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IMMOBILIZED HEPARINASE: PRODUCTION, PURIFICATION, AND APPLICATION IN EXTRACORPOREAL THERAPY

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There are nearly 20,000,000 perfusions involving extracorporeal medical machines performed each year (1,2). The artificial kidney is employed several times weekly by over 100,000 persons each year (1). Open-heart operations employing cardio-pulmonary bypass are an everyday occurrence at many medical centers (2). Less often employed is the membrane oxygenator for pulmonary support in the critically ill infant (3). Still other applications of extracorporeal devices, such as the artificial liver, are in a conceptual stage (4).

In every case, systemic levels of heparin, which fully anticoagulate the patient, are required for the operation of extracorporeal medical devices. However, the resulting circulating concentration of heparin results in a high incidence of hemmorhagic complications, particularly in patients at risk of bleeding and for certain longer term perfusions. The severity of hemmorhage may vary from mild mucosal oozing to massive intracranial, gastrointestinal, genitourinary, and intrathoracic bleeding (5,6). The incidence of hemmorhage during heparinization is reported to be from 8 to 33% (7). Six to 10% of patients develop coagulation abnormalities with excessive bleeding following open-heart surgery; and this percentage increases drastically with the use of long-term pulmonary support with membrane oxygenators (3). Bleeding complications occured 10-19% of the time using either low dose heparin or regional heparin regimens in hemodialysis of patients with increased risk of hemorrhage (8); and nearly 25% of all patients suffering from acute renal failure are subject to increased bleeding risk. The incidence of partial clotting in the

dialyzer was 3-5% with either low dose heparin or regional heparinization (8). In addition to hemorrhage, there are a number of other complications associated with heparinization, particularly when the drug is administered over a long period. Some of these complications include alopecia (9) and interference with bone repair (10), leading in some cases to severe decalcifying bone disease (11).

Because of the problems associated with systemic heparinization, a number of approaches have been explored to solve this problem. Attempts at regional heparinization, by infusing heparin into the machine and trying to reverse it with a heparin antagonist such as protamine, have met with little clinical success (8). Protamine only reversibly neutralizes heparin probably by occupying its highly charged regions. It causes hypotension as well as anticoagulation; but most importantly its anti-heparin affect is quite variable (12). Some investigators have used very low dose heparin successfully to minimize the amount of anticoagulation to which the patient is subject (8). There has also been a great deal of research devoted to the creation of blood compatible materials (13), including materials with heparin (14) or other substances (15) bonded to their surface. Machines could be fabricated from these materials; and theoretically no anticoagulant would be needed. The development of heparin substitutes has also been explored (16). At present however, there is no ready solution to the problem of using extracorporeal devices requiring blood perfusion without anticoagulating the patient.

We are proposing a new approach which may enable heparin to be removed specifically from the blood after it has served its purpose in the extracorporeal device and before it is returned to the patient. This approach consists of a blood filter, containing immobilized heparinase, which could be placed at the effluent of any extracorporeal device. Theoretically, this would allow the extracorporeal machine to be anticoagulated while the patient was not. The significance of the proposed heparinase filter is that it may open new possibilities in extracorporeal treatment. Among the potential advantages include a) the possibility of obviating the need of potentially toxic neutralizing substances such as protamine (17) (i.e., the filter could be used at the termination of the perfusion in place of current protamine reversal techniques) and b) the opportunity of heparinizing the extracorporeal system without simultaneous heparinization of the patient (i.e., the filter could be used continuously in a manner analogous to regional heparinization by replacing the protamine influx with the filter). The filter would also permit the use of higher heparin concentrations in extracorporeal devices.

The primary objective of our research is to determine if immobilized heparinase is capable of removing heparin from blood.

The amount of data on heparinase has until now been limited and the methods for producing it are inadequate for large scale use. Therefore, the principal focus of our research has been on conducting feasibility studies on heparinase production, purification, immobilization, and initial trials of filters of immobilized heparinase with human blood and in dogs. These studies are reported here.

HEPARINASE PRODUCTION

The limitations of earlier procedures (18) to produce heparinase were a) non reproducability, b) extreme expense, c) not conducive to high yields, and d) not suitable for scale up. In order to solve these problems, we have conducted two types of studies: a) one aimed at the kinetics of microbial growth and heparinase production and at optimizing the conditions (e.g., harvest time) of fermentation and b) one aimed at developing a simple defined low cost medium for heparinase fermentation.

The wild-type strain of *Flavobacterium heparinum* produces a cell associated heparinase during growth only when heparin is supplied to the growth medium as an inducer. The inducer was provided at the time of innoculation of the sterile medium. Our findings were that growth was initially exponential and heparin was rapidly taken up by the cell at a rate of 1.1 g/g cell/hr. Enzyme specific activity began to increase just as heparin uptake was finishing and increased at a volumetric rate of 375 units/L/hr. One unit is defined as one mg of heparin degraded/hr. At the onset of the stationary growth phase, enzyme production stopped; and a deactivation was observed resulting in a 86% loss of total activity within 4 hr. Thus, understanding the kinetics of enzyme production, and implementing timely harvest, were critical to obtaining highly active heparinase. By implementing the conditions of induction and harvest outlined above, nearly a 100 fold increase in maximum volumetric productivity was obtained compared to previous studies. Many fermentations have been performed yielding an enzyme level on the average of 9600 units of heparin/L fermentor broth, demonstrating the reliability of this method (19).

In earlier procedures a complex media involving trypticase-soybroth, vitamins, heparin, and mineral salts was used. Results in this complex medium were difficult to reproduce and difficult to interpret. We conducted a step-by-step investigation of the factors critical to the fermentaiton. Our findings were as follows: a) nineteen compounds (other than heparin) were tested for their ability to induce heparinase. Of these, only hyaluronic acid, heparin monosulfate, maltose, and N-acetyl D-glucosamine induced *Flavobacteria heparinum* to produce heparinase. None induced at levels higher than heparin. b) The nutritional requirements in-

clude an absolute requirement for histidine and growth stimulation by methionine. No other amino acids and no vitamins were required. c) A defined growth medium was developed consisting of glucose, ammonium sulfate, potassium and sodium phosphate, magnesium sulfate, trace minerals, heparin, histidine, and methionine. d) The nitrogen source (ammonium sulfate) optimum concentration for growth was 0.5 g/L. e) The optimum temperature for growth was 27°C. f) The optimum concentration of initial glucose (carbon source) for growth was 8 g/L. g) Above a sodium chloride concentration of 4.8 g/L (as sodium) the growth rate was diminished by 0.04 hr^{-1}/g sodium added. h) The optimum phosphate concentration for growth was 20 mM (19).

Using the defined media developed in these studies, the volumetric heparinase production, cell growth rate, and densities all increased. A typical production run resulted in a 10 fold increase (compared to our optimized complex medium) in total enzyme obtained to 96,000 units/L fermentor broth. This fermentation has been repeated many times demonstrating the reliability of the method. Furthermore, there is not a rapid loss of the enzyme at the end of the fermentation in this medium (19).

PURIFICATION AND CHARACTERIZATION OF HEPARINASE

The objectives of our work on the purification of heparinase were two-fold: a) to adapt previous purification schemes (20) to large scale production, and b) to purify heparinase to homogeneity. 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 disrupted soncially; the nucleic acids were precipitated with 12.5 mg/ml protamine sulfate; and the protein solution was added to 4 g hydroxylapatite/g protein. The hydroxylapatite bound protein was then washed stepwise in a batch procedure with increasing concentrations of sodium chloride and sodium phosphate (from 0 M and 0.01 M to 0.50 M and 0.25 M, respectively). The resulting enzyme preparation (HA) was obtained in 0.125 M NaCl and 0.07 M sodium phosphate wash (21).

For the second goal, we tried affinity chromatography. 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. Over 30 sulfated substances were screened; and three synthetic heparin substitutes polyvinylsulfate (PVS), polyanethole sulfonate (PAS), and polystyrene sulfonate (PSS) were found to meet these requirements. These are the first heparinase inhibitors to be discovered. The inhibitory effect of PVS ($K_i = 3.0 \times 10^{-8}$ M; MW $\sim$ 10,000) was lost as PVS was hydrolyzed. An affinity column was prepared by

immobilizing partially hydrolyzed PVS on epoxy-activated Sepharose (22). Heparinase (HA purified) was bound to this column and released at either pH 11 or 4 to give 500% enrichment in specific enzyme activity with 5-10% total activity recovery.

Isoelectric focusing (IEF) also was tried for obtaining highly pure heparinase. The enzyme was loaded onto prefocused acrylamide gel at pH 7.0. After isoelectric focusing the enzymatic $\pm$ 0.5 to give a specific activity of about 5000 units/mg protein and an enrichment of 50 fold.

The purification of heparinase was 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. Heparinase has a MW of 51,000 $\pm$ 6,000 as judged by Sephadex G-200 gel exclusion chromatography. SDS gel electrophoresis of isoelectric focused heparinase showed only two bands, one at 80,700 MW and one at 45,700 $\pm$ 1,600. The latter band fell within the range of that obtained by Sephadex chromatography, further suggesting the accuracy of the 51,000 $\pm$ 6,000 value obtained by that method.

The enzyme is very specific, acting only on heparin (K_m = 4.2×10^{-5} M) and slightly on heparin monosulfate. Over 30 very similar polysaccharides, including other glycosaminoglycans, were tested. We also found that heparinase acts endolytically as an α-1,4-eliminase cleaving heparin (M.W. $\sim$ 10,000) at 9-10 sites. A computer model has been developed and used successfully to predict the degradation kinetics of heparin by heparinase (23).

IMMOBILIZED HEPARINASE

Immobilization of heparinase should prevent the enzyme from circulating and thereby reduce or eliminate immunological problems. Heparinase was immobilized to Sepharose using a variation (21) of the procedure of March *et al.* (24) at a yield of 91%. The heparinase-Sepharose had enhanced thermal stability, which was especially noticeable at low temperature storage of this enzyme. At 4°C the immobilized enzyme had a half life of denaturation of >3600 hr compared with 125 hr for the native enzyme at the same temperature. The greater stability of the immobilized enzyme is also seen at higher temperatures (21).

In initial trials with blood we took a 100 cc Bentley blood transfusion filter, packed it with 50 cc of Sepharose-heparinase, and fluidized the Sepharose by pumping it through a U-tube interconnected to the filter at a flow rate 6 times higher than blood was being pumped through the same filter (Fig. 1). This

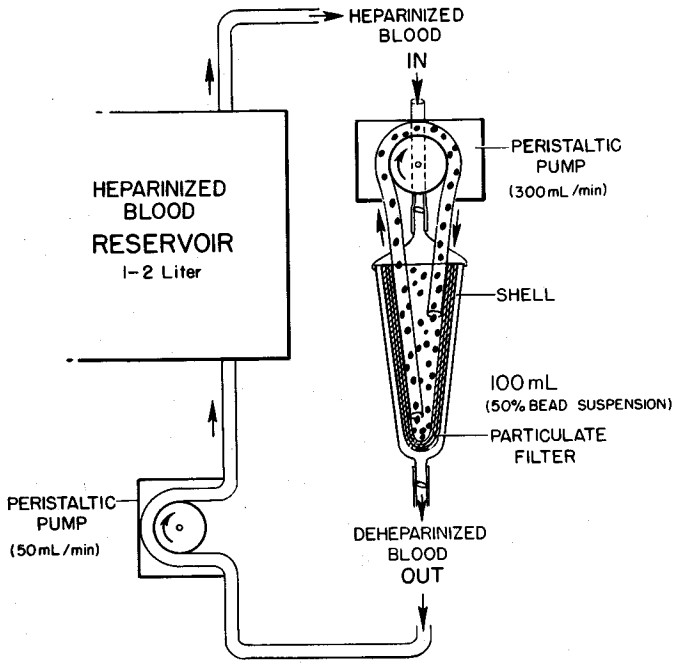

Fig. 1. Heparinase reactor.

fluidization prevented the Sepharose from packing and preventing blood flow. Using this reactor, two sets of experiments were conducted. In one set, whole human blood (2 units) was obtained from Boston's Children's Hospital Blood Donor Service and anticoagulated at 100 units heparin/ml. This was much higher than clinical levels; but gave a more difficult test. The blood was stored at 37°C during use. Blood was passed through the filter at 50 ml/min. Samples were taken after each of 6 passes and prior to heparinization, centrifuged at 4500 rpm for 20 min; and the plasma was assayed by the APTT (25), and Azure A (26) methods. Sixty percent of heparin anticoagulant activity was gone by 6 min or 3 passes by the APTT. The Azure A test was performed as a control to insure that heparin was actually being lost and that the results obtained were not merely due to damage to coagulation factors. Azure A measures the concentration of heparin for all heparin segments of hexasaccharide or larger (27). The reason the Azure A test does not show zero heparin activity is that a few of hexasaccharide or larger are present, as shown by both HPLC and gel permeation chromatography and by digestion of heparin by heparinase in buffer. These segments show Azure A binding activity but do not possess anticoagulant activity as measured by APTT. Control filters containing Sepharose without heparinase showed no effect as judged by either assay.

We performed a similar test on an 11 kg dog, which was anesthetized and catheterized through the carotid artery and vein with a Scribner Shunt fitted with silastic tubing, which interfaced directly with the heparinase reactor. The dog was administered 4500 units of heparin. Blood samples were taken and anlayzed by 3 assays: APTT (25), whole blood recalcification time (26), and Azure A (26). As shown in Fig. 2, within 2 min or 1 pass through the filter, nearly all anticoagulant activity was lost. The Azure A test again was used as a control assay. The controls showed no detectable effect (Fig. 2). An *in vivo* half life of 2 hr for heparin was measured in the dog. The dogs appeared healthy and were still alive 3 month after the deheparinization experiments.

We have also taken the heparinase degradation products from these filters and have subjected them to toxicity tests using the *S. typhimurim* mutagenesis assay (28). No mutagenicity was observed even with concentrations 1000-fold in excess of those we would anticipate clinically. Heparin was also negative in this assay. Some platelet loss was noted using this filter; this may be circumvented, however, by a different choice of biomaterials for the heparinase filter, co-immobilization of other compounds (e.g., prostacylin) to the filter, or a different filter design.

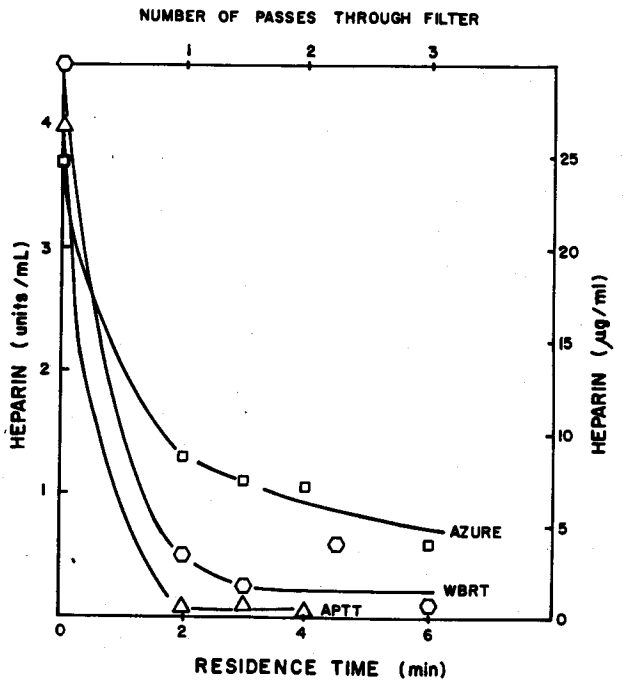

Fig. 2. Heparin levels in blood of dogs.

Although the filter is at an early stage of development, it is clear that such a filter could have broad generality. At present there exist blood filters, in some cases as large as 2000 cc, that are routinely used at the termination of extracorporeal procedures to remove aggregates formed during these procedures. It is possible that heparinase could be bound to these filters. In fact, one attractive feature of the application of immobilized heparinase is that the filter would only be used in situations where the blood must leave the patient and where existing biomaterials already interface with blood at the desired location. Thus, the eventual application of this process might not require any additional inconvenience to the patient or additional apparatus for the physician.

REFERENCES

1. SCHREINER, G. E. *Trans. Am. Soc. Art. Int. Org. 26:* 9 (1980).
2. KENNEDY, R. H., KENNEDY, M. A., MCGOON, D. C., PLUTH, J. R. & NOBREGA, F. T. *J. Thorac. Cardiovasc. Surg. 80:* 702 (1980).
3. FLETCHER, J. R., MCGEE, A. E., MILLS, M., SNYDER, K. C. & HERMAN, C. M. *Surgery 80:* 214 (1976).
4. GLABMAN, S., GERONEMUS, R., VONALBERTINI, B., KAHN, T., MOUTOSSIS, G. & BOSCH, J. P. *Trans. Am. Soc. Art. Int. Org. 25:* 394 (1979).
5. BASU, D., GALLUS, A., HIRSCH, J. & CADE, J. *New Eng. J. Med. 287:* 324 (1972).
6. GILSON, S. *Throm. Diath. Hemorh. 6:* 152 (1961).
7. GERVIN, A. S. *Surg. Gyn. Obstet. 140:* 789 (1975).
8. SWARTZ, R. D. & PORT, F. K. *Kidney Int. 16:* 513 (1979).
9. HIRSCHBOECK, J., MADISON, F. & PISCIOTTA, A. *Am. J. Med. Sci 227:* 279 (1954).
10. STINCHFIELD, F., SANKAVAN, B. & SAMILSON, R. *J. Bone Joint Surg. 38:* 270 (1956).
11. GRIFFITH, G., NICHOLS, G., ASHER, J. & FLANAGEN, B. *J. Am. Med. Assoc. 193:* 91 (1965).
12. JASTRZEBSKI, J., HILGARD, P. & SYKES, M. K. *Card. Res. 9:* 691 (1975).
13. KOLFF, W. J. & STELLWAG, F. *Ann. N.Y. Acad. Sci. 283:* 443 (1977).
14. LEININGER, R. I., FALB, R. D. & GRODE, G. A. *Ann. N.Y. Acad. Sci. 146:* 11 (1968).
15. KUSSEROW, B. K., LARROW, R. & NICHOLS, J. *Trans. Am. Soc. Artif. Int. Org. 17:* 1 (1971).
16. BERGLIN, E., HANSSON, M. A., TGER-NILSSON, A. C. & WILLIAM-OLSSON, G. *Thromb. Res. 9:* 81 (1976).
17. JACQUES, L. B. *Canad. Med. Ass. J. 108:* 1291 (1973).
18. HOVINGH, P. & LINKER, A. *J. Biol. Chem. 245:* 6170 (1970).

19. GALLIHER, P. M., COONEY, C. L., LANGER, R. & LINHARDT, R. *Appl. Envir. Micro. 41:* 360 (1981).
20. LINKER, A. & HOVINGH, P. *Biochemistry 11:* 53 (1972).
21. LANGER, R., LINHARDT, R. J., GALLIHER, P. M., FLANAGAN, M., KLEIN, M. & COONEY, C. L.in "Biomaterials: Interfacial Phenomena and Applications" (S. Cooper, A. Hoffman, B. Ratner, and N. A. Peppas, eds.), Advances in Chemistry Series, in press.
22. VRETBALD, P. *Biochim. Biophys. Acta. 434:* 169 (1976).
23. LINHARDT, R. J., FITZGERALD, G. L., COONEY, C. L. & LANGER, R. *Biochim. Biophys. Acta.*, in press.
24. MARCH, S. C., PARIKH, I. & CUATRECASAS, P. *Anal. Biochem. 60:* 149 (1974).
25. LANGDELL, R. D. in "Thrombosis and Bleeding Disorders," (N. U. Bank, F. K. Beller, E. Deutsch, E. F. Mammen, eds.) Academic Press, New York (1971).
26. HOFFBERG, S., thesis, M.I.T. (1981).
27. DIETRICH, C. P. *Biochem. J. 108:* 647 (1968).
28. SKOPEK, J. R., LIBER, H. L., KADEN, D. A. & THILLY, W. G. *Proc. Natl. Acad. Sci. USA 75:* 4465 (1978).