Blood Deheparinization Using Immobilized Microbial Heparinase. R.J. Linhardt, R. Langer, C.L. Cooney, P.M. Galliher, M.M. Flanagan, S.M. Hoffberg, Massachusetts Institute of Technology, Department of Nutrition and Food Science, Cambridge, MA 02139.

INTRODUCTION

Extracorporeal 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 (1,2,3). There is the prospect of even longer perfusion times with machines such as the membrane oxygenator. In such cases, the drawbacks of systemic heparinization are multiplied (4). A number of approaches 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 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 which would allow the full heparinization of the extracorporeal device yet which could enable elimination on-demand of heparin in the patient's bloodstream. This approach consists of a blood filter containing immobiized 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 report 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.

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 one thousandfold increase in volumetric heparinase production has been obtained over previously reported results 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 effect 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 innoculation of the sterile medium. Growth was initially exponential and heparin was rapidly taken up by the cell at a rate of 1.1 g/gcell-hr (Figure 2). Enzyme specific activity began to increase just as heparin uptake was finishing and increased at a volumetric rate of 375 units/L-hour. 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 hours. To avoid this deactivation it was crucial to understand the kinetics of enzyme production. Timely

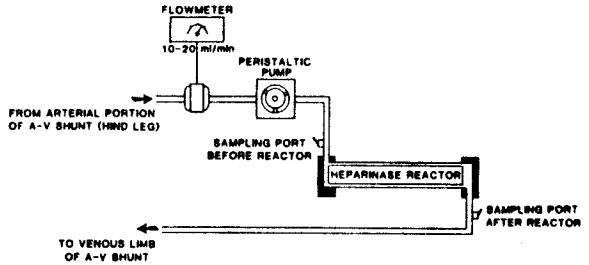

Figure 1: Proposed heparin circuit. The extracorporeal device could be a renal dialysis unit or a pump-oxygenator. The heparinase reactor could be part of a blood filter to be used either continuously (in which case heparin would be added continuously at the start of the circuit) or at the end of an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

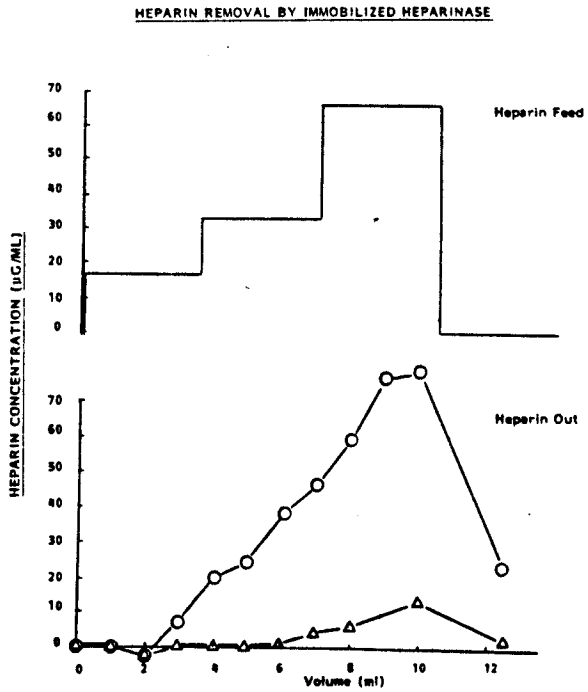

Figure 4: Heparin removal from a protein/salt solution by a 1.5 cc column packed with Sepharose-immobilized heparinase. The upper portion of this figure represents the stepped increase of heparin input. The bottom portion represents the measure of heparin output from both the Sepharose-immobilized enzyme (Δ) and the heat denatured Sepharose-immobilized enzyme (o).

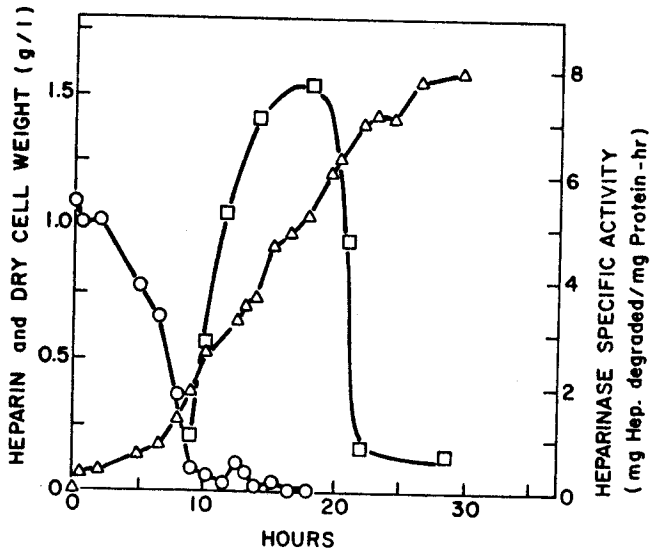

Figure 2: Results of a typical fermentation on complex medium showing heparin (O), heparinase specific activity (□), and dry cell weight (△) as a function of time as determined in a 2-liter fermenter.

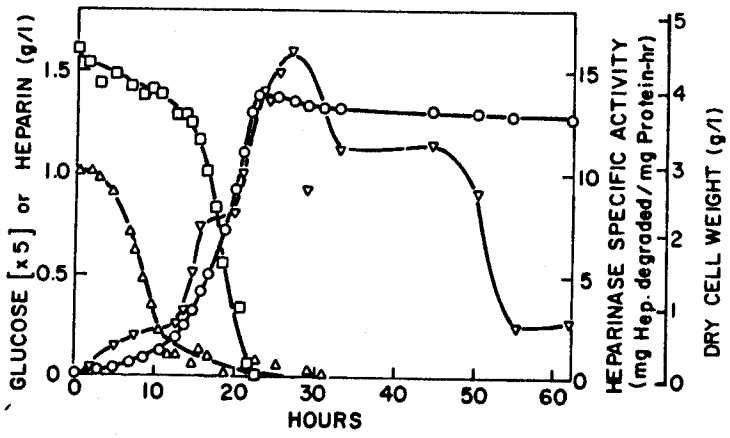

Figure 3: Results of a typical fermentation on defined medium showing dry cell weight (O), glucose (□), heparin (△), and heparinase specific activity (▽) as a function of time in a 2-liter fermenter.

harvest was thus important to obtaining highly active heparinase. Fifteen fermentations have been performed all yielding a total enzyme level on the average of 9600 units of heparinase/Liter of fermentor broth demonstrating the reliability of this method.

In order to better understand 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 found to be 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), $(NH_4)_2SO_4$ (nitrogen source), K_2HPO_4, Na_2HPO_4, L-Histidine, L-Methionine, trace salts, $MgSO_4.7H_2O$. A 30% increase in growth rate was observed using this medium. Volumetric heparinase production was increased 4 fold over the complex medium production to 1480 units/L-hour. Additionally, higher cell densities were routinely obtained in this defined medium. A typical production run using 20 g/L of glucose results in a 10 fold increase in total enzyme obtained to 96,000 units/L fermentor broth. This fermentation has been repeated 8 times to date demonstrating the reliability of this method. In addition, the product heparinase is more stable as no rapid loss of the enzyme occurs in this medium (Figure 3). This 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 $(NH_4)_2SO_4$ concentrations were found to be 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 found to be 29°C whereas optimum temperature for enzyme production was found to be 24°C. The maximum PO_4 concentration not deleterious to growth was found to be 20 mM.

Other methods of increasing the specific heparinase production of Flavobacteria are currently 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 2 fold 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 two-fold; 1) to adapt previous purification schemes of Hovingh and Linker (16) 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 x g was resuspended at 100 mg/ml protein in 0.01 M phosphate buffer pH 7.0 and dissrupted sonically, the nucleic acids were precipitated with 12.5 mg/ml of protamine sulfate and the protein solution was added to 4g of hydroxylapatite per g-protein. The hydroxylapatite bound protein was then washed stepwise with increasing concentrations of sodium chloride 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 has been explored. In preliminary experiments, we found that 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; polyvinylsulfate (PVS), polyanethole sulfonate (PAS) and polystyrene sulfonate (PSS) were found to meet these requirements. The inhibitory effect of PVS (Ki = 3.0×10^{-8}M; MW ~ 10,000) appeared to be linked to the

presence of sulfate groups because it was found that inhibition was lost when PVS was hydrolyzed. An affinity column was prepared by immobilizing partially hydrolyzed PVS on Epoxy-activated Sepharose (25). Heparinase (HA purified) 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 (18).

Isoelectric focusing (IEF) has also been applied towards the HA purified enzyme to obtain highly pure heparinase. The enzyme is loaded onto a pre-focused acrylamide gel at pH 7.0. After isoelectric focusing the enzymatic activity can be recovered at pH 8.5 ± 0.5. The resulting enzyme has a specific activity of about 5000 units/mg protein having undergone an enirchment of 50 fold (18).

The purification of heparinase has been followed by SDS-gel electrophoresis. The crude sonicate gave > 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 1.

TABLE 1 - Heparinase Purification

	Sp. Activity (mg. of Hep. degraded/ mg. Prot-h) (15)	Mg. Prot.	# Major SDS Bands
Whole Cells	4.3	1000	-
Sonnicate	7.9	730	20
Protamine precipitate	12.5	480	-
Hydroxylapatite purified	88	45	3
Affinity chromatography	2,000	-	-
Isoelectric focusing	5,000	-	2

Properties of Heparinase

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

The enzyme is very specific, acting only on heparin ($Km = 4.2 \times 10^{-5}M$) and heparin monosulfate. Heparinase acts endolytically as an α 1,4 eliminase cleaving heparin (MW ≅ 10,000) at 9 to 10 sites (18).

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 bovine serum albumin or polylysine (0.05%) and glycerol (7.5%) to permit 100% activity recovery on freeze-thawing.

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

Studies of the effect of pH on HA heparinase activity and stability determined that the activity maxima is pH 5.8, while the stability maxima is a pH 7.0.

HA heparinase has an activity maxima at 30°C but greater stability at lower temperature; $t_{1/2}$ denaturation at 4°C = 125 h and $t_{1/2}$ denaturation at 30°C = 25 h.

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, cellulose, and polyacrylamide. Low levels of activity recovery have been found on poly-2-hydroxyethylmethacrylate (polyHEMA). The other supports tested gave either no activity recovery or only barely detectable levels of activity.

At present, we are continuing our efforts to immobilize heparinase to support materials in the hopes of achieving 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 noticable 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. 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 maxima is shifted to a slightly higher temperature, from T = 30°C for the free enzyme to T = 37°C for the Sepharose-immobilized enzyme. 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.

The apparent Km of the immobilized enzyme is $1.2 \times 10^{-3}M$ (this can be determined by analyzing the kinetic data using a Lineweaver-Burk Plot) This Km is considerably higher than the Km determined for the free enzyme ($Km = 4.2 \times 10^{-5}M$).

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°C for 30 min. In one set of experiments, both active and denatured immobilized heparinase were loaded into 2 columns both with a 1.5 cc bed volume. Solutions of heparin, BSA (60 mg/ml) and salts were passed through each column at a flow rate of 0.5 ml/min. The concentrations of the non-heparin species were chosen to mimic physiologic concentrations and the flow rate was chosen to be in the same order of magnitude range as those used in renal dialysis units. The heparin levels in the solution increased in stepwise fashion from 15 µg/ml to 75 µg/ml as shown in Figure 4. 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. Even at 66 µg/ml the heparin was largely removed by the active heparinase column while the denatured heparinase column had no effect. Clinically used levels of heparin are on the order of 5-10 µg/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 one hundred 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 (0.5cc) at a flow rate of 0.5 ml/min. 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 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 an ongoing program in our laboratory to develop a system for removing heparin in extracorporeal therapy. Because the amount of data on heparinase has until now been limited and the methods of producing it inadequate for large scale use (16), the principal 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 (16), 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 31% activity recovery and excellent stability, and 5) demonstrating that columns as small as 1.5 cc can remove clinically used quantities of heparin in aqueous medium and in blood.

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 liters whereas those used in renal dialysis are only several ml. We might speculate that 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. It could ideally 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 conntinuously added to the extracorporeal unit either by infusion (2) or controlled release (19). 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 (20). It should be noted that 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 further develop the heparin removal system.

LITERATURE CITED

1. Gervin, A.S.; Surg. Gyn. Obst., 140, 789 (1975).
2. Swartz, R.D.; Port, F.K.; Kidney Int., 16, 513 (1979).
3. Basu, D.; Gallus, A.; Hirsh, J.; and Cade J.; New Eng. J. Med., 287, 324 (1972).
4. Fletcher, J.R.; McKee, A.E.; Mills, M.; Snyder, K.C.; and Herman, C.M.; Surgery, 80, 214 (1976).
5. Jastrzebski, J.; Hilgard, P.; and Sykes, M.K.; Card. Res., 9, 691 (1975).
6. Berglin, E.; Hansson, M.A.; Teger-Nilsson, A.C.; and William-Olsson, G.; Thromb. Res., 9, 81 (1976).
7. Schmer, G.; Trans. Amer. Soc. Artif. Int. Organs, 18, 321 (1972).
8. Lagergran, M.R.; and Eriksson, J.C.; Trans. Amer. Soc. Artif. Int. Organs, 17, 10 (1971).
9. Leininger, R.I.; Falb, R.D.; and Grode, G.A.; Ann. N.Y. Acad. Sci., 146, 11 (1968).
10. Yen, S.P.S.; and Rembaum, A.; J. Biomed. Mat. Res. Symposium, 1, 83 (1971).
11. Hersh, L.S.; Gott, V.C.; and Najjar, F.; J. Biomed. Mat. Res. Symposium, 3, 85 (1972).
12. Rea, W.J.; Whitley, D.; and Eberle, J.W.; Trans. Amer. Soc. Artif. Int. Organs, 18, 316 (1972).
13. Kusserow, B.K.; Larrow, R.; and Nichols, J.; Trans. Amer. Soc. Artif. Int. Organs, 17, 1 (1971).
14. Kolff, W.J.; and Stellwag, F.; Ann N.Y. Acad. Sci., 283, 443 (1977).
15. Galliher, P.M.; Cooney, C.L.; Langer, R.; Linhardt, R.J.; J. Applied Environ. Micro., in press (1980).
16. Linker, A.; Hovingh, P.; Methods in Enzymol., 28, 902 (1972).
17. Vretblad, P.; Biochem. Biophys. Acta, 434, 169 (1976).
18. Linhardt, R.J.; Langer, R.; Cooney, C.L.; Galliher, P.M.; Flanagan, M.M.; Hoffberg, S.M.; Unpublished data.
19. Langer, R.; Chem. Eng. Commun., 6, 1 (1980).
20. Jastrzebski, J.; Sykes, M.K.; Woods, D.G.; Thorax, 29, 534 (1974).

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health grant number GM25810. The authors wish to thank G. Fitzgerald for his technical assistance.