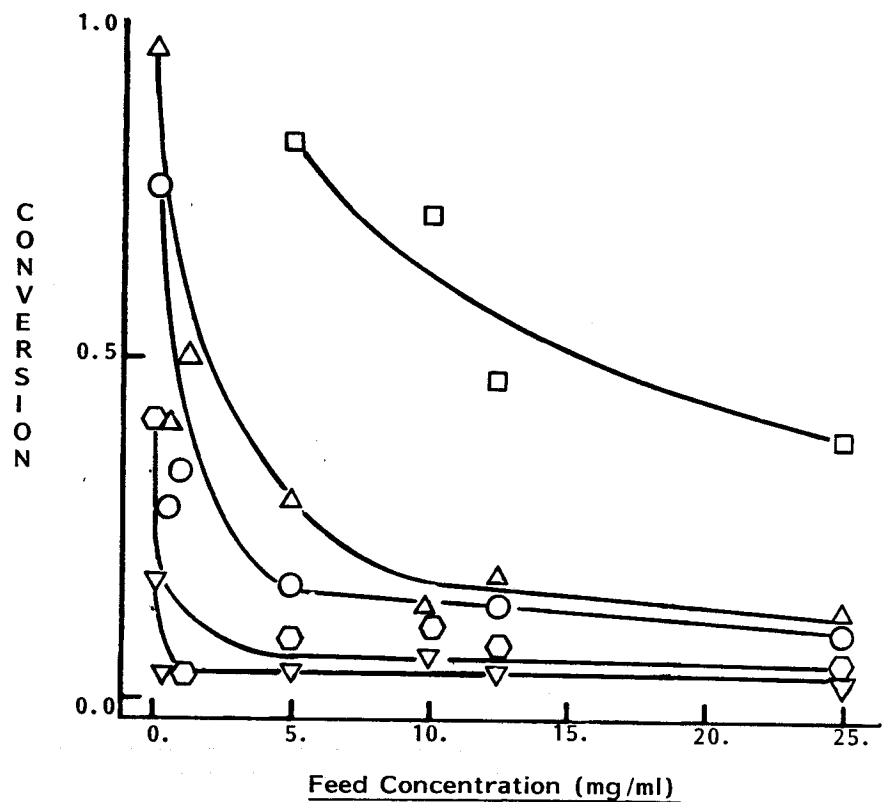


Figure 6. The conversion of heparin to products as the result of passing a sodium acetate buffer (pH 7.0, 0.25M) containing heparin through a 1.5-mL Sepharose-heparinase column. The conversion was measured as both a function of feed concentration (x-axis) and flow rate (mL/min). Key:  $\square$ , 0.1;  $\triangle$ , 0.2;  $\circ$ , 0.3;  $\hexagon$ , 0.4;  $\nabla$ , 0.5.

mg/mL), and salts were passed through each column at a flow rate of 0.5 mL/min. The concentrations of the nonheparin species were chosen to mimic physiological concentrations. The heparin levels in the solution increased in stepwise fashion from 15  $\mu\text{g/mL}$  to 75  $\mu\text{g/mL}$ , as shown in Figure 7. As the heparin level in the input solutions increased, the difference in the heparin recovered at the outlet of the control and active columns also increased (Figure 7). Even at 66  $\mu\text{g/mL}$  the heparin was largely removed by the active

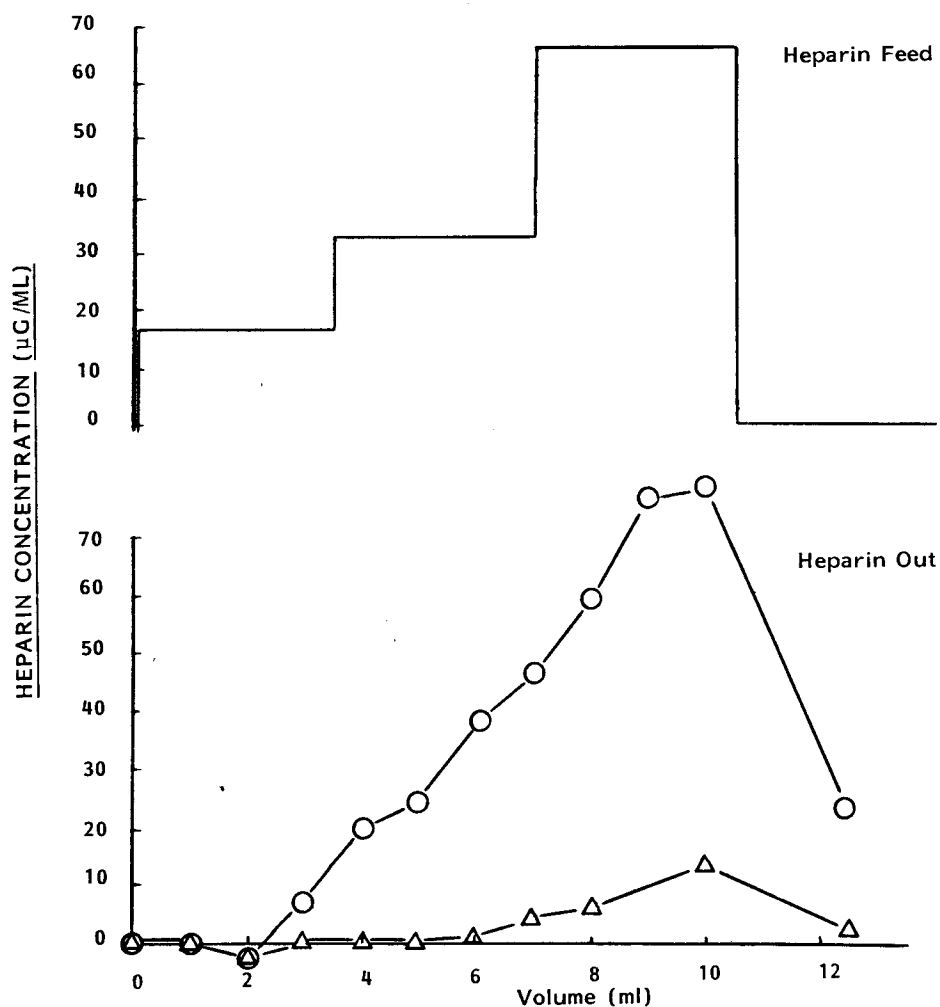


Figure 7. Heparin removal from a protein/salt solution by a 1.5-mL column packed with Sepharose-immobilized heparinase. The upper portion represents the stepped increase of heparin input; the bottom portion measures heparin output from both the Sepharose-immobilized enzyme ( $\Delta$ ) and the heat-denatured Sepharose-immobilized enzyme ( $\circ$ ).

heparinase column, while the denatured heparinase column had no effect. Clinically used levels of heparin are on the order of 5–10  $\mu\text{g}/\text{mL}$ .

In a second experiment, the effect of both heparin concentration and flow rate on heparin degradation was examined. The same size column as was used in the previous experiment was employed. As shown in Figure 6, at low flow rates this small column was fully capable of degrading very large quantities of heparin (more than 100-fold in excess of clinically used amounts) in a single pass.

We have just begun a series of experiments in which citrated rabbit blood, heparinized at a level of 10 units/mL (153 units/mg), was passed through a Sepharose–heparinase column (0.5mL) at a flow rate of 0.5 mL/min (Figure 8). After 5 min the blood leaving the bottom of the column was sampled and assayed for heparin by whole blood clotting time and Factor  $X_a$  heparin assays. In the active column, 50% of the heparin was removed. However, when the same heparinized blood was treated with a control column, less than a 5% decrease in anticoagulant activity was observed.

### *Discussion*

These studies provide initial data for developing a system to remove heparin in extracorporeal therapy. Because the amount of data on heparinase has been limited, until now, and the methods of producing it inadequate for large scale use (24), the focus of our research thus far has been on developing the necessary technology for enzyme production and purification. The principal contributions of our studies have been (1) increasing production levels of heparinase by over 1000-fold (15) from previously published procedures (24), (2) purifying heparinase by over 1000-fold from the crude cell extracts, (3) characterizing the properties of heparinase and isolating the first heparinase inhibitors, (4) immobilizing the enzyme with 91% activity recovery and excellent stability, and (5) demonstrating that columns as small as 1.5 mL can remove clinically used quantities of heparin in aqueous medium and in blood.

The development of the heparin removal system is still at an early stage. Work currently is being directed toward (1) completing the purification of heparinase, (2) immobilizing heparinase to additional supports, and (3) testing the blood compatibility and effectiveness of heparinase reactors in vitro and in vivo.

One of the critical factors in our research has been the adaptation and use of multiple assays to follow heparinase activity. Particularly important were assays (e.g., Azure A) used in monitoring the fermentation and early stages of purification. By utilizing three different approaches for assaying heparin (disappearance of heparin, appearance of reaction products, and disappearance of heparin's biological activity), the occurrence of any arti-

facts in the production and purification procedures, and activity tests, was avoided.

While our studies on heparinase production and purification have been encouraging, less success has been achieved in the immobilization procedures (Table II). Studies are in progress to understand better the important parameters in immobilization procedures and in establishing new supports. Initial results indicate that a noncharged support with a high surface area is best (Table II). Additionally, our preliminary evidence is that high levels of heparinase ( $> 1$  mg/mL) and the presence of substrate in the immobilization reaction enhance the recovery of immobilized enzyme activity.

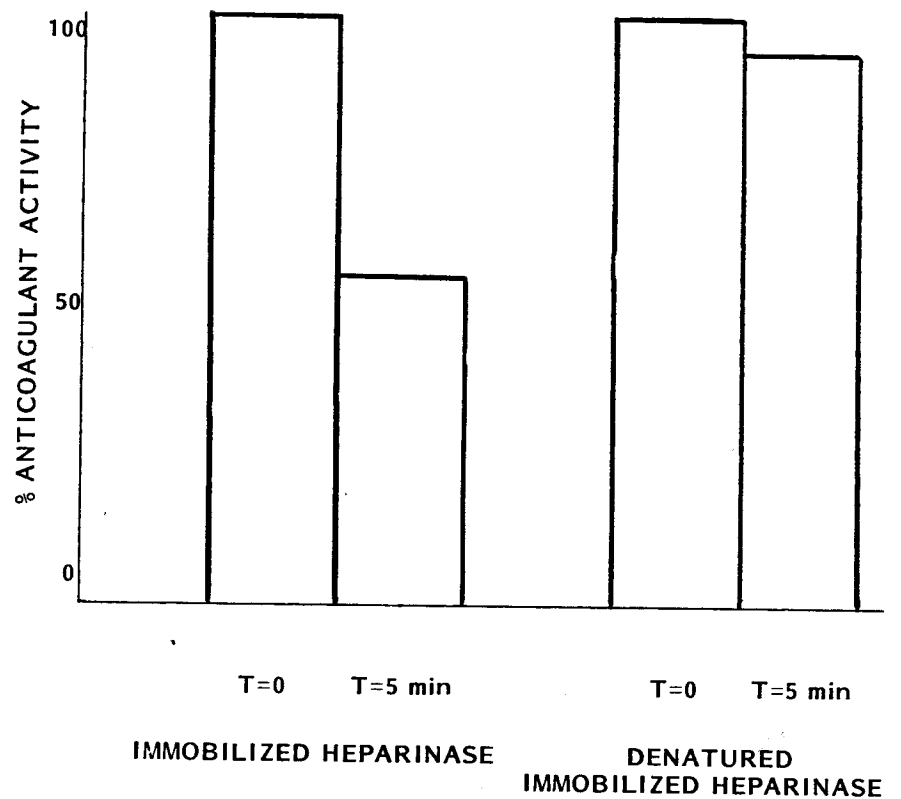


Figure 8. *In vitro* heparin removal from citrated rabbit blood by passing heparin through a 500- $\mu$ L Sepharose-heparinase column at a flow rate of 0.5 mL/min. The anticoagulant activity as measured by both Factor X<sub>a</sub> and whole blood clotting time is shown for the untreated blood and for the blood cycled through either the natured or denatured Sepharose-heparinase column for 5 min.

Heparinase immobilized on a negatively charged support probably will result in substrate repulsion, and thus reduced activity due to the strong negative charge of heparin. This result may, in fact, explain the apparent poor activities of some of the immobilized heparinase preparations listed in Table II.

The initial tests of immobilized heparinase on heparinized blood were limited to short time periods ( $< 5$  min). At later times, apparent decreases in heparin levels were observed in the control columns, although at a slower rate than with the active column. This effect may be due to blood damage occurring on the column. Such damage by Sepharose is not unexpected (37), and research is in progress to use either a different support with better blood compatibility or a Sepharose column with a lower bed-to-blood volume ratio.

At present, synthetic blood filters are routinely placed at the effluent of extracorporeal devices such as the pump-oxygenator or artificial kidney to remove clots or aggregates formed during the perfusion. The filters used in oxygenators can be as large as 2 L, whereas those used in renal dialysis are only several milliliters. With further development, heparinase could be immobilized to polymers in these filters. In this case, the filter could remove both clots and heparin.

The use of such a filter is anticipated to be general. Ideally it could be used during the entire operation to prevent high levels of heparin from ever entering the patient. Such an approach would require that heparin be added continuously to the extracorporeal unit either by infusion (2) or controlled release (38). The filter could be used at the very end of the perfusion to neutralize heparin, similar to the way protamine is currently used. However, the specificity of heparinase and the fact that it is immobilized could eliminate the toxic effects caused in some cases by protamine (39). The toxicity of heparinase reaction products and immunological effects remain to be tested. Nevertheless, the results obtained to date indicate that small volumes of immobilized heparinase can remove clinically used levels of heparin. Although work is still at an early stage, many of the feasibility questions (e.g., stability, activity) of the heparinase reactor have been addressed, and work is in progress to develop further the heparin removal system. Finally, we hope that the studies conducted will not only act as a first step towards developing a heparinase reactor, but that they will also aid and encourage other studies using this enzyme to examine the structure of heparin (40), to develop new assays for heparin (41), and to understand better the action of eliminase enzymes (42).

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