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An Investigation of Heparinase Immobilization

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

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

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ABSTRACT

A systematic investigation of the parameters that affect the efficiency of immobilizing heparinase onto cyanogen bromide activated crosslinked 8% agarose beads was conducted. Two experimental measures, the "fraction bound" and the "fraction retained," were used to monitor the coupling efficiency. The fraction bound is the portion of the total initial enzyme that is bound to the agarose gel. The fraction retained is the fraction of bound enzyme that is active. The product of the two measures indicates the coupling efficiency. The activity of the immobilized heparinase was measured under conditions free of both internal and external mass transfer limitations, and thus, the fraction retained represents the true immobilized enzyme activity.

Increasing the degree of activation of the beads results in an increase in the fraction bound, the fraction retained, and consequently, the coupling efficiency. As the ratio of enzyme solution to gel volume increases from 1.5 to 2.2, the fraction bound remains constant but the fraction retained decreases (heparinase concentration; 0.15 mg/mL and degree of activation; 9.5 μmol of cyanate esters/g of gel). At volume ratios greater than 2.2, both the fraction bound and the fraction retained decline continuously. Changing the heparinase concentration in the coupling solution changes the coupling efficiency in a manner similar to that of the volume ratio change.

When heparin is added during the coupling process, the fraction bound declines as the heparin concentration increases, whereas the

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

fraction retained increases up to a heparin concentration of 12 mg/mL and decreases thereafter. When arginine, lysine, and glycine are used to block the unreacted cyanate ester groups after the coupling process, the immobilized heparinase shows different pH optima of 6.5, 6.9, and 7.2, respectively. Based upon these findings, a protocol to optimize heparinase immobilization is developed.

Index Entries: Heparinase immobilization; fraction bound; fraction retained; coupling efficiency; cyanogen bromide activation; crosslinked 8% agarose beads; degree of activation; blocking agents.

INTRODUCTION

A reactor containing immobilized heparinase has previously been shown to be useful in removing heparin and thereby preventing heparin-induced bleeding complications when the anticoagulant is used in extracorporeal perfusions (*1,2*). The enzyme was immobilized onto crosslinked 8% agarose gel beads that were activated with cyanogen bromide (CNBr) (*3,4*). Although heparinase was successfully immobilized onto these beads, a systematic study of the immobilization process was not conducted. In order to maximize the efficiency of heparinase immobilization and at the same time minimize any waste of heparinase, it is essential to investigate the factors that affect the binding and the retention of heparinase activity so that the immobilization procedure can be optimized.

We present herein a systematic investigation of the parameters that affect the immobilization of heparinase to a CNBr activated support material. Crosslinked 8% agarose beads were chosen as the support material because when compared with other polysaccharide support materials (e.g., Sephadex) that are suitable for CNBr activation, agarose beads possess an optimal pore size and surface area for heparinase immobilization (*5*). In addition, crosslinked 8% agarose beads are mechanically strong and have been used for the ex vivo heparinase reactor studies (*2*).

The activity of the enzyme after immobilization can be influenced by both the distribution of the enzyme within the porous suport and the conditions under which the enzyme is assayed. Thus, to measure the true fraction retained, diffusional limitations and the concentration profile of the enzyme within the support must be taken into consideration.

The binding kinetics of heparinase to CNBr activated agarose has been studied previously (*3*). Under the conditions utilized in the current investigation heparinase is uniformly distributed within the agarose gel. The reaction kinetics of the immobilized heparinase and assay conditions have also been determined (*4*). Under the conditions employed in the current study, internal and external mass transfer limitations have been completely eliminated (*4*). Thus, the enzyme activity measured is the true activity of immobilized enzyme.

Two experimental measures, the "fraction bound" and "fraction retained," are used to monitor the efficiency of heparinase immobilization. The fraction bound is defined as the portion of the total initial enzyme that is bound to the support material. The fraction retained is defined as the fraction of bound enzyme that is catalytically active. The product of the two measures represents the ratio of bound active enzyme to the total amount of enzyme initially available for immobilization and indicates the overall coupling efficiency. Since the two experimental measures are independent ones and may vary in a different fashion, the overall coupling efficiency accounts for variability in both these measures.

The parameters involved in the present study include the degree of activation of the support (i.e., μmol cyanate esters per weight agarose), the ratio of heparinase solution to agarose bead volume, the enzyme concentration in the coupling solution, and the amount of heparin added during the coupling procedure. In addition, the effects of the blocking agents which were used to block any unreacted cyanate ester groups after heparinase immobilization were also examined.

Based upon these findings, a protocol to optimize heparinase immobilization and to minimize the waste of the enzyme was developed. The systematic approach discussed in this paper may be followed to develop protocols for the preparation of other immobilized enzymes, especially when the samples are prepared in large quantities and an optimized process is necessary.

EXPERIMENTAL

Materials

Heparin was purchased from Hepar Industries. Bio-Rad Coomassie dye reagent, hydroxylapatite, and Biogel A 1.5 (8% agarose) were from Biorad Laboratories. 2,3-dibromopropanol was from Aldrich Chemicals. Cyanogen Bromide was purchased from Fisher Scientific Company. All other chemicals were reagent grade and water was distilled and deionized. All immobilizations were conducted in 16 mL polypropylene tubes obtained from Nalgene.

Methods

Assays

Protein concentrations were measured by the method of Bradford (*6*). The activity of free heparinase was assayed by following the appearance of the double bonds of the heparin degradation products at 232 nm (*7*). The activity of immobilized heparinase (i.e., on crosslinked agarose beads) was measured using a procedure described previously (*4,8*). A unit of

heparinase activity is defined as the amount of enzyme required to produce 1 μmol of double bonds/min (*7*).

Heparinase Preparation

Cultures of *Flavobacterium heparinum* were grown according to the procedure of Galliher et al. (*10*). After harvest, the cells were disrupted with a homogenizer, and the homogenate was treated with protamine sulfate to remove the nucleic acids present (*11*). Heparinase was then purified from the homogenate by a combination of batch hydroxylapatite and QAE-Sephadex chromatography, according to a previously described method (*12*). The enzyme solution was concentrated by ultrafiltration at 4°C for 2–3 h, using an Amicon Model 8400 Ultrafiltration Cell equipped with a YM30 membrane. After ultrafiltration, the enzyme had a specific activity of 0.45–1.2 U/mL and a protein concentration of 0.10–0.28 mg/mL.

The heparinase solution thus prepared was nearly free from all the other catalytic enzyme contaminants (*12*). The catalytically pure heparinase has been shown to have identical kinetic properties to the homogeneous heparinase (*12*). In addition, the protein contaminants present have been shown to have no effect on the binding of heparinase to CNBr activated agarose (*3*). Heparinase content was determined from the densitometry trace of the SDS electrophoresis gel of the preparation.

Preparation of Crosslinked 8% Agarose

Biogel 1.5 (8% agarose) beads were crosslinked twice with 2,3-dibromopropanol, according to the procedure described previously (*9*).

Heparinase Immobilization

The immobilization process consists of two steps: the activation of the agarose matrix with cyanogen bromide, and the coupling of heparinase to the activated gel. A detailed description of both steps is listed below. Unless otherwise stated, this general procedure was followed throughout the entire study.

Activation of the Agarose Beads

The crosslinked 8% agarose beads were activated according to a modified procedure of March et al. (*13*). One hundred mL of beads were washed with 500 mL of chilled distilled water and resuspended in 100 mL of the distilled water. To the suspension, 200 mL of chilled 2 M sodium carbonate solution were added. After a short period of equilibration, 40 mL of CNBr in acetonitrile (1 g/mL) were added, and the mixture was vigorously agitated in a fume hood for 5 min. The reaction mixture was quickly filtered, washed with 1000 mL of chilled distilled water, and then with 500 mL of chilled 1 mM HCl.

A 150 mg of sample of suction-dried beads was assayed for the degree of activation according to a previously described procedure (*9*). One g of suction dried resin corresponds to 0.5 gm dry weight. The activated

beads thus prepared normally contain 10–20 μmol of cyanate esters per gram of the suction-dried resin. To prepare beads possessing different degrees of activation, different volumes of the CNBr solution were used.

Coupling of Heparinase

Coupling was carried out by adding two volumes of the above prepared heparinase solution to one volume of the activated beads. The bottle containing the reaction mixture was agitated for 10 h at 4°C using an orbital rocking table (Boekel, W. Germany). The beads were filtered on a coarse glass filter and the supernatant was assayed for heparinase activity. To block the unreacted cyanate ester groups, the beads were suspended in two volumes of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.2 M lysine (i.e., the blocking agent) and 0.5 M NaCl. The suspension was incubated overnight at 4°C.

After the incubation, the beads were washed with three volumes of chilled 0.1 M $NaHCO_3$, 0.5 M NaCl buffer (pH 8.3), three volumes of 0.0725 M NaH_2PO_4, 0.125 M NaCl buffer (pH 7.0), and then resuspended in the latter buffer. The washings and a sample of beads were assayed for heparinase activity. The volume of beads was determined by spinning the suspension at 4°C, 2000 g for 5 min in a graduated polystyrene centrifuge tube.

When the effect of heparin in the coupling solution was examined, heparin powder was added to the enzyme solution and the same coupling process was followed. When the effect of using different blocking agents was examined, agents such as glycine and arginine were used to replace lysine in the blocking process, and identical procedures were followed.

Estimation of the Fraction Bound, Fraction Retained, and Coupling Efficiency

The fraction bound (F_b) was calculated as follows:

$$F_b = (V_i A_i - V_s A_s - V_w A_w) / (V_i A_i)$$

where V_i was the volume of the original enzyme solution, A_i was the activity of the original enzyme solution, V_s was the volume of the supernatant, A_s was the activity of the supernatant, V_w was the volume of the washings, and A_w was the activity of the washings.

The fraction retained (F_r) was computed as

$$F_r = V_b A_b / (F_b V_i A_i)$$

where V_b was the total volume of beads and A_b was the activity of the beads.

The coupling efficiency (CE) was calculated as

$$CE = F_b F_r = V_b A_b / V_i A_i$$

RESULTS AND DISCUSSION

Control experiments in which heparinase was incubated with non-activated agarose were conducted to determine the loss of heparinase activity caused by agitation and thermal deactivation. No loss of enzymatic activity ($N=6$) was observed over the 10-h period. All losses of heparinase activity from the coupling solution with activated beads are therefore attributed to the immobilization procedure.

If the functional groups at the active site of the enzyme are involved in the coupling, it may result in a conformational change of the enzyme, rendering it catalytically inactive. To protect the active site, enzymes are often immobilized in the presence of their substrate or competitive inhibitors (*14*). We have chosen to use heparin to protect the active site of heparinase because of its availability and high water solubility.

The effect of adding heparin at different concentrations to the coupling solution is given in Fig. 1. Heparin was maintained in the concentration range of 6–90 mg/mL. At these levels the enzyme is kept saturated with the substrate since the heparin concentrations are much larger than 0.12 mg/mL, which is the K_m of the free enzyme (*11*). All other immobilization parameters were held constant.

The maximum fraction bound occurred with no added heparin and declined with increasing concentrations of heparin added. The differences in the fraction bound are statistically significant ($P<0.01$) except between heparin concentrations of 6 and 12 mg/mL. The decrease in fraction bound with increasing heparin concentration suggests that heparin competes with heparinase for binding sites on the activated agarose beads. The coupling of heparin to the agarose beads may be attributed to the amino groups on the heparin molecule that allow heparin to bind (*14*). It is important to note that even at the lowest heparin concentration (6.0 mg/mL), the molar ratio of heparin to heparinase is 240 to 1 and heparin is present in a large excess.

The fraction retained increased with increasing heparin concentration up to a level of 12 mg/mL, and decreased with further increases in heparin concentration (Fig. 1). The initial rise in the fraction retained suggests that heparin offers protection to the active site during the coupling process. The decline in activity with higher heparin levels, however, may result from the increase in bound heparin. Heparin molecules are highly negatively charged and their attachment to the gel surface may create a microenvironment unfavorable to heparin degradation. The bound heparin will electrostatically repel soluble heparin and prevent it from approaching the bound enzyme.

The drop in the fraction bound and the rise in the fraction retained observed at heparin concentrations between 0 to 12 mg/mL are statistically significant ($P<0.01$). However, at these heparin levels, there is no statistical difference in the coupling efficiency. The increase in the fraction retained observed with adding small amounts of heparin is offset by the

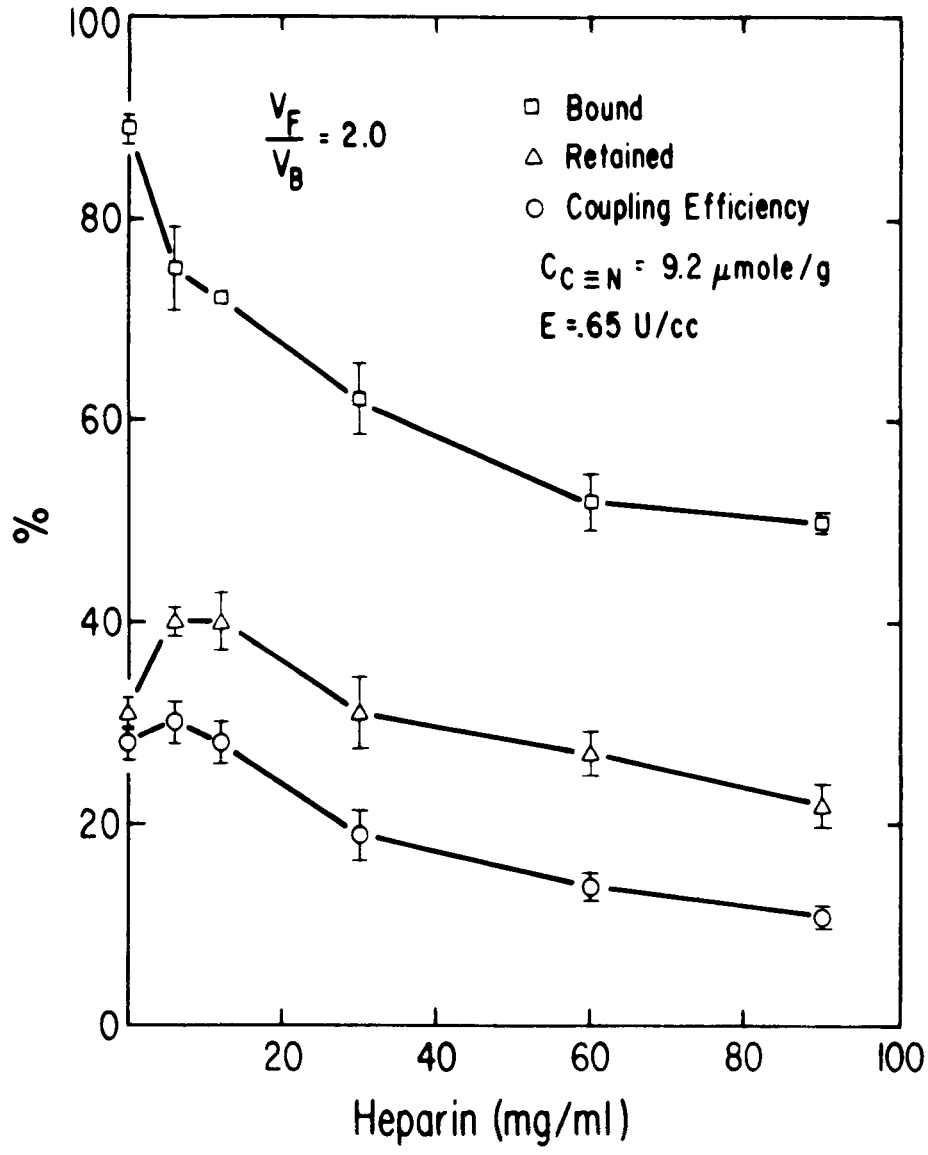

Fig. 1. The effect of heparin on the immobilization of heparinase. The symbols of $C_C \equiv N$, E, and V_F/V_B used in this and the following figures (Figs. 2-5) represent the cyanate ester concentration, the enzyme concentration (in U/mL), and the volume ratio of enzyme solution to beads, respectively. Unless otherwise stated, the pH and temperature in this and the following figures were 7.0 and 4°C, respectively. In addition, each point was the average of two independent experiments, and all assays were conducted in triplicate.

decrease in the fraction bound, and as a consequence, the addition of heparin results in no net effect on the overall coupling process. However, at heparin levels greater than 12 mg/mL, the overall coupling efficiency drops at a statistically significant rate ($P < 0.02$) and the addition of heparin has a negative effect.

The effect of adding heparin was further studied with beads containing a higher degree of activation. The cyanate ester concentration on the beads was increased from 9.2 μmol/g in Fig. 1 to 13.5 μmol/g in Fig. 2. It was assumed that at a higher cyanate ester concentration the likelihood of multipoint attachments of the enzyme to the agarose would be increased. Under such conditions, the effect of protecting the active site of the enzyme with heparin would be more obvious. Heparin concentrations were maintained between 0–6 mg/mL, since, as shown in Fig. 1, values outside of this range had either no effect or a negative effect on the immobilization.

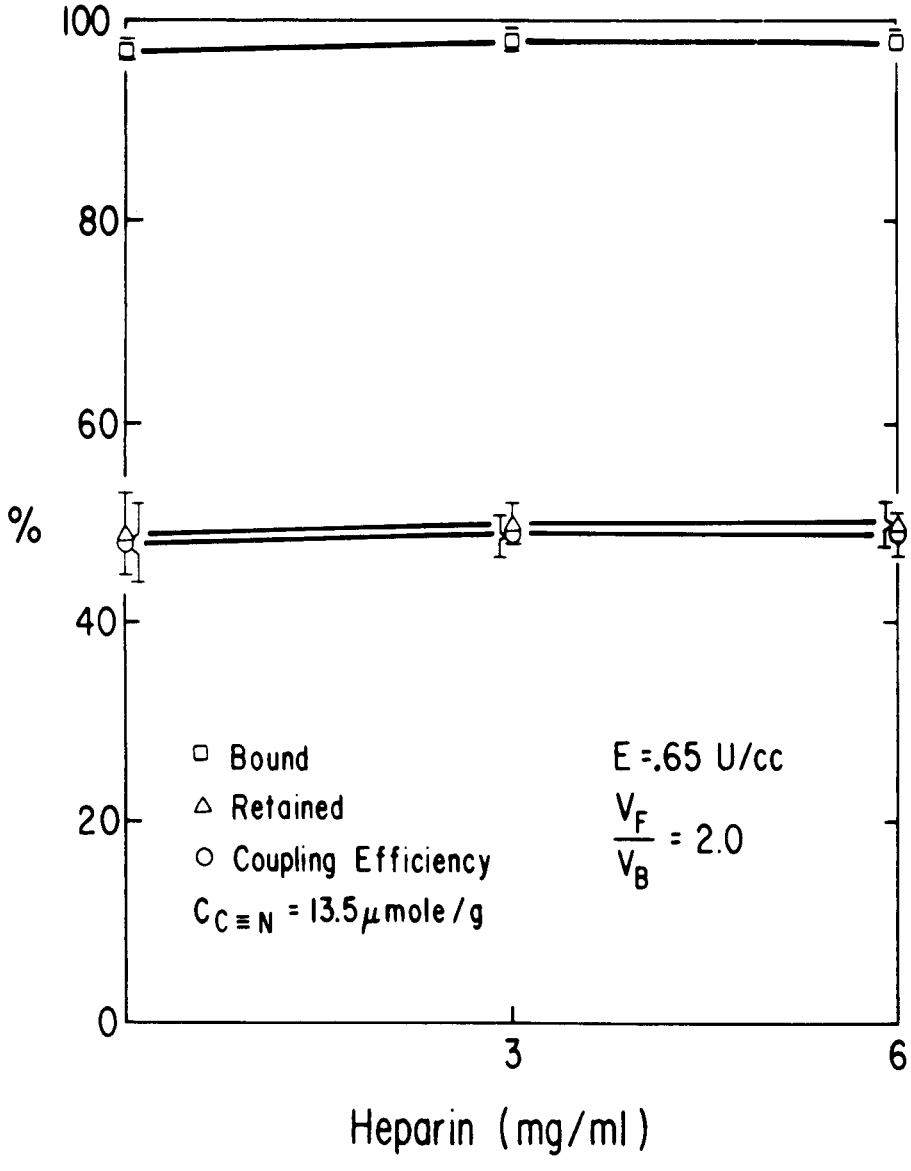

Fig. 2. The effect of heparin on the immobilization of heparinase. The cyanate ester concentration was 13.5 μmol/g.

There was no significant variation in the fraction bound, fraction retained, and the coupling efficiency over the heparin range tested (Fig. 2, $P < 0.05$). The increase in available cyanate esters apparently negated the effect of heparin competing with heparinase for binding.

Unlike the data shown in Fig. 1, the fraction retained in Fig. 2 remained constant over the heparin concentrations studied. The reasons for this behavior are not clear and any suggested interpretation must be speculative. As discussed previously, heparin could offer some protection to the active site of heparinase during the coupling process and bound heparin may also stabilize the immobilized enzyme. These effects would result in an increase in the fraction retained as opposed to what was observed. However, a high cyanate ester concentration would introduce a high level of bound heparin. This could impart a net negative charge on the beads, and alternatively, decrease the level of the fraction retained. Thus the constancy in the fraction retained shown in Fig. 2 may be the consequence of these two opposite effects offsetting each other. It could also mean that at high cyanate ester concentrations more heparin would be required to issue the protection to the enzyme active site.

The coupling efficiency, which was constant over the heparin range studied, was a direct consequence of the fraction bound and the fraction retained. It therefore appears that at a high cyanate ester concentration of 13.5 μmol/g, the addition of heparin has no statistically significant effect.

The effect of varying the cyanate ester concentration is given in Fig. 3. Three different cyanate ester concentrations (5.6, 9.2, and 13.5 μmol/g) were examined. All other immobilization parameters were held constant. The fraction bound and the fraction retained increased with increasing cyanate ester concentration, causing the overall coupling efficiency to increase as well.

At these conditions, the cyanate esters were always present in at least a 900-fold excess of the enzyme. Therefore, the drop in the fraction bound at lower cyanate ester concentrations was not owing to a shortage of unreacted esters. The results may be more properly interpreted on the basis of the kinetics of the coupling process. The coupling kinetics increase with increasing cyanate ester concentration (3). Since the experiment was conducted over a fixed time period, the gel with the highest ester concentration will have bound the most enzyme and have a larger fraction bound.

As more cyanate ester groups are added to the gel, multipoint attachments are favored. The possibility of coupling amino groups near or in the enzyme active site to the gel increases. The conformation of the enzyme is more likely to be altered and may inactivate the enzyme. This would decrease the fraction retained as opposed to the experimentally observed rise. Thus, if multipoint attachments were occurring they were not sufficient to decrease the immobilized enzyme activity.

A second explanation for the experimental finding may be related to the lysine buffer that was used after the enzyme coupling step. For these experiments, lysine buffer was added to the gel to block the unreacted

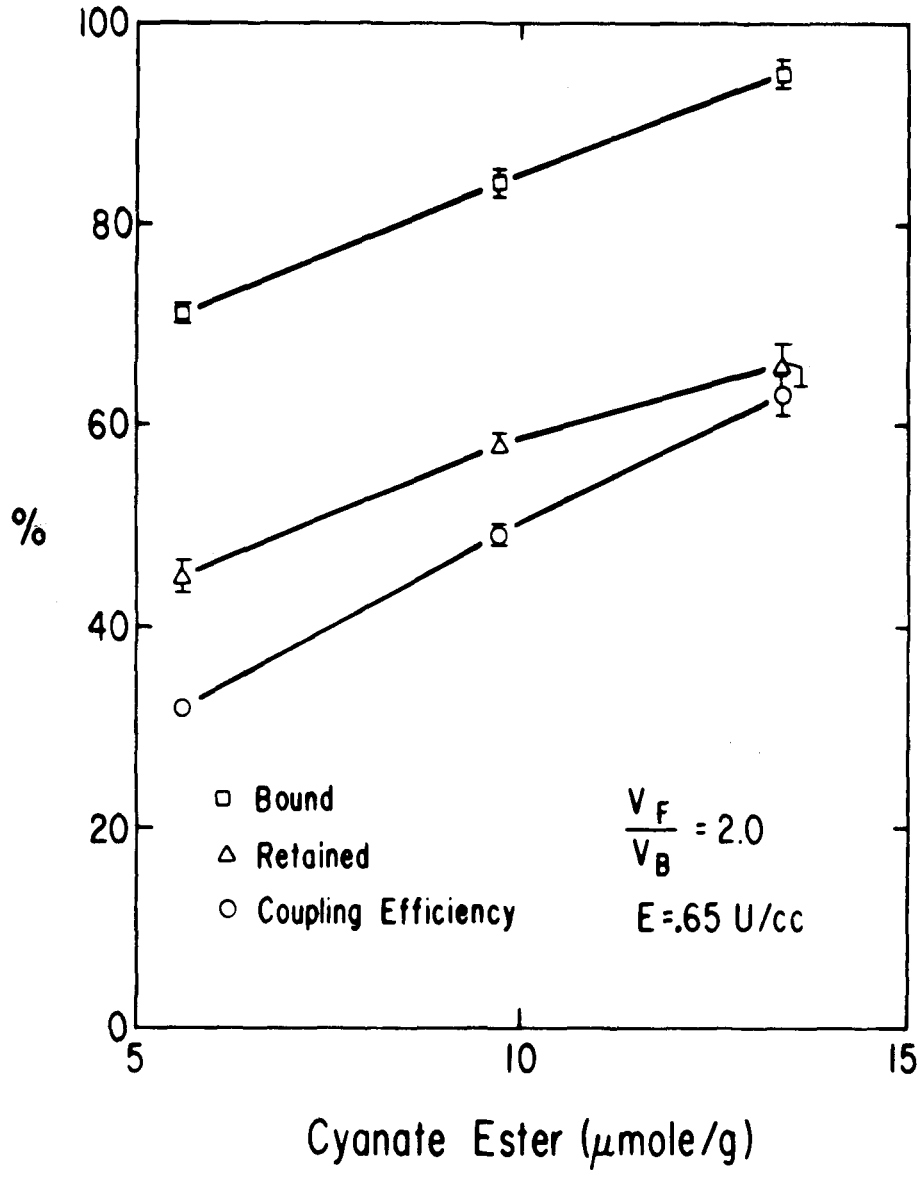

Fig. 3. The effect of cyanate ester concentration on the immobilization of heparinase. No heparin was added during the coupling process.

cyanate groups. It has been shown that lysine enhances the amount of heparinase activity retained as compared to no lysine or the use of other amino acids such as glycine (*5*). Rosenberg (*14*) has also reported that lysine residues are responsible for the binding of heparin to antithrombin. From these results it appears that heparin has a strong affinity for lysine. Those beads that had a high degree of activation will have more unused cyanate esters remaining on the gel at the end of the coupling step. More lysine can be attached to the gel, creating a microenvironment favorable to the substrate heparin.

The effect of the volume ratio of enzyme solution to beads is given in Fig. 4. All other immobilization parameters were held constant. Up to a volume ratio of 2.2, there was no statistically significant difference in the fraction bound ($P < .05$). However, at a volume ratio greater than 2.2, the fraction bound declined continuously. The fraction retained decreased with increasing values of the volume ratio. The overall coupling efficiency decreases in a manner parallel to that of the retained fraction. As pre-

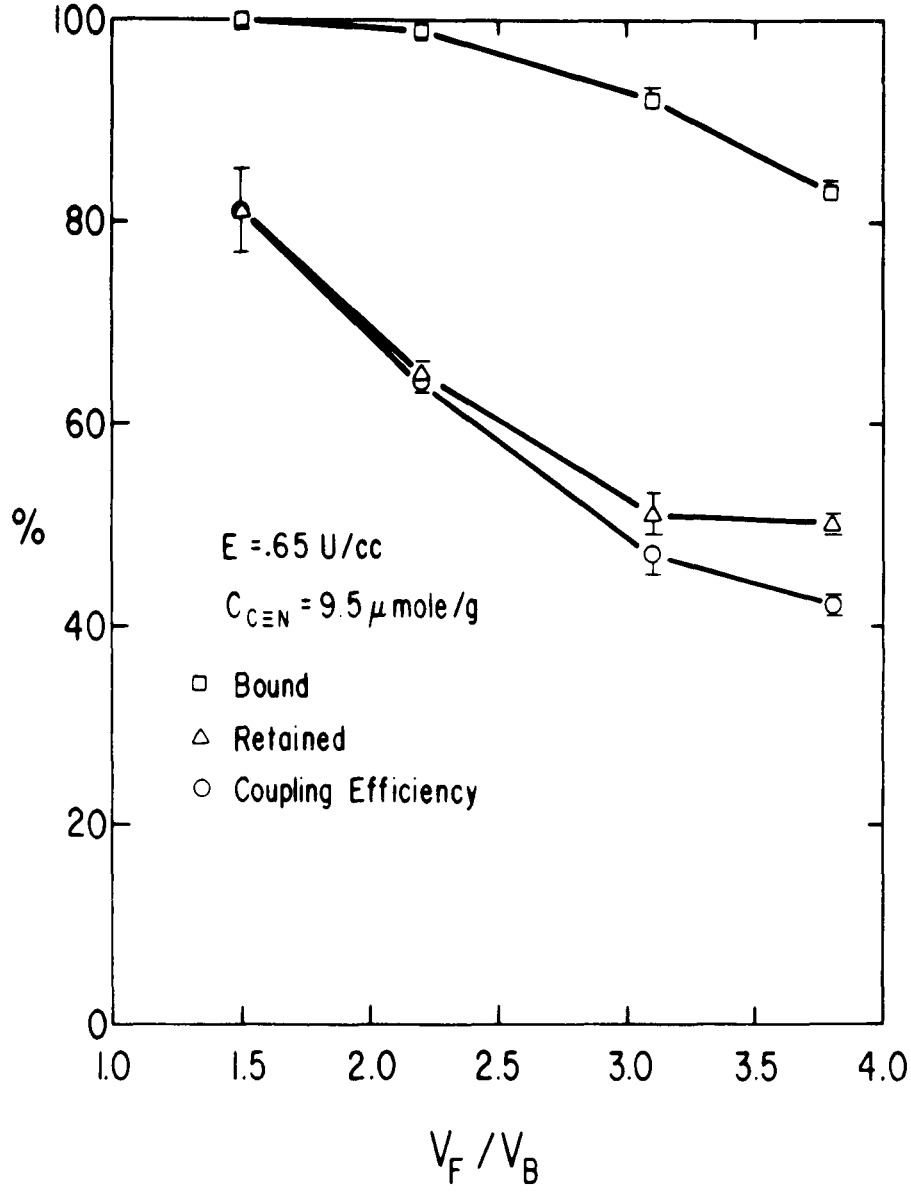

Fig. 4. The effect of the volume ratio of enzyme solution to beads on the immobilization of heparinase. No heparin was added during the coupling process.

viously discussed, the drop in the bound fraction was not owing to the lack of cyanate ester groups. As the volume ratio was increased, the total amount of enzyme loaded onto the support was greater. A higher percentage of the cyanate esters become sterically inaccessible to the unbound enzyme. This presumably results from direct shielding of the esters by a bound molecule, space limitations, or electrostatic effects. Together with multipoint attachments, the cyanate ester concentration that is available to react is much lower and the rate of binding slows down. As more enzyme binds, it becomes more difficult for subsequent free enzyme molecules to couple. Since the experiment was conducted over a fixed time period, the slowdown in binding rate causes a net drop in the fraction bound.

The drop in the fraction retained at higher ratios may result from unfavorable electrostatic interactions and space limitations arising from enzyme crowding. Such crowding prevents enzyme molecules from maintaining their native active conformation and can result in a drop of the fraction retained (*15*).

If high bound enzyme concentrations were responsible for the drop in the fraction bound and the fraction retained, the same qualitative behavior should be seen when the gel was overloaded with enzyme by increasing the heparinase concentration of the immobilization solution. The effect of the enzyme concentration on the immobilization is given in Fig. 5. The volume ratio was maintained at a constant value of 2.0, whereas the enzyme concentration in the solution was varied from 0.45 to 1.2 U/mL. As shown in Fig. 5, the same qualitative behavior was observed on the fraction bound, fraction retained, and coupling efficiency. As the enzyme concentration in the coupling solution was increased, the fraction bound and the fraction retained decreased. These results are consistent with our conclusion that in the presence of a high level of bound enzyme the steric hindrance between enzyme molecules will impede their catalytic activity.

The effect of using different blocking agents is shown in Fig. 6. When arginine, lysine, and glycine were used to block the unreacted cyanate ester groups after heparinase immobilization, the immobilized heparinase showed different pH optima of 6.5, 6.9, and 7.2, respectively. It is reported that free heparinase has a pH optimum of about 6.5 (*11*). The pH optimum of the immobilized heparinase appears to shift toward a more alkaline value with increasing the acidity of the blocking agents used. These results are consistent those of Goldstein et al. (*15*), which suggest that an alkaline pH shift upon immobilization is indicative of the enzyme-support possessing a net negative charge. When the amino groups of the blocking agents are covalently linked to the support, it will provide the gel surface with a more negatively charged environment.

SUMMARY

Based on the above findings, a protocol to optimize heparinase immobilization and to minimize the waste of the enzyme was developed.

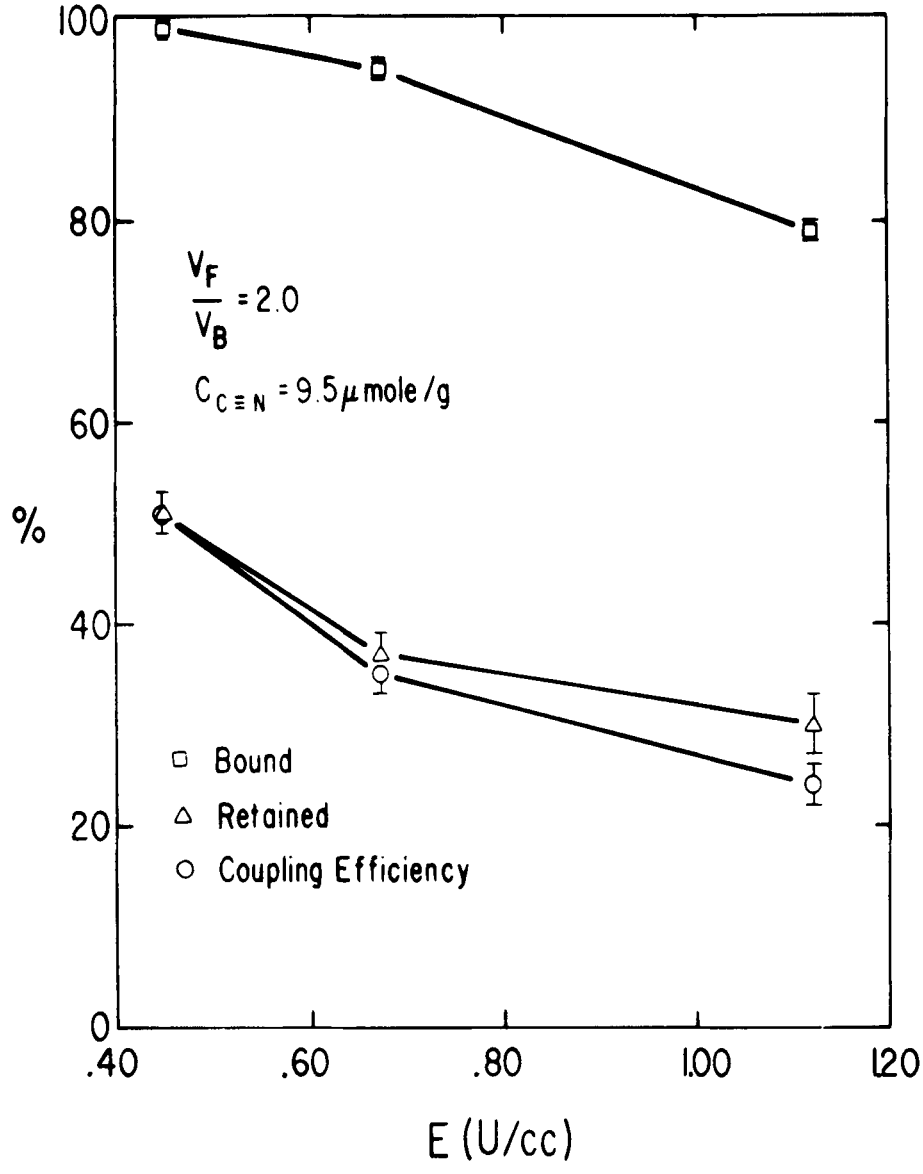

Fig. 5. The effect of enzyme concentration on the immobilization of heparinase. The volume ratio of enzyme solution to beads was maintained at 2.0. No heparin was added during the coupling process.

The protocol suggests the use of a highly activated gel containing 10–15 μmol/g of cyanate esters, a low volume ratio of enzyme to beads (e.g., a ratio of 1.5) or a low heparinase concentration (e.g., 0.45 U/mL), and no heparin during the coupling process. Although glycine provides the immobilized heparinase with an optimum pH closer to the physiological pH (this is the pH for the ex vivo testing of the heparinase reactor), lysine is suggested to be used as the blocking agent. This is because lysine provides a more favorable environment for heparin degradation.

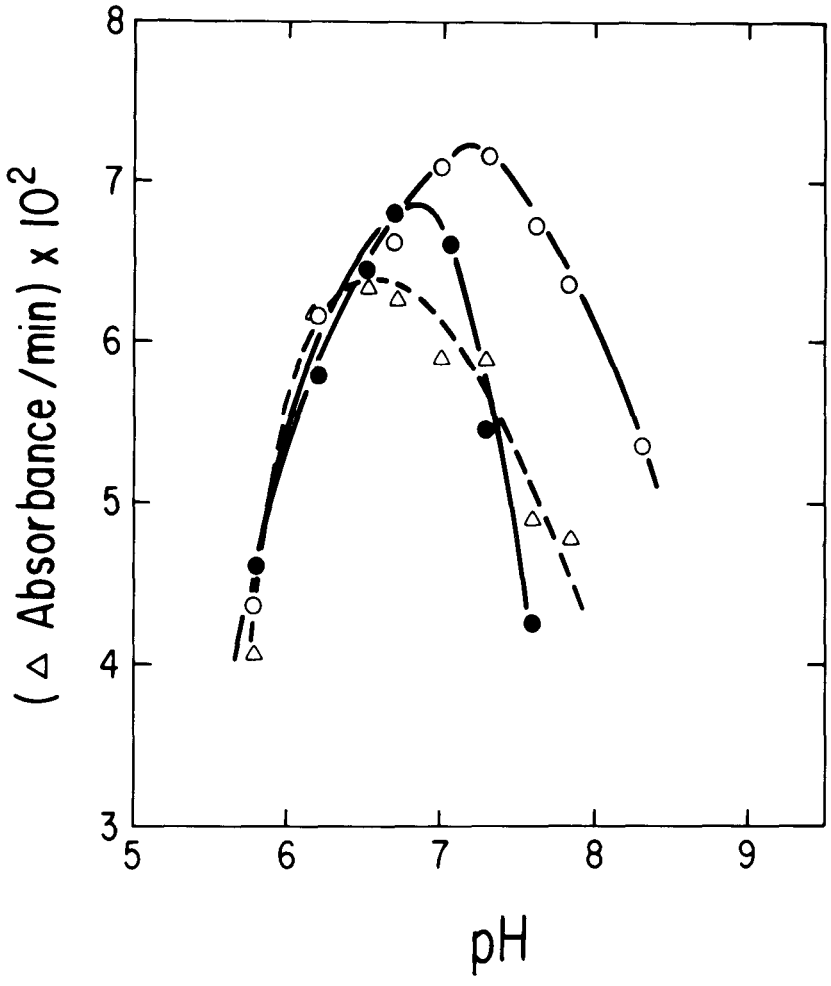

Fig. 6. The effect of blocking agents on the pH activity optimum of the immobilized heparinase. Heparinase activity (y-axis) was indicated by the change in the ultraviolet absorption at 232 nm, according to the procedure described in the Methods section. No heparin was added during the immobilization process.
△ Aginine ● Lysine ○ Glycine

Recently, immobilized enzymes (or proteins) have been found useful in many applications including those for industrial processing, chemical purification, therapeutic detoxification, and pharmaceutical and waste processing (*16*). In most cases, the enzyme costs are high and the production of the immobilized samples is on a large scale. Under these circumstances a well-defined procedure which can optimize the immobilization efficiency and minimize enzyme waste would be essential. The approach discussed in this paper may not only be useful for heparinase but perhaps for other enzymes as well.

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