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Affinity chromatography of sulphated polysaccharides separately fractionated on antithrombin III and heparin cofactor II immobilized on concanavalin A–Sepharose☆

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ABSTRACT

Three sulphated polysaccharides, dermatan sulphate, fucan and heparin, were fractionated according to their affinity towards antithrombin III (ATIII) and heparin cofactor II (HCII), the two main physiological thrombin (IIa) inhibitors. Both inhibitors were immobilized on concanavalin A–Sepharose, which binds to the glycosylated chains of the proteins while the protein-binding site for the polysaccharide remains free. Each polysaccharide was fractionated into bound and unbound fractions either for ATIII or HCII. The eluted fractions were tested for their ability to catalyse ATIII/IIa and HCII/IIa interactions. The possible presence of a unique binding site for ATIII and HCII, on each sulphated polysaccharide, was also studied.

INTRODUCTION

Heparin is the most widely used anticoagulant drug for the prevention and treatment of thrombosis. This polysaccharide acts as a catalyst in thrombin (IIa) inhibition by antithrombin III (ATIII). A highly conserved well defined pentasaccharide sequence in the heparin molecule binds to ATIII [1,2]. Heparin can also catalyse the effect of another plasmatic inhibitor of thrombin, heparin cofactor II (HCII) [3]. No defined structure of heparin responsible for the interaction with HCII is known. Other sulphated polysaccharides are also able to catalyse thrombin inhibition by HCII: these include dermatan sulphate, which is specific for HCII [4], heparan sulphate [4], fucan derived from marine algae [5] and dextran sulphate [6]. Several studies on the interaction of these anticoagulant polysaccharides with HCII and also with ATIII are in progress. Structural domains of the inhibitors that

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are involved in the binding mechanism to sulphated polysaccharides are now quite well described [7–11]. In contrast, except in the case of the heparin pentasaccharide mentioned above, very little is known concerning the binding region on the polysaccharide structure. It was thus interesting to fractionate different sulphated polysaccharides known to catalyse the reaction of thrombin inhibition and to isolate molecules with a higher affinity for ATIII and HCII.

We first studied the influence of the ionic strength on the adsorption of the sulphated polysaccharides on ATIII and HCII immobilized on concanavalin A–Sepharose columns; secondly, we determined the catalytic activities of the eluted sub-fractions towards the reaction of thrombin inhibition. Then, the high-affinity fractions for ATIII were passed through the HCII column, and the high-affinity fractions for HCII through the ATIII column, in crossed experiments, to investigate the ATIII and HCII binding site on the polysaccharide molecules.

EXPERIMENTAL

Materials

Three different native dermatan sulphates (DS) were studied: DS MF701 (extracted from bovine intestinal mucosa) from Mediolanum Farmaceuti (Milan, Italy), and two samples extracted from porcine intestinal mucosa: DS p1106 from Institut Choay (Gentilly, France) and DS NF112 from Pharmuka (Gennevilliers, France). Three resulphated dermatan sulphates, DS1, DS2 and DS3, with a sulphur content of 7.8, 10 and 11.5%, respectively (compared with 6% for native DS), were kindly supplied by Dr. Mardiguian from Pharmuka. Heparin was purchased from LEO (Montigny-le-Bretonneux, France) and fucan was prepared as previously described [5]. Human purified HCII (10 U/mg) was purchased from Diagnostica Stago (Asnières, France) human purified ATIII (5 IU/mg) from Kabi Vitrum (Stockholm, Sweden), concanavalin A (con A)–Sepharose from Pharmacia (Bois d'Arcy, France), human purified thrombin and CBS 34–47, a specific chromogenic substrate for thrombin, from Diagnostica Stago. All polysaccharide activities were measured on a Beckman DU 7000 spectrophotometer. For the colorimetric assay of sulphated polysaccharides, 1,9-dimethyl-methylene blue (DMB) was provided by Serva Feinbiochemica (Heidelberg, Germany).

Binding of HCII and ATIII to conconavalin A–Sepharose columns

Two chromatographic columns (13 cm × 1.5 cm I.D.) were prepared: 4.5 ml of con A–Sepharose 4B were equilibrated with 0.05 *M* NaCl in 0.02 *M* Tris–HCl (pH 7.4) (buffer A) for the HCII column, and 9.5 ml of con A–Sepharose were equilibrated with 0.15 *M* NaCl in 0.1 *M* Tris–HCl (pH 7.4) (buffer B) for the ATIII column, at 4°C. Then, ATIII (36 mg) and HCII (6 mg), previously dialysed against their respective buffers, were applied to the respective columns at a flow-rate of 2.0 ml/h. The columns were then washed with two column volumes of appropriate buffer to remove any unbound protein. Eluates were tested by HCII and ATIII activity assays.

Fractionation of polysaccharides on ATIII and HCII concanavalin A–Sepharose

A 1-mg amount of each sulphated polysaccharide was applied to the ATIII and HCII concanavalin A columns equilibrated with either buffer A or buffer B. The flow-rate was 10 ml/h. Eluted polysaccharides were detected by a UV detector at 206 nm. The elution was performed either by step or by a saline gradient ranging from 0.05 to 1 *M* NaCl in 0.02 *M* Tris–HCl, or 0.15 to 1 *M* NaCl in 0.1 *M* Tris–HCl buffer (pH 7.4). Fractions of bound and unbound polysaccharides were pooled separately. The polysaccharide concentrations were measured by a colorimetric assay, and the catalytic activities by a chromogenic assay. Each chromatographic experiment was performed three times. Unfractionated polysaccharides were designated UF-polysaccharide, bound and unbound fractions were designated B and UB. The affinity gels were recycled with a washing buffer at 2 *M* NaCl in 0.02 or 0.15 *M* Tris–HCl, then the gels were equilibrated with buffers A or B until the baseline (A_{206}) was reached.

Colorimetric assay of the sulphated polysaccharides

The concentrations of the polysaccharides in the eluent were determined using a slightly modified version of the method of Farndale *et al.* [12]. To a 100-μl aliquot of sample containing the polysaccharide was added 1 ml of DMB colour reagent. After gentle mixing, the absorbance at 525 nm was read immediately. The A_{525} value was compared with a standard curve established by using the same volumes of solutions containing 2–18 μg/ml of the same polysaccharide.

Antithrombic activities of the sulphated polysaccharides

All activities were determined by measuring the residual thrombin with a chromogenic substrate, CBS 34-47, after catalysis of the thrombin inhibition reaction by the polysaccharide. Each fraction was tested for its ability to catalyse thrombin inhibition either by purified ATIII and/or HCII (designated ATIII/IIa or HCII/IIa activity).

Antithrombic activity of the polysaccharides mediated by ATIII. To 20 μl of sample were added 75 μl of ATIII (0.1 IU/ml); after 30 s (in the case of heparin) or 90 s (in the case of fucan) incubation time, at 37°C, 75 μl of human thrombin (6 U/ml) were added. After 15 s (for heparin) or 30 s (for fucan) inucation time, 75 μl of chromogenic substrate were added and Δ absorbance/min was measured at 405 nm. From the absorbance, activities were calculated using a standard curve covering the ranges 0.2–1 μg/ml for heparin or 1–8 μg/ml for fucan.

Antithrombic activity of the polysaccharides mediated by HCII. To 20 μl of sample were added 75 μl of HCII (0.15 U/ml); after 15 s (in the case of heparin), 2 min (in the case of DS) or 1 min (in the case of fucan) incubation time, at 37°C, 75 μl of human thrombin (6 U/ml) were added. After 15 s (for heparin) or 1 min (for fucan and DS) incubation time, 75 μl of chromogenic substate were added and Δ absorbance/min was measured at 405 nm. From the absorbance, activities were calculated using a standard curve covering the range 1–6 μg/ml for heparin, 0.5–3 μg/ml for DS and 0.5–4 μg/ml for fucan.

Definition of the units used for antithrombic activities. Except for the activity of heparin mediated by ATIII, where international units were used (IU/mg), the other polysaccharidic activities were defined in unit/ml (U/ml) where 1 μg/ml corresponds arbitrarily to 1 unit/ml. As native DS has no activity towards ATIII, the fractionated DS activities towards ATIII/IIa reaction were measured by an ATIII/IIa assay in which the standard curve was established with heparin as catalyst.

RESULTS AND DISCUSSION

Control experiments

The absence of binding of the sulphated polysaccharides to the con A–Sepharose was first checked. The polysaccharides were applied to a con A–Sepharose column, which was washed with either buffer A or buffer B. Elution of the sulphated polysaccharides adsorbed on the con A–Sepharose was performed with a Tris buffer containing 2 *M* NaCl. With heparin, 51.5% of the molecules were adsorbed on con A–Sepharose when using buffer A (and only 1.6% with buffer B); the two other polysaccharides did not interact with the lectin, whatever the buffer used. DS is essentially not adsorbed on the lectin: 95% and 99% were recovered in the washing buffers A and B, respectively. Of the fucan molecules, 10% were adsorbed with the buffer A, and only 2.5% with the buffer B. These results suggest that sulphated polysaccharides have very weak ionic interactions with con A. It can be concluded that the polysaccharides tested in our work have a negligible binding to the con A–Sepharose matrix, under the conditions used.

Immobilization of HCII and ATIII on concanavalin A–Sepharose

Concanavalin A, a vegetable lectin, has two binding sites with high affinity for α-glycosyl residues, specifically for glucosyl and mannosyl. Covalently immobilized on Sepharose, this protein has already been used as a chromatographic ligand to bind ATIII and HCII by their carbohydrate chains [13–15]. After application of AT-

TABLE I

ANALYSIS OF HEPARIN FRACTIONATED BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ATIII- AND HCII-CON A (BUFFER B)

Active fraction[a]	Recovery (%)	ATIII/IIa		HCII/IIa	
		Activity[b] (IU/mg)	Relative activity[c]	Activity[d] (U/mg)	Relative activity
UF-Hep	100	150	1	975	1
UB-ATIII	75	85	0.56	896	0.92
B-ATIII	25	265	1.8	1218	1.25
UB-HCII	90	96	0.64	685	0.7
B-HCII	10	211	1.4	1415	1.45

[a] UF-Hep = unfractionated heparin; UB-ATIII = heparin fraction unbound to ATIII; B-ATIII = heparin fraction bound to ATIII; UB-HCII = heparin fraction unbound to HCII; B-HCII = heparin fraction bound to HCII.

[b] Heparin/ATIII/IIa assay.

[c] Relative activity = activity of fractionated polysaccharide/activity of unfractionated polysaccharide.

[d] Heparin/HCII/IIa assay.

III and HCII to the two columns, neither ATIII nor HCII activities were detected in the washing buffer. The amount of bound inhibitor was 3.8 mg for ATIII and 1.3 mg for HCII per ml of con A–Sepharose gel.

Affinity chromatography of sulphated polysaccharides on immobilized HCII and ATIII

The overall recoveries of each sulphated polysaccharide, after affinity chromatography, were 91% for heparin, 88% for native DS, 90% for oversulphated DS and 86.5% for fucan. For a better understanding, the overall recoveries were arbitrarily referred to as 100%.

Affinity chromatography of heparin on ATIII- and HCII-con A

On ATIII-con A. In the presence of buffer B, *ca.* 25% of the heparin bound to ATIII and was eluted by 1 *M* NaCl (Table I). These results are quite similar to those reported previously [16]. The activities of unfractionated, bound and unbound heparin fractions, measured by catalysis of ATIII/IIa, were 150, 265 and 85 IU/mg, respectively. The ATIII/IIa activity of the UB-ATIII fraction was decreased while the HCII/IIa activity was almost unchanged compared with that of unfractionated heparin. Similarly, the ATIII/IIa activity of the B-ATIII fraction was enhanced *ca.* 2-fold while it was slightly increased when mediated by HCII (1.2-fold).

On HCII-con A. In the presence of buffer B, 10% of the heparin was adsorbed on the HCII column (Table I). The anticoagulant activities mediated by HCII were 975, 1415 and 685 U/mg for unfractionated, bound and unbound heparin, respectively. The HCII/IIa and ATIII/IIa activities of B- and UB-HCII fractions were in a similar ratio, ATIII and HCII being weakly catalysed by the UB fraction, whereas they were efficiently catalysed by the B fraction. This suggests that 10% of the heparin molecules that bind to HCII can potentiate ATIII and must have the ATIII binding site in their sequence. However, it is not evident whether this heparin fraction binds to HCII by the ATIII binding site, to a contiguous site or to an overlap region.

Crossed experiments were done with the subfractions obtained after the affinity chromatographies: bound and unbound fractions isolated from the ATIII column were passed through the HCII column. Whereas 62% of the B-ATIII fraction was adsorbed on the HCII column, only 2% of the UB-ATIII fraction bound to the same col-

TABLE II

ANALYSIS OF NATIVE DERMATAN SULPHATES FRACTIONATED BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ATIII- AND HCII-CON A

Active fraction		Buffer A				
		Recovery (%)	ATIII/IIa		HCII/IIa	
			Activity[a] (IU/mg)	Relative activity	Activity[b] (U/mg)	Relative activity
UF-DS	Choay	100	<1	1	985	1
	Pharmuka	100	<1	1	965	1
	Mediolanum	100	<1	1	955	1
B-HCII	Choay	50	<1	–	1840	1.9
	Pharmuka	25	1.9	>1.9	1750	1.8
	Mediolanum	33	<1	–	1425	1.5
B-ATIII	Choay	8	3.3	>3.3	3660	3.7
	Pharmuka	1	10.5	>10.5	2500	2.6
	Mediolanum	7	1.4	>1.4	1514	1.6
		Buffer B				
		Recovery (%)	ATIII/IIa		HCII/IIa	
			Activity[a] (IU/mg)	Relative activity	Activity[b] (U/mg)	Relative activity
UF-DS	Choay	100	<1	1	985	1
	Pharmuka	100	<1	1	965	1
	Mediolanum	100	<1	1	955	1
B-HCII	Choay	11.5	3.7	>3.7	6500	6.6
	Pharmuka	3.5	8.8	>8.8	4160	4.3
	Mediolanum	7	3.7	>3.7	3765	3.9
B-ATIII	Choay	0	–	–	–	–
	Pharmuka	0	–	–	–	–
	Mediolanum	0	–	–	–	–

[a] Heparin/ATIII/IIa assay.
[b] DS/HCII/IIa assay.

umn. In the same way, 90% of the B-HCII fraction was absorbed on the ATIII column, and 26% of the UB-HCII fraction bound to this column. These results seem to show that the binding sites of ATIII and HCII are strongly dependent and localized on the same molecules.

Affinity chromatography of native dermatan sulphates on HCII- and ATIII-concanavalin A

On HCII-con A. Based on our previous results [17], adsorption of native DS was performed using first the buffer A. Under these conditions, 25–50% of the DS fractions (depending on the native DS) were adsorbed on the HCII column, and the HCII/IIa activity was enhanced *ca.* 2-fold compared with that of unfractionated DS (Table II).

When buffer B was used, only 3.5–11.5% of the sample was adsorbed. In that case the HCII/IIa activities were greatly increased, from 4- to 6.6-fold. The bound DS fraction from Choay

TABLE III

SALINE STRENGTH REQUIRED FOR THE DESORPTION OF SULPHATED POLYSACCHARIDES FROM AT-III- AND HCII-CON A

	HCII-con A	ATIII-con A
Native dermatan		
Choay-Mediolanum	0.38–0.37 *M*	–[a]
Pharmuka	0.30 *M*	–[a]
Fucan	0.5 –0.65 *M*	0.2 –0.3 *M*
Heparin[b]	0.1 –0.5 *M*	0.60–0.70 *M*

[a] Below detection limit.
[b] Literature data.

showed a greater potency than those from Pharmuka and Mediolanum. Thus, at high ionic strength, a small fraction of native DS has a very high affinity for HCII. Surprisingly, these B-HCII fractions exhibited an ATIII/IIa activity of 3.7–8.8 IU/mg, which is very low compared with unfractionated heparin (150 IU/mg). Griffith and Marbet [15] fractionated DS on an HCII column but, contrary to our results, the bound fraction was devoid of ATIII/IIa activity. The three DS were also eluted from the HCII column by a linear saline gradient. The elution of DS Choay, Mediolanum and Pharmuka occurred, respectively, at 0.38, 0.37 and 0.30 *M* NaCl (Table III), which is quite similar to the ionic strength used to elute heparin from the HCII column (0.10–0.50 *M* NaCl [15]).

On ATIII-con A. Fractionation of DS on the ATIII column was obtained but only at a low ionic strength (buffer A) and with a poor recovery (1–8%, Table II). This affinity for ATIII is not due to the presence of contaminating heparin, because DS was previously chemically treated by nitrous acid. The very weak adsorption of DS on the ATIII affinity gel seems to indicate that DS has no or very limited specificity towards ATIII. However, the ATIII/IIa activities of the bound fractions are increased, showing that a few molecules of DS have an affinity, though very low, for the inhibitor (1.4–10.5 IU/mg), compared with the activity of unfractionated heparin (150 IU/mg). When their potency towards HCII is measured, the B-ATIII fractions have a greater activity (*ca.* 1.6- to 3.7-fold) than the B-HCII fractions at the same ionic strength. DS Pharmuka seems to contain molecules with a higher AT-III affinity than the two others, because an AT-III/IIa activity of 10.5 IU/mg was obtained.

At high ionic strength (buffer B), no DS fractions were adsorbed on the ATIII column.

Desorption from the ATIII column with a saline gradient was not possible, because of the weak adsorption of this polysaccharide on the ATIII columns (1–8% distributed in multiple fractions and not detectable by colorimetric assay).

Affinity chromatography of oversulphated dermatan sulphates on ATIII- and HCII-con A

Three dermatan sulphates obtained from Pharmuka, which were previously chemically oversulphated [18], were fractionated on the HCII and ATIII columns (Table IV). First, we can notice that the UF-oversulphated DS have an increased ATIII/IIa activity compared with the native DS. DS3 had an ATIII/IIa activity of 76 IU/mg, which is half of the heparin activity. Similarly, the HCII/IIa activities of these UF-oversulphated DS are also greatly increased by 28- to 431-fold, compared with the native DS. To compare their fractionation to that of native DS, the same buffers were used.

With buffer A, the three oversulphated DS were totally adsorbed on both the ATIII and HCII columns. Thus, increasing the number of sulphate groups on the sugar residues leads to a very high affinity towards HCII and ATIII.

With buffer B, a great difference appeared between HCII and ATIII. Whereas the three oversulphated DS were totally bound on HCII, only 10–13.5% was adsorbed on ATIII. The ATIII/IIa activities of the bound ATIII fractions are weakly increased (1.05- to 2.2-fold), compared with the UF-oversulphated DS, whereas the HCII/IIa activities of these bound fractions are increased 2.2- to 4.1-fold. These results suggest that, even at high sulphur content, DS preserves its specificity for HCII. However, the oversulphation also leads to interactions with ATIII that do

TABLE IV

ANALYSIS OF OVERSULPHATED DERMATAN SULPHATES FRACTIONATED BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ATIII- AND HCII-CON A

Active fraction		Recovery with buffer A (%)	Buffer B				
			Recovery (%)	ATIII/IIa		HCII/IIa	
				Activity[a] (IU/mg)	Relative activity	Activity[b] (U/mg) (10^3)	Relative activity
UF-DSS	DS1	100	100	16.5	1	28	1
	DS2	100	100	18	1	21.5	1
	DS3	100	100	76	1	425	1
B-HCII	DS1, DS2 and DS3	90	99	–	1	–	–
B-ATIII	DS1	99	10	32	1.9	91	3.2
	DS2	94	10.5	40	2.2	89	4.1
	DS3	98	13.5	79	1.05	927	2.2

[a] Heparin/ATIII/IIa assay.
[b] DS/HCII/IIa assay.

not occur with native DS. The B-ATIII fractions of the oversulphated DS have a much lower affinity for ATIII than has the corresponding fraction of heparin.

Affinity chromatography of fucan on HCII- and ATIII-con A

With buffer A, fucan was strongly adsorbed on both columns, at least 88% of the fucan being found on both immobilized inhibitors (Table V).

On HCII-con A. With buffer B, 47% of the fucan were strongly bound on the HCII support (Table V). The HCII/IIa and the ATIII/IIa activities of the UB-HCII fraction were decreased, relative to those of the UF-fucan, whereas they were increased in the case of the bound fraction. The

TABLE V

ANALYSIS OF FUCAN FRACTIONATED BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ATIII- AND HCII-CON A

Active fraction	Recovery from buffer A (%)	Buffer B				
		Recovery (%)	ATIII/IIa		HCII/IIa	
			Activity[a] (U/mg)	Relative activity	Activity[b] (U/mg)	Relative activity
UF-fucan	100	100	900	1	970	1
UB-HCII	7	53	90	0.1	497	0.51
B-HCII	93	47	1510	1.7	1463	1.7
UB-ATIII	12	77	296	0.33	398	0.41
B-ATIII	88	23	2770	3.08	2961	3.05

[a] Fucan/ATIII/IIa assay.
[b] Fucan/HCII/IIa assay.

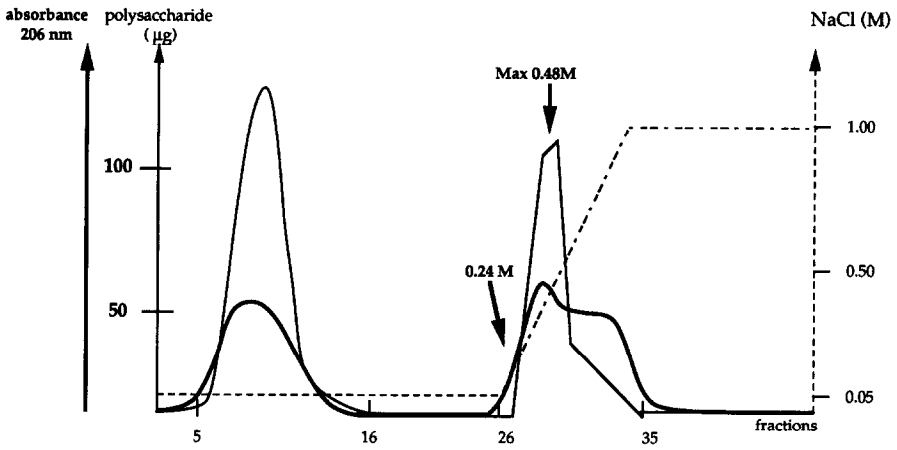

Fig. 1. Affinity chromatography of fucan on the ATIII column (buffer A).

weaker activity of the B-HCII fraction (compared with that of the B-ATIII fraction) can be explained by the fact that a partial fractionation of fucan occurred, because half of the fucan was bound to HCII, including perhaps molecules with an intermediate affinity for HCII, so that a decrease of the specific activity of the fractions was observed. By increasing the ionic strength of the buffer, it would have been possible to obtain a better fractionation of fucan on the immobilized HCII.

The fact that the UB-HCII fraction did not potentiate ATIII, whereas the B-HCII fraction had an equal potency on both inhibitors, seems to show that the binding of ATIII and HCII involves the same molecules of fucan. This was confirmed with the UB-ATIII fraction, which only weakly catalyses HCII. Catalysis of the HCII/IIa reaction was observed with both bound fractions (as shown in Table V).

On ATIII-con A. About 23% of fucan was bound on the ATIII column, a percentage similar to the recovery of heparin. It is interesting to notice that Nishino *et al.* [19] observed no adsorption of fucan on an ATIII column, with a buffer of 0.2 *M* NaCl in 0.1 *M* Tris–HCl. This is not surprising because, with our saline gradient, fucan was desorbed at *ca.* 0.2 *M* NaCl (Fig. 1). It suggests that the limit of adsorption of fucan was reached with our ATIII column, so that a fucan fraction of very high affinity towards ATIII was isolated. Desorption of the fucan from the HCII column with a saline gradient was obtained at 0.5 *M* NaCl, which is a higher ionic strength than that used for the desorption of native DS.

Crossed experiments were also carried out: no adsorption occurred on the ATIII columnn with the UB-HCII fraction, whereas 80% of the B-ATIII fraction bound to the HCII column. As for the heparin molecules, it seems that the binding sites of the two inhibitors are strongly dependent and localized on the same fucan molecules.

CONCLUSION

Native and oversulphated dermatan sulfates, fucan and heparin are sulphated polysaccharides that can catalyse the inhibition of thrombin by ATIII and HCII. Although the interaction of heparin with ATIII is now well understood, the interactions of the other polysaccharides with the two inhibitors, and between heparin and HCII, are still under investigation. We have fractionated DS, fucan and heparin into bound and unbound fractions towards HCII and ATIII. The bound fractions had a catalytic activity towards thrombin inhibition greatly enhanced compared with that of the unfractionated polysaccharide.

About 25% of heparin and fucan bind to ATIII with a high affinity. However, the chromatographic profiles of these two polysaccharides, when applied to the HCII column are different: while 10% of heparin binds to HCII, half of the fucan molecules bind to this inhibitor. However, it seems that, for these two polysaccharides, the binding sites of ATIII and HCII are localized on the same molecules. When native DS were fractionated, fractions having high affinity for HCII were isolated at high ionic strength. The HCII/IIa activities of these fractions were strongly increased, the relative activities obtained rising to 6.6-fold and the specific activity increasing from 985 to 6500 U/mg. Surprisingly, we were able to obtain a small fraction of DS with affinity for ATIII. However, the yield and the thrombin inhibitory activity of this fraction mediated by ATIII were low compared with those of the fractions eluted from the HCII column. Finally, oversulphation of native DS reinforces the affinity for HCII but also leads to the development of a measurable affinity towards ATIII. However, when a high ionic strength is used, a predominant affinity of the oversulphated DS for HCII is shown and, moreover, the high affinity fractions for ATIII of the oversulphated DS still exhibit their potency towards HCII.

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