# Heparinase Immobilization

## Characterization and Optimization[a]

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### INTRODUCTION

Heparinase from *Flavobacterium heparinum* has been found to be useful in numerous applications. These include the structural elucidation of heparin, new bioassays for heparin, the investigation of the anticoagulant mechanism, and the preparation of low molecular weight heparin anticoagulants and antitumor agents.[1-3] Recently, our laboratory has demonstrated a potential therapeutic application of heparinase by placing a reactor containing immobilized heparinase at the termination of extracorporeal therapy for blood deheparinization.[4,5] In order to test such a possibility, we have conducted a thorough investigation regarding heparinase immobilization.[6-8] Various immobilization techniques, as well as different support materials, have been examined for heparinase immobilization.[6,7] Among these techniques and support materials, cross-linked (CL) 8% agarose activated with cyanogen bromide (CNBr) has been selected for further studies of heparinase immobilization. This is done mainly because CL-8% agarose beads are mechanically strong enough to withstand the high operating pressure encountered in our *in vivo* deheparinization studies.[5] In addition, agarose beads permit high retention of heparinase activity after immobilization.[5,7] A systematic investigation of the parameters affecting the binding and retention of heparinase activity on CL-8% agarose beads has been conducted in order to optimize the immobilization procedure and to minimize any waste of heparinase.[8] These parameters include the degree of activation of the support, the volume ratio of enzyme to beads, the enzyme concentration in the coupling solution, and the amount of heparin added during the coupling procedure. Based upon these findings, a protocol to optimize heparinase immobilization and to minimize the waste of the enzyme has been developed. We also have characterized the immobilized heparinase with regard to its kinetics, as well as to the surface charge of the beads.

Herein, we present a detailed review of the developments concerning heparinase immobilization. The protocol developed in this report for heparinase immobilization

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currently is being adopted in our laboratory to prepare large quantities of immobilized heparinase for the *in vivo* extracorporeal blood deheparinization studies.

## RESULTS AND DISCUSSION

### *Immobilization of Heparinase*

#### *Characterization*

Heparinase was immobilized onto a variety of support materials using different activation techniques.[6,7] The supports giving the highest levels of immobilized activity included the following: CNBr-activated Sepharose 4B (50–91% activity immobilized), Sephadex (5–56%), and regenerated cellulose hollow fibers (4%); carbodiimide-activated or active ester–activated CM-Sephadex (4%), CM-cellulose (1%), and polyacrylamide (PAN) (35%); and epoxy-activated (oxirone) acrylic beads (1%). The highly negatively charged supports (e.g., CM-Sephadex) resulted in very low levels of immobilized heparinase activity, even when a large amount of protein was immobilized. The reduced activity observed was possibly due to the electrostatic repulsion between the support material and the highly negatively charged substrate, heparin. On the other hand, macroporous supports such as Sepharose and Sephadex gave the highest activity retention. Further examination of the Sephadex G series (including Sephadex G-15, 50, 75, 100, 150, and 200) of controlled pore size showed that increasing pore size increased the level of total activity that could be recovered on immobilization.[6] When the pore size was increased from Sephadex G-15 to that of Sephadex G-150, the total activity recovery on the gel was increased by nearly tenfold. However, further increases in pore size from Sephadex G-200 to Sepharose 4B resulted in only a slight increase in the level of immobilized heparinase activity. There was almost no change in activity recovery when heparinase was immobilized onto 4%, 6%, 8%, or 10% agarose beads.[5,7] Cross-linking of these agarose beads also resulted in no effect on the level of total immobilized heparinase activity.[5,7]

Cross-linked 8% agarose beads activated with CNBr were selected for further studies of heparinase immobilization because the beads were mechanically strong so as to withstand the high blood flow rates (>250 mL/min) normally encountered in an extracorporeal procedure. They have been selected for our *in vivo* deheparinization studies.

#### *Optimization*

A systematic investigation of the parameters that affected the efficiency of coupling heparinase onto CL-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 beads. The fraction retained is the fraction of the bound enzyme that is active. The product of the two measures indicates the coupling efficiency.

The effect of adding substrate (i.e., heparin) during the coupling procedure to protect the active site of heparinase is shown in TABLE 1. Heparin was maintained in

TABLE 1. The Effect of Heparin on the Immobilization of Heparinase[a]

| Heparin Concentration (mg/mL) | Fraction Bound (%) | Fraction Retained (%) | Coupling Efficiency (%) |
|---|---|---|---|
| 0 | 89 ± 3 | 31 ± 3 | 28 ± 3 |
| 6 | 75 ± 8 | 40 ± 3 | 30 ± 4 |
| 12 | 72 ± 1 | 40 ± 6 | 28 ± 4 |
| 25 | 62 ± 7 | 31 ± 7 | 19 ± 5 |
| 60 | 52 ± 5 | 27 ± 4 | 14 ± 3 |
| 90 | 50 ± 2 | 22 ± 4 | 11 ± 2 |

[a]The heparinase concentration in the coupling solution was 65 units/mL. The ratio of enzyme volume to bead volume was 2.0.

the concentration range of 6–90 mg/mL, which is far above the $K_m$ value (0.12 mg/mL) of the free enzyme.[9] The cyanate ester concentration on the activated beads was controlled at 9.2 μmoles/g beads. As shown in TABLE 1, the maximum fraction bound occurred with no added heparin and then declined with increasing concentrations of heparin added. The decline in fraction bound at high heparin concentrations suggested that heparin competed with heparinase for binding sites on the activated agarose beads. Heparin also contained amino groups that could bind with the cyanate ester groups on the activated beads. However, the fraction retained increased with increasing heparin concentration up to a level of 12 mg/mL and then decreased with further increases in heparin concentration. The initial rise thus suggested that heparin could possibly give protection to the active site of heparinase during the coupling process, whereas the decline in activity at higher heparin levels could possibly result from the increase in bound heparin on the gel. Heparin molecules were highly negatively charged and their binding to the gel surface could create a microenvironment that was repulsive for the free heparin to approach the bound heparinase. The increase in the fraction retained was offset by the decrease in the fraction bound and, as a consequence, this resulted in no net effect on the overall coupling efficiency.

The effect of varying the cyanate ester concentration is given in TABLE 2. The fraction bound and the fraction retained increased with increasing cyanate ester concentrations. Because both the fraction bound and the fraction retained increased at higher cyanate ester concentrations, the overall coupling efficiency also increased proportionally. It is important to note that the amounts of cyanate ester groups were always present in at least a 500-fold excess of the enzyme in all cases. Therefore, the drop in the fraction bound at lower cyanate ester concentrations was not due to the

TABLE 2. The Effect of Cyanate Ester Concentration on the Immobilization of Heparinase[a]

| Cyanate Ester Concentration (μmoles/g beads) | Fraction Bound (%) | Fraction Retained (%) | Coupling Efficiency (%) |
|---|---|---|---|
| 5.6 | 71 ± 2 | 45 ± 3 | 32 ± 1 |
| 9.6 | 84 ± 2 | 58 ± 2 | 49 ± 2 |
| 13.5 | 95 ± 2 | 66 ± 4 | 63 ± 3 |

[a]The heparinase concentration in the coupling solution was 65 units/mL. The ratio of enzyme volume to bead volume was 2.0.

TABLE 3. The Effect of the Ratio of Enzyme Volume to Bead Volume on the Immobilization of Heparinase[a]

| Volume Ratio | Fraction Bound (%) | Fraction Retained (%) | Coupling Efficiency (%) |
|---|---|---|---|
| 1.5 | 100 ± 1 | 80 ± 8 | 80 ± 5 |
| 2.2 | 99 ± 1 | 65 ± 3 | 64 ± 3 |
| 3.1 | 92 ± 2 | 51 ± 4 | 47 ± 4 |
| 3.8 | 83 ± 2 | 50 ± 2 | 42 ± 2 |

[a]The cyanate ester concentration on the beads was 9.5 μmoles/g beads. The heparinase concentration in the coupling solution was 65 units/mL.

shortage of unreacted esters. Instead, it was due to a respective drop in the coupling kinetics at lower cyanate ester concentrations.

The effect of the volume ratio of enzyme to beads is given in TABLE 3. The cyanate ester concentration on the gel was 9.5 μmoles/g beads. Up to a volume ratio of 2.2, there was no significant difference in the fraction bound. However, at a volume ratio greater than 2.2, the fraction bound declined continuously. Once again, the drop in the fraction bound was not due to the lack of cyanate ester groups. As the volume of the enzyme increased, the total amount of enzyme loaded onto the support became greater. Due to the shielding effect, as well as the space limitation and the electrostatic effect on the cyanate esters by the bound enzyme molecules, a higher percentage of cyanate esters became sterically inaccessible to the unbound (i.e., free) heparinase. This essentially slowed down the rate of coupling. Because the experiment was conducted over a fixed time period, the slowdown in coupling rate caused a net drop in the fraction bound. Therefore, despite the increase in the total amount of bound enzyme, the fraction bound decreased at higher volume ratios. In addition, TABLE 3 shows that the fraction retained decreased with increasing volume ratios. This could result from unfavorable electrostatic interactions and space limitations arising from enzyme crowding. Such crowding could prevent enzyme molecules from maintaining their native active conformation and could result in a drop of the fraction retained. The overall coupling efficiency, as a consequence of the drop in the fraction bound and the fraction retained, decreased with increasing volume ratios.

If high bound enzyme concentrations were responsible for the drop in the fraction bound and the fraction retained, then the same qualitative behavior should be seen when the gel was overloaded with enzyme by increasing the heparinase concentration in the coupling solution. The effect of enzyme concentration on the immobilization is

TABLE 4. The Effect of Enzyme Concentration on the Immobilization of Heparinase[a]

| Heparinase Concentration (units/mL) | Fraction Bound (%) | Fraction Retained (%) | Coupling Efficiency (%) |
|---|---|---|---|
| 45 | 98 ± 2 | 51 ± 3 | 50 ± 2 |
| 67 | 95 ± 2 | 37 ± 6 | 35 ± 6 |
| 112 | 79 ± 2 | 30 ± 6 | 24 ± 6 |

[a]The cyanate ester concentration on the beads was 9.5 μmoles/g beads. The ratio of enzyme volume to bead volume was 2.0.

given in TABLE 4. The volume ratio was maintained at a constant value of 2.0, whereas the enzyme concentration in the coupling solution was varied from 45 units/mL to about 112 units/mL. As shown in TABLE 4, the same qualitative behavior was observed on the fraction bound and the fraction retained. As heparinase concentration was increased, both the fraction bound and the fraction retained decreased. Furthermore, these results have been found to be consistent with our earlier conclusion that the steric hindrance between enzyme molecules could impede their catalytic activity in the presence of a high level of bound enzyme.

Therefore, based on the above findings, a protocol to optimize heparinase immobilization and to minimize the waste of heparinase has been developed. The protocol suggests the use of highly activated gel containing 10–15 μmoles of cyanate esters per gram of beads, a low volume ratio of enzyme to beads (e.g., a ratio of 1.5) or a low heparinase concentration (e.g., 90 units of heparinase per volume of beads), and no heparin during the immobilization. At present, this protocol is being followed in our laboratory to prepare the heparinase-bound beads for the *in vivo* blood deheparinization experiments.

### *Characterization of the Immobilized Heparinase*

Characterization of the immobilized heparinase showed that the enzyme had a $K_m$ of 0.15 ± 0.03 mg/mL and an activation energy of 10.3 ± 0.57 kcal/mol. These values were statistically indistinguishable from the values of the free enzyme.[9] However, the effects of salt on the activity of the immobilized enzyme contrasted with those observed for the free enzyme.[9] The activity optimum for the immobilized heparinase occurred in the range of 0–0.1 M NaCl, but it was insensitive to salt concentration within this range. For the free enzyme, the activity increased with increasing salt up to a maximum that occurred at 0.1 M NaCl and then it declined thereafter.

When arginine, lysine, and glycine were used to block unreacted cyanate ester groups after heparinase immobilization, the immobilized heparinase showed different pH optima of 6.5, 6.9, and 7.2, respectively. In comparison with the value of pH 6.5 for the free enzyme,[9] the pH optimum of the immobilized heparinase appeared to shift towards a more alkaline value by increasing the acidity of the blocking agents used. Hence, these results suggested that all the enzyme-bound supports possessed a net negative charge. Moreover, when the amino groups of the blocking agents could be linked covalently to the support, they provided the surface of the support material with a more negatively charged environment.

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