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DERMATAN SULFATE AND HEPARIN CAN BE FRACTIONATED BY AFFINITY FOR HEPARIN COFACTOR II[1]

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Commercial preparations of dermatan sulfate and heparin were applied to a concanavalin A-agarose to which heparin cofactor II had been noncovalently bound. Small amounts of both mucopolysaccharides bound to the column with relatively high affinity. Heparin and dermatan sulfate which were eluted from the affinity column catalyzed the inhibition of thrombin by heparin cofactor II to a greater degree than did the respective unfractionated mucopolysaccharides. Dermatan sulfate did not catalyze thrombin inhibition by antithrombin III. The results suggest that heparin cofactor II differs from antithrombin III with respect to the mucopolysaccharide binding site.

There are, at least, two proteins in human plasma that inhibit thrombin at an accelerated rate in the presence of heparin: antithrombin III (1-3) and heparin cofactor II (4-9). Heparin cofactor II is not precipitated by antibody directed against antithrombin III and appears to be larger in molecular weight than antithrombin III (7). In the presence and absence of heparin, the heparin cofactor II/thrombin reaction rate is slower than the antithrombin III/thrombin reaction rate (7,8). Heparin cofactor II, when compared to antithrombin III, does not appear to inhibit factor Xa (4,5,7-9), plasmin and trypsin (9) at appreciable rates. Antithrombin III and heparin cofactor II, therefore, appear to differ significantly in both structural and functional properties.

We have been interested in the mechanism of action of heparin in catalyzing the antithrombin III/thrombin reaction. We have shown that the heparin-catalyzed antithrombin III/thrombin reaction is saturable with respect to both thrombin and antithrombin III (10). In recent work we have found that the heparin cofactor II/thrombin reaction, catalyzed by either heparin or dermatan sulfate, is saturable with respect to both thrombin and heparin cofactor II

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(Marbet and Griffith, unpublished). Thus, two different inhibitor/thrombin reactions are accelerated by heparin by, what appears to be, a similar mechanism of action of heparin.

The present study was undertaken to 1) determine the relative activities of gel-filtered heparin and dermatan sulfate fractions in catalyzing the heparin cofactor II/thrombin reaction and 2) determine if heparin and dermatan sulfate can be fractionated by affinity for heparin cofactor II. The results indicate that the structural properties of heparin required for catalyzing the heparin cofactor II/thrombin reaction are similar, but not identical, to the structural properties of heparin required for catalyzing the antithrombin III/thrombin reaction.

EXPERIMENTAL PROCEDURES

N^{α}-p-Tosyl-L-glycyl-L-prolyl-L-argine-p-nitroanilide (TosGlyProArgNaN) was purchased from Boehringer Mannheim. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene) was purchased from Aldrich. Porcine mucosal heparin (165 USP units/mg), which was essentially devoid of protein, and heparin molecular weight standards were generously provided by Dr. G. van Dedem and E. Coyne, Diosynth B.V. (Oss, The Netherlands). Dermatan sulfate (hog skin chondroitin sulfate type B, Lot 1601) was purchasd from Miles Laboratories, Inc. Concanavalin A-agarose was purchased from Sigma. Human alpha-thrombin (3,600 NIH units/mg) and human antithrombin III were prepared as described previously (11). Human heparin cofactor II was purified to homogeneity, as judged by SDS-polyacrylamide gel electrophoresis (12), essentially as described by Tollefsen and coworkers (7).

Mucopolysaccharide fractionation by heparin cofactor II affinity chromatography. Approximately 6 mg of heparin cofactor II was noncovalently bound to a 1.1 cm x 4.0 cm column of concanavalin A-agarose equilibrated with 0.02 M Tris HCl (pH 7.0), 0.05 M NaCl. Ten mg of mucopolysaccharide in 10 ml of 0.02 M Tris HCl (pH 7.0), 0.05 M NaCl was applied to the column which was then washed with $\geq$ 50 ml of the same solution. Elution of mucopolysaccharide from the column was accomplished by increasing the NaCl concentration of the buffer in a stepwise manner. Mucopolysaccharide obtained from the column in this manner did not inhibit thrombin in the absence of added heparin cofactor II indicating that dissociation of heparin cofactor II from the column was negligible.

Mucopolysaccharide activity determination. Heparin and dermatan sulfate activities were evaluated by determining the rate of thrombin (10 nM) inhibition by antithrombin III (300 nM) or by heparin cofactor II (200 nM) in the presence of heparin (10 ng/ml) or dermatan sulfate (50 ng/ml). Heparin and dermatan sulfate concentration, by weight, was determined by uronic acid assay as described previously (13). Inhibitor/thrombin reactions were initiated by adding thrombin to a solution containing 0.1 M triethanolamine (pH 8.0), 0.1 M NaCl, 0.1% polyethylene glycol (PEG M_r = 6,000-7,500), inhibitor and mucopolysaccharide. At time intervals after the addition of thrombin, samples were removed and added to a solution containing 0.1 M triethanolamine, 0.1 M NaCl, 0.1% PEG, 0.5 mg/ml polybrene and 0.15 μM TosGlyProArgNaN. The hydrolysis of TosGlyProArgNaN was terminated by the addition of acetic acid. The amount of TosGlyProArgNaN hydrolyzed, determined by absorbance at 400 nm, was proportional to the thrombin concentration.

Thrombin inhibition followed apparent pseudo first order kinetics over the time course studied. The initial reaction velocity (v_i) was calculated by multiplying the observed pseudo first order rate constant by the initial

thrombin concentration. In the absence of mucopolysaccharide, the initial reaction velocities were 1.3 nM thrombin/min for the antithrombin III/thrombin, (AT/T), reaction and 0.075 nM thrombin/min for the heparin cofactor II/thrombin, (HC/T), reaction. The initial reaction velocity for the (unfractionated) heparin-catalyzed antithrombin III/thrombin reaction was 8.3 nM thrombin/min. The initial reaction velocities for the heparin cofactor II/thrombin reactions catalyzed by (unfractionated) heparin and (unfractionated) dermatan sulfate were 1.0 nM thrombin/min and 0.38 nM thrombin/min, respectively. The reaction velocity due to catalysis was obtained by subtracting the reaction velocity determined in the absence of mucopolysaccharide from the observed reaction velocity in the presence of mucopolysaccharide. The relative specific activity of a given mucopolysaccharide fraction was calculated by dividing the inhibitor/thrombin reaction velocity obtained in the presence of fractionated mucopolysaccharide by the reaction velocity obtained in the presence of an equal weight of unfractionated mucopolysaccharide. The term AT/T-activity refers to the mucopolysaccharide activity with respect to catalysis of the antithrombin III/thrombin reaction. The term HC/T-activity in turn, refers to the mucopolysaccharide activity with respect to catalysis of the heparin cofactor II/thrombin reaction.[2]

RESULTS

Sephadex G-200 gel filtration of heparin and dermatan sulfate. Fractions obtained by gel filtration of heparin and dermatan sulfate were assayed for HC/T-activity. With both mucopolysaccharides, the HC/T-activity peak was different from the uronic acid peak. These results are shown in Figure 1. The relative specific HC/T-activity of heparin decreased gradually from fraction number 40 to fraction number 60, then decreased more rapidly thereafter. The relative specific AT/T-activity of heparin followed a similar pattern. These results are summarized in Table 1. The relative specific HC/T-activity of dermatan sulfate increased from fraction number 35 to fraction number 50 and was essentially constant thereafter.

Mucopolysaccharide fractionation by heparin cofactor II affinity chromatography. Heparin and dermatan sulfate were fractionated by heparin cofactor II affinity chromatography. As shown in Figure 2, the majority of both of the mucopolysaccharides was recovered in the first four fractions. Heparin and dermatan sulfate in these fractions had measurably lower relative specific activities. Small amounts of heparin eluted from the column with 0.1 M NaCl. This material had essentially the same activity as the respective unfractionated material (i.e. relative specific activity $\approx$1.0) and appeared to correspond to nonspecifically adsorbed material obtained by passing heparin or dermatan sulfate through concanavalin A-agarose (not shown). Heparin eluting from the column with 0.25 M NaCl had a slightly higher relative specific activity, but the highest relative specific activity material was obtained with 0.5 M NaCl. The relative specific HC/T-activity was greater than the relative specific AT/T-activity for heparin

[2]USP units have not been used to avoid any implication that the activities determined as described above bear any relationship to anticoagulant activities.

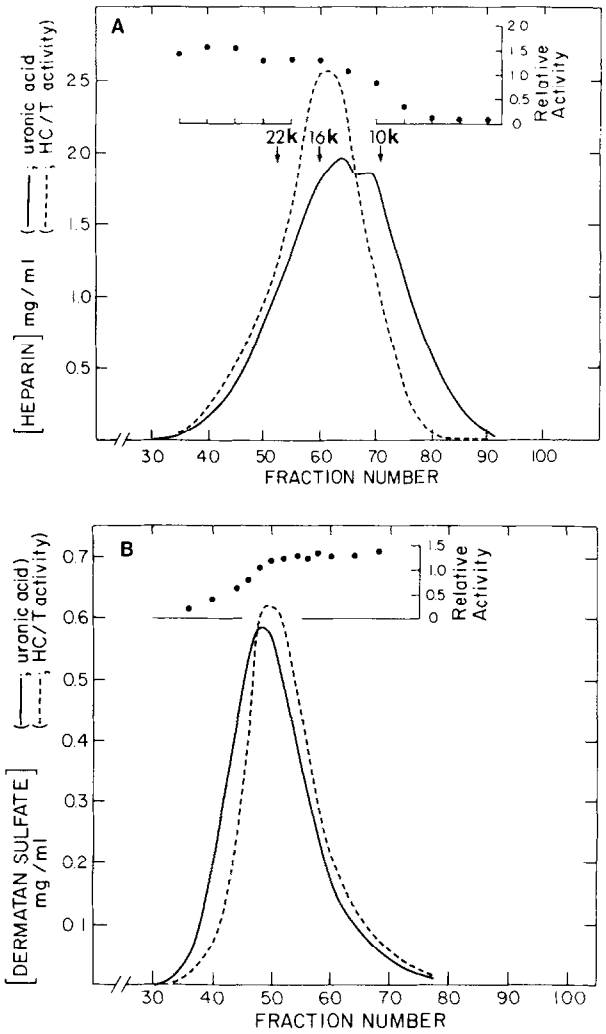

Figure 1. Sephadex G-200 gel filtration of heparin and dermatan sulfate. Heparin (Panel A, 265 mg) and dermatan sulfate (Panel B, 50 mg) were applied to a 2.5 cm x 93 cm Sephadex G-200 column equilibrated with 0.05 M Tris HCl (pH 7.4), 0.15 M NaCl. Fractions (5.2 ml) were collected at a flow rate of 25 ml/n. Arrows indicate the elution position of heparin molecular weight standards. Samples from the indicated fractions were assayed for uronic acid (——) and HC/T-activity (---) as described in Experimental Procedures. Insets. HC/T-activity of fraction relative to HC/T-activity of unfractionated mucopolysaccharide.

eluting with 0.5 M NaCl. Heparin recovered in the first four fractions was recycled through the column two additional times and the distributions of HC/T- and AT/T-activities determined for each cycle. The results are summarized in Table 2.

The relative specific HC/T-activity of dermatan sulfate increased as the NaCl concentration used to elute material from the column was increased. The highest activity dermatan sulfate fractions were devoid of AT/T-activity.

TABLE 1. Relative Specific Activities of Gel-filtered Heparin

Fraction number	Relative Specific Activities HC/T	AT/T
40	1.40	1.75
45	1.50	1.75
50	1.25	1.55
55	1.25	1.40
60	1.25	1.20
65	1.05	1.00
70	0.80	0.80
75	0.30	0.30
80	0.10	0.05
85	0.05	0.05

[a]Gel-filtered heparin fractions were assayed for HC/T- and AT/T activities as described in Experimental Procedures. Fraction numbers correspond to the data shown in Fig. 1A.

DISCUSSION

In 1976, two studies were reported indicating that the anticoagulant activity of heparin resides in a fraction of heparin which binds to antithrombin III with relatively high affinity (14,15). Subsequent work has shown that the antithrombin III binding site within the heparin molecule is composed of a unique oligosaccharide sequence (16-18). The anticoagulant activity of heparin also appears to be related to its apparent molecular weight (19) and to its charge density (20,21). In general, the anticoagulant activity of heparin parallels the activity of heparin with respect to catalyzing the antithrombin III/thrombin reaction.

In the present study we have found that the relative specific HC/T-activities of gel-filtered heparin fractions decreases with increasing effluent volume from the column. The HC/T-activity peak was, however, more or less symmetrical with a midpoint corresponding to an apparent molecular weight of 15,000. Similar results were obtained when the same gel-filtered heparin fractions were measured for AT/T-activity. To a certain extent, then, the apparent molecular weight dependence for heparin activity appears to be related to the amount of low molecular weight (inactive) heparin 'contaminating' high molecular weight heparin fractions. This is consistent with the study reported by Laurent and coworkers (19) where it was shown that the percentage of HA-heparin (i.e. heparin with high affinity for antithrombin III) in gel-filtered heparin fractions decreases with effluent volume from the column. Interestingly, it was also found (19) that the specific activity (units/mg) of HA-heparin was essentially constant over the range of fractions corresponding to apparent molecular weights between 7,800 and 25,000. These observations suggest that the majority of heparin with catalytic activity (HC/T and AT/T reactions) is from a relatively uniform molecular weight class of heparin.

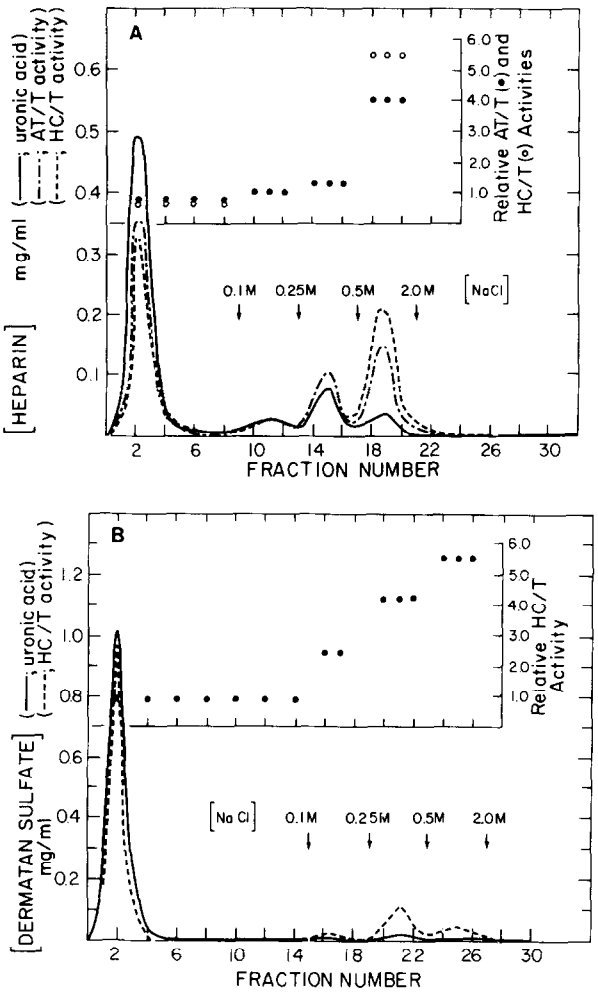

Figure 2. Mucopolysaccharide fractionation by heparin cofactor II affinity chromatography. Heparin (Panel A) and dermatan sulfate (Panel B) were fractionated by heparin cofactor II affinity chromatography as described in Experimental Procedures. Fractions (6.45 ml) were collected at a flow rate of 1.25 ml/min. Arrows indicate the points at which the NaCl concentration was increased. Samples were assayed for uronic acid (——), HC/T-activity (---) and AT/T-activity ('"'), as described in Experimental Procedures. Insets. Activity of fraction relative to activity of unfractionated mucopolysaccharide.

We have also found that heparin can be fractionated by affinity for heparin cofactor II. The relative specific HC/T-activity of bound heparin decreased as the heparin was recycled through the column. While this result may reflect heterogeneity in the heparin preparation with respect to binding affinity for heparin cofactor II, an increased proportion of nonspecific heparin binding to the column cannot be excluded as an explanation. The relative specific AT/T-activity of bound heparin also decreased as heparin was recycled through the column. One observation, of possible significance, was that the ratio of AT/T-activity to HC/T-activity of heparin eluting with 0.5 M NaCl increased from 0.71

TABLE 2. Recoveries and Relative Specific Activities of Heparin Fractionated by Heparin Cofactor II Affinity Chromatography[a]

Pools[c]	Recoveries[b]			Relative Specific Activities	
	Percentage by weight	Percentage by HC/T-Activity	Percentage by AT/T-Activity	HC/T	AT/T
Run 1					
Flow through	74.5	45.9	53.4	0.66	0.72
0.10 M NaCl	6.0	5.6	5.8	1.0	1.0
0.25 M NaCl	13.6	17.1	17.4	1.3	1.3
0.50 M NaCl	5.9	31.4	23.4	5.6	4.0
Run 2					
Flow through	73.2	28.3	50.5	0.25	0.48
0.10 M NaCl	8.8	18.3	14.3	1.34	1.20
0.25 M NaCl	10.6	26.3	17.3	1.60	1.20
0.50 M NaCl	7.4	27.0	17.8	2.30	1.80
Run 3					
Flow through	59.3	35.5	35.5	0.14	0.24
0.10 M NaCl	13.7	16.1	13.7	0.27	0.41
0.25 M NaCl	20.2	29.4	33.0	0.34	0.67
0.50 M NaCl	6.7	19.0	18.1	0.66	1.10

[a]Heparin was fractionated by affinity for heparin cofactor II as shown in Fig. 2A. The total recovery of heparin by weight (uronic acid) and by activity (HC/T and AT/T) was essentially 100% (± 4%) for each run. For Run 1, 8.4 mg of heparin was applied to the column. The relative HC/T- and AT/T-activities were, by definition, 1.0. For Run 2, 'flow-through' heparin (5.5 mg) from Run 1 was applied to the column. For Run 3, 'flow through' heparin (3.7 mg) from Run 2 was applied to the column. For Runs 2 and 3, the relative specific HC/T- and AT/T-activities of the heparin applied to the column correspond to the activities indicated for the 'flow through' heparin obtained in Runs 1 and 2 respectively.

[b]Recoveries are expressed in terms of the percentage of heparin (by weight and activity) applied to the column for the given run.

[c]Heparin was pooled according to the NaCl concentration required to elute the heparin from the column. For example, for Run 1 (shown in Fig. 2A), the 'flow through' heparin pool corresponded to fractions 1-9. The 0.10 M NaCl, 0.25 M NaCl and 0.50 M NaCl heparin pools corresponded to fractions 10-13, 14-17 and 18-21, respectively.

to 1.67 as the heparin was recycled through the column. This suggests that heparin cofactor II and antithrombin III bind to different sites on the heparin molecule. It is likely that there is an overlap in the population of heparin molecules which can bind the two proteins.

Commercial dermatan sulfate catalyzes the heparin cofactor II/thrombin reaction and, to some extent, the antithrombin III/thrombin reaction (22). In the present study we found that dermatan sulfate could be fractionated by affinity for heparin cofactor II. The highest activity dermatan sulfate we obtained did not accelerate the rate of thrombin inhibition by antithrombin III. We have also found that dermatan sulfate which has been run through an antithrombin III-agarose column retains essentially 100% of its HC/T-activity. We conclude from these results that the HC/T-activity in the dermatan sulfate preparation we have studied is not due to contamination by heparin.

Heparin and dermatan sulfate are two structurally different mucopolysaccharides. Both mucopolysaccharides catalyze the heparin cofactor II/thrombin reaction. The ability of a mucopolysccharide to catalyze an inhibitor/protease reaction is, therefore, not unique to heparin. It is apparent that the inhibitor must bind to the mucopolysaccharide for protease inhibition to be accelerated. Further investigation is required to determine the specific oligosaccharide sequence which is necessary to bind heparin cofactor II.

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