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FRACTIONATION OF HEPARIN BY AFFINITY CHROMATOGRAPHY ON COVALENTLY-BOUND HUMAN α-THROMBIN[1]

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SUMMARY

Commerical heparin, 135 USP units/mg, was fractionated by human α-thrombin-agarose affinity chromatography. Heparin was applied to an α-thrombin-agarose column equilibrated with 0.01 M Tris HCl (pH 7.4). Unbound heparin was washed from the column with the equilibration buffer. Bound heparin could be eluted with buffer containing 0.025 M NaCl. The specific activity of bound heparin was as great as 500 USP units/mg. Gel filtration was used to fractionate the heparin into molecular size classes. Low molecular weight heparin, with an average specific activity of 100 USP units/mg, was applied to the α-thrombin-agarose column. Gel filtration of the unbound heparin indicated that larger heparin molecules been selectively removed by the α-thrombin-agarose column. Bound heparin had a specific activity of 270 units/mg. Kinetic results of N-α-tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide hydrolysis by α-thrombin in the presence of heparin correlated with the anticoagulant activity.

Since the discovery of the anticoagulant activity of heparin by McLean (1), the mechanism by which heparin exerts its effect *in vivo* has been sought. Brinkhous *et al.* found that a plasma component was essential to the mechanism (2). Recently it has been shown that antithrombin III and thrombin form an apparent covalent complex associated with the inactivation of thrombin and that the rate of complex formation and concomitant thrombin inactivation is greatly accelerated by heparin (3). Subsequently it has been demonstrated that antithrombin III also inactivates Factor XII_a (4), IX_a (5) and X_a (6); these reactions are also accelerated by heparin. At the present time, it is not clear as to whether all of these reaction are critical in the *in vivo* action of heparin.

There have been several reports demonstrating that crude heparin can be separated into high and low activity fractions on the basis of its affinity for antithrombin III (7, 8, 9). Low activity heparin either does not bind to antithrombin or does so very poorly. The correlation between the anticoagulant

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activity and the binding of heparin to antithrombin has been taken as evidence that heparin binding to antithrombin is the premier event in the formation of the inactive heparin/thrombin/antithrombin complex. Kinetic data has recently appeared, however, which is not consistent with this sequence and suggests that the formation of a heparin/thrombin complex takes precedence (10). This would be particularly important for the identification of the structural properties of heparin which are essential for anticoagulation and for the in vitro evaluation of the anticoagulant activities of different heparin preparations.

In the present investigation we studied the interaction of heparin with immobilized human α-thrombin. It was postulated that the binding of heparin to thrombin-agarose would be dependent on the anticoagulant activity if the thrombin heparin interaction takes precedence in the antithrombin/heparin inactivation mechanism. Our results support this premise.

MATERIALS AND METHODS. Ultrogel AcA 54 was purchased from LKB. Bovine fibrinogen (65% clottable) was obtained from Miles Laboratories. Polyethylene glcol (M.W. 6,000-7,500) was purchased from J.T. Baker Chemical Co. Sepharose 4B was obtained from Pharmacia Fine Chemicals. N-α-p-tosyl-L-glycyl-L-prolyl-L-arginine-p-nitroanilide (TosGlyProArgNaN) was purchased from Boehringer-Mannhein. Sodium heparin was purchased from Grand Island Biological Co.

Human α-thrombin. Human α-thrombin was prepared as described previously[2] (11). The specific activity was 3,200-3,800 NIH units per mg. Protein was estimated using an $E_{280}^{0.1\%} = 1.75$ ml.mg^{-1}.cm^{-1} (12). Enzyme amidase activity was determined by measuring the rate of hydrolysis of TosGlyProArgNaN in a 0.1 M triethanolamine (TEA, pH 8.0), 0.1% polyethylene glycol (PEG 6000) solution at room temperature.

Heparin assays. The heparin concentration was determined by the carbazole method described by Dische (13). The optical density at 535 nm of an unknown solution was converted to mg/ml heparin from a standard curve prepared by assaying heparin solutions of known concentration. The anticoagulant activity of heparin solutions was determined by clotting time inhibition. A 10 μg/ml solution of heparin was prepared in 0.15 M Tris HCl (pH 8.0), 0.1% PEG 6000 containing human α-thrombin. After preincubating $\geq$ 30 s, 0.1 ml was removed and added to 0.2 ml of a fibrinogen (5 mg/ml, 0.15 M Tris HCl, pH 8.0); normal citrated plasma (1:1) solution at 37° and the clotting time determined. Each determination was done in triplicate. Longer heparin/thrombin preincubation had no effect, nor were the results altered if the heparin was added to the plasma solution first. The

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[2]Prothrombin complex concentrate used for the preparation of thrombin in these studies was provided by the American Red Cross National Fractionation Center with the partial support of the National Institutes of Health Grant No. HL 07255

units of anticoagulant activity were determined from a standard curve prepared by assaying various amounts of unfractionated heparin (135 USP units/mg) as above.

α-Thrombin-agarose. Human α-thrombin was coupled to CNBr-activated agarose by the following method (14). Washed agarose was suspended in an equal volume of deionized water and chilled at 4° in an ice water bath. Sodium carbonate (2 M) was added 1:1, followed by 1 ml of acetonitrile-CNBr solution (2 g CNBr). The suspension was stirred rapidly for two min then the activated agarose was washed on a sintered glass funnel with 10 volumes of cold 0.1 M $NaHCO_3$ (pH 8.5), water, and 0.1 M $NaHCO_3$ (pH 8.5). The activated beads were suspended in an equal volume of 0.1 M $NaHCO_3$ (pH 8.5). Human α-thrombin, 15 ml, (1.0 mg/ml) which was dialyzed against 1.0 l of 0.1 M $NaHCO_3$ (pH 8.5) was added to 30 ml of the agarose suspension. The mixture was kept suspended for 18 h at 4° by constant rotation. The α-thrombin-agarose was then washed with 5 vol of 0.1 M $NaHCO_3$, water and 0.01 M Tris·HCl (pH 7.4). The protein concentration in the wash was determined in order to estimate the amount of thrombin bound to the agarose.

Heparin fractionation by affinity chromatography on covalently-bound human α-thrombin. A 15 ml bed volume α-thrombin-agarose column (1.5 x 10 cm) equilibrated with .01 M Tris HCl (pH 7.4) was used. The protein content was estimated to be 0.5 mg/ml. In all cases, heparin solutions were dialyzed against the same buffer prior to application. After application of the heparin solution, the column was shut off for 2 h. The column was then washed with starting buffer and then eluted with buffer containing NaCl, as described in the figure legends. The column was regenerated by washing with 10 bed vol of 2 M NaCl followed by 0.5 l of 0.01 M Tris HCl (pH 7.4).

RESULTS. Fractionation of heparin by α-thrombin-agarose affinity chromatography. Heparin was applied to an α-thrombin-agarose column and fractionated as shown in Fig. 1A. The low ionic strength required to elute bound heparin was suprising in light of similar affinity experiments with antithrombin where 0.5 M NaCl was required to dissociate the heparin (8, 9). Bound heparin had a specific activity 1.5-fold higher than bound heparin indicating that binding was selective. On a molar basis, however, six moles of heparin (mol. wt. 12,000) were bound to the column per mole of α-thrombin, suggesting a significant degree of ion-exchange, rather than actual affinity chromatography. The heparin bound to thrombin-agarose was chromatographed a second time as shown in Fig. 1B. The gradient used to elute the heparin was much shallower with 100% recovery of the anti-coagulant activity in the eluate between 0.02-0.05 M NaCl. Only 54% of the uronic acid applied to the column was recovered in the peak. The remaining uronic acid, devoid of any anticoagulant activity, was not recovered. A similar

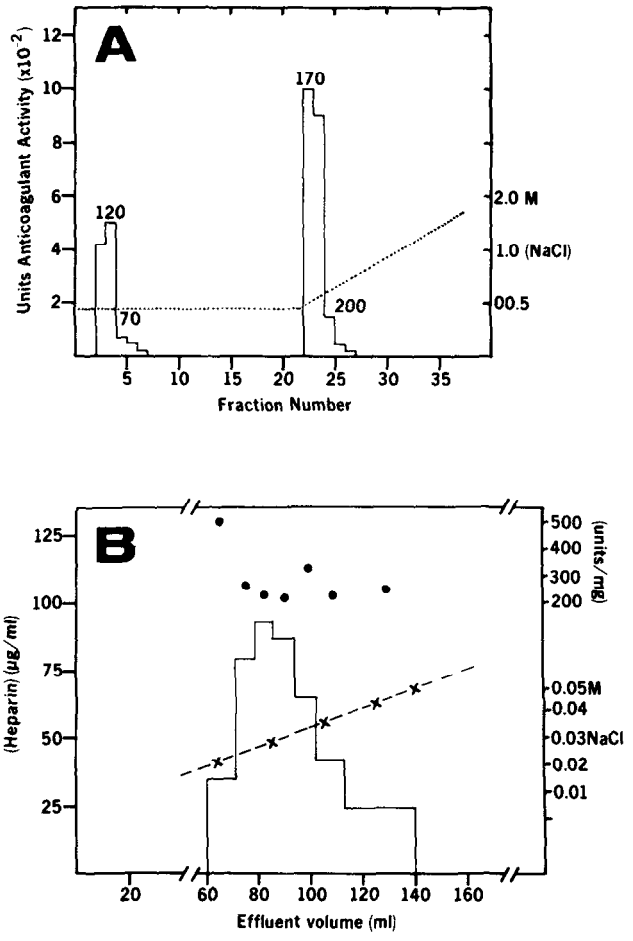
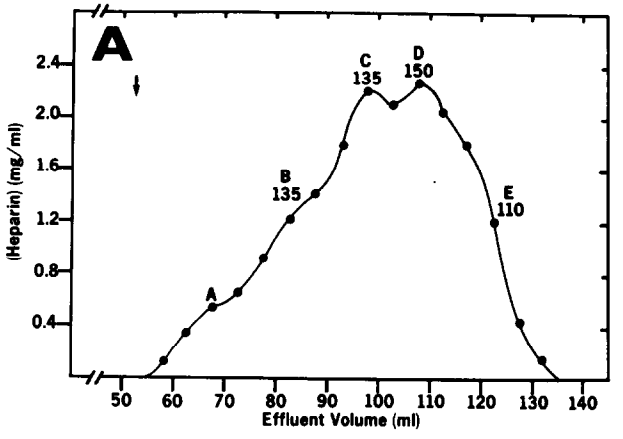
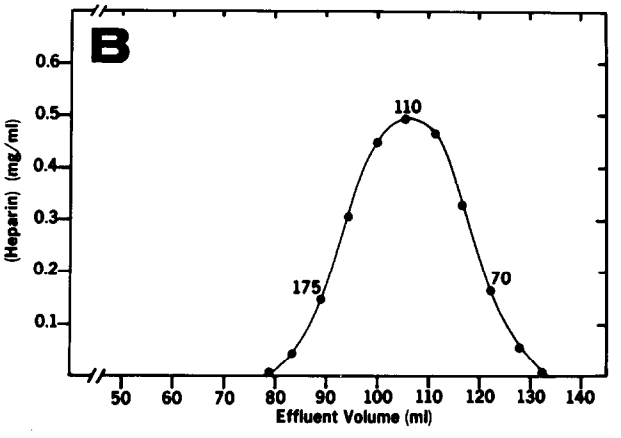


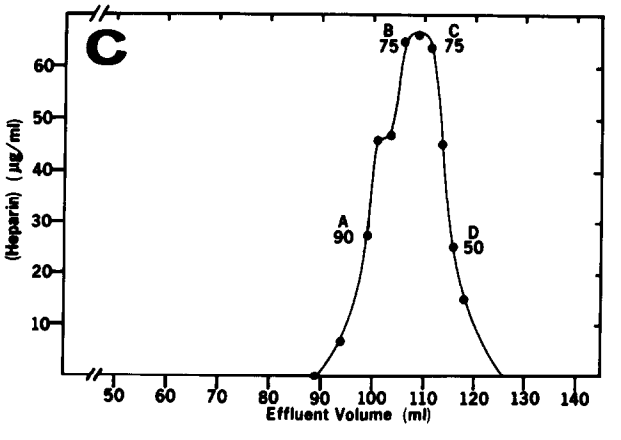
Figure 1. Human α-thrombin-agarose affinity chromatography. A) A 5.0 ml heparin solution (5.0 mg/ml) was applied to the column as described in Methods. The column was washed with 0.01 M Tris HCl (pH 7.4) then eluted with a salt gradient in the same buffer. The specific activities of several fractions are indicated (USP units/mg). Fraction volume ca 5.0 ml. B) Heparin from fractions 23 and 24 (panel A) were dialyzed against 0.01 M Tris HCl (pH 7.4) then applied to the affinity column. Washing and elution was carried out as described in A except that the salt gradient was shallower.

observation was made by Hook et al. (8) using an antithrombin-agarose column to fractionate heparin. The average specific activity of bound heparin was 2-fold higher than unfractionated heparin. The molar ratio of bound heparin to α-thrombin was approximately 1.5:1.0.

Fractionation of low molecular weight heparin by α-thrombin-agarose affinity chromatography. Heparin was fractionated by gel filtration prior to affinity chromatography as shown in Fig. 2A. Low molecular weight heparin was divided


A
(Heparin) (mg/ml)
2.4
2.0
1.6
1.2
0.8
0.4
A
B
135
C
135
D
150
E
110
50 60 70 80 90 100 110 120 130 140
Effluent Volume (ml)


B
(Heparin) (mg/ml)
0.6
0.5
0.4
0.3
0.2
0.1
110
175
70
50 60 70 80 90 100 110 120 130 140
Effluent Volume (ml)


C
(Heparin) (µg/ml)
60
50
40
30
20
10
B
75
C
75
A
90
D
50
50 60 70 80 90 100 110 120 130 140
Effluent Volume (ml)

into two fractions. One fraction was gel filtered a second time as shown in Fig. 2B. The other fraction was applied to the α-thrombin-agarose column which was then washed as described previously. Unbound heparin which appeared in the wash was gel filtered as shown in Fig. 2C. The results show that region E heparin is part of the trailing edge of region D heparin. Gel filtration of region D heparin resulted in the appearance of a minor peak at a volume of 98 ml, on the shoulder of the major peak at 108 ml. This profile was reversed when region C heparin was gel filtered. These results attest to the ability of gel filtration to reproducibly separate heparin into size classes. After adsorption of region E heparin with thrombin-agarose, the gel filtration elution profile shown in Fig. 2C reveals that the larger heparin molecules have been selectively removed.

Kinetic analysis of fractionated heparins. Several heparin samples were tested to evaluate their effect on the hydrolysis of TosGlyProArgNaN by α-thrombin. Both high and low activity heparins were effective in increasing the affinity of thrombin for substrate as shown in Table I. High activity heparin was slightly more effective (ca 30%) than lower activity samples. This difference, although consistently observed, seemed significantly smaller than would be predicted from the difference in specific activities. High activity heparin (ca 300 units/mg) had a 2-fold greater affinity for α-thrombin than low activity heparin (ca 75 units/mg) based on the enhancement of synthetic substrate binding.

DISCUSSION. The binding properties of heparin to human α-thrombin have been used to fractionate crude heparin. Heparin which bound to α-thrombin-agarose was consistently higher in specific activity than heparin which did not bind. A 2.5-fold increase in specific activity of the heparin used in the present investigation was obtained by this method. A comparable increase in the specific activity of heparin has also been achieved by antithrombin-affinity techniques (7, 8).

The low NaCl concentration ($\leq$ 0.05 M) required to elute heparin from α-thrombin-agarose in the present study can be contrasted with the high NaCl concentration ($\geq$ 0.05 M) used to dissociate heparin-antithrombin complexes

Figure 2. Ultrogel AcA 54 gel filtration of heparin. A) A 1.0 ml (100 mg/ml) solution of crude heparin was applied to a 1.5 x 90 cm Ultrogel AcA 54 column equilibrated with 0.1 M Tris HCl (pH 7.4). The arrow indicates the position corresponding to the void volume. Specific activities of several regions are indicated (USP units/mg). Recovery of uronic acid was 98%. B) Heparin obtained in the effluent volume between 115-135 ml (panel A) was pooled and gel filtered as in A. C) Heparin obtained in the effluent volume between 93-117 ml (panel B) was adsorbed with α-thrombin-agarose then gel filtered as in A.

TABLE I

Heparin effect on the hydrolysis of TosGlyProArgNaN by human α-thrombin. Human α-thrombin was added to assay solution containing 0.05 μg of heparin, 0.1 M TEA (pH 8.0), 0.1% PEG 6000, and 5 x 10^{-5}M TosGlyProArgNaN. The final enzyme concentration was 7.2 x 10^{-10}M in a total volume of 1.05 ml. The progress curve of substrate hydrolysis was followed spectrophotometrically at 410 nm and the Km and Vmax values determined using the integrated rate equation.

Sample[a]	Specific[b] Activity (USP units/mg)	Km (X 10^6 M)	Vmax[c] (nmoles/min)
A	90	5.3, 5.1	61, 60
B	75	5.1, 5.0	54, 58
C	75	6.5, 6.5	61, 59
D	50	5.9, 5.5	60, 58
A'	350	4.5, 4.1	56, 56
crude heparin	135	5.1, 4.9	54, 52

a) Samples A, B, C and D correspond to the gel filtration fractions shown in Fig. 2B. Sample A' corresponds to heparin which was bound by α-thrombin-agarose.

b) Anticoagulant activity.

c) Corrected to 1 nmole of human α-thrombin.

(8, 9). This is a preplexing observation since the affinity of heparin for α-thrombin is considerably greater than its affinity for antithrombin (15). It is possible that the coupling of α-thrombin to CNBr-activated agarose alters the conformation of the protein such that its affinity for heparin is reduced. In a previous communication from our laboratory, thrombin-agarose was shown to have a lower fibrinogen clotting activity than unbound thrombin (16). This suggests that steric factors hinder the interaction of thrombin-agarose with macromolecular ligands.

The precise mechanism by which heparin enhances the rate of formation of the inactive thrombin/antithrombin complex is not clearly understood. It is apparent that high activity heparin can bind selectively to either antithrombin or thrombin, which suggests that specific binding sites for heparin exist on each macromolecule. Alternatively, thrombin or antithrombin may possess non-

specific binding regions which coincidentally select for high activity heparin, but do not play a role in the enzyme inactivation process. In the present report, larger heparin is selectively bound to α-thrombin-agarose. It is conceivable that heparin binding to thrombin is simply a size dependent phenomenon and larger heparin happens to possess the anticoagulant properties. The enzyme kinetic data, however, indicate that high-activity heparin obtained by affinity for α-thrombin-agarose has a greater effect on α-thrombin amidase activity than does low activity material. This correlation supports the postulate that the heparin/thrombin interaction takes precedence in the formation of the inactive ternary complex (10). Whether the interaction of heparin with antithrombin is important is not known. The results of antithrombin modification with methylisourea suggest that heparin does interact with antithrombin during the inactivation of thrombin (3). It has been shown, however, that high activity heparin isolated by its affinity for antithrombin does not differ significantly in chemical composition from low activity heparin (7, 8). This suggests the difference between high and low activity heparin resides in the carbohydrate sequence or in the size of the heparin molecule.

The present investigation has shown that high activity heparin can be obtained by fractionation of crude heparin on covalently-bound human α-thrombin. This is important evidence in support of kinetic data indicating that the formation of the heparin/thrombin complex takes precedence in the rapid inactivation of thrombin by antithrombin in the presence of heparin (10).

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