



Regular Article

# A small fraction of dermatan sulfate with significantly increased anticoagulant activity was selected by interaction with the first complement protein

Graciela C. Calabrese<sup>a</sup>, María F. Alberto<sup>b</sup>, Rosario Tubio<sup>a</sup>,  
Mariela M. Marani<sup>a</sup>, Marta E. Fernández de Recondo<sup>a</sup>,  
María Lazzari<sup>b</sup>, Eduardo F. Recondo<sup>c,\*</sup>

<sup>a</sup>*Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, Ciudad Autónoma de Buenos Aires, 1113, Argentina*

<sup>b</sup>*Departamento de Hemostasia y Trombosis, Academia Nacional de Medicina, Ciudad Autónoma de Buenos Aires, Argentina*

<sup>c</sup>*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 4° piso, Ciudad Autónoma de Buenos Aires, 1428, Argentina*

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## KEYWORDS

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## ABSTRACT

Dermatan sulfate (DS) is a member of the family of structurally complex, sulfated, linear heteropolysaccharides called glycosaminoglycans (GAGs). It has a similar structure to heparin and heparan sulfate (HS), but with acetylgalactosamine replacing glucosamine, and the uronic acid moiety, mainly iduronic, joined 1 → 3 to the hexosamine. We are studying the relationships between structure and activities of dermatan sulfate, in particular those associated with the thrombin inhibition mediated by heparin cofactor II (HCII). As we have demonstrated with heparin, a small fraction of dermatan sulfate was isolated by precipitation with the first component of the complement system, under very specific conditions of low ionic

*Abbreviations:* AT, antithrombin; C1, the first protein complex of the complement system; Con A, Concanavalin A; DS, Dermatan sulfate; free DS, free Dermatan sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HCII, heparin cofactor II; LMWH, low molecular weight heparin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; pp DS, DS specifically recognized by C1; TRIS, (hydroxymethyl) aminomethane; T, thrombin; UFH, unfractionated heparin.

\* Corresponding author. Juncal 2186, 6° P, Buenos Aires (1125) Argentina. Tel.: +54-11-4824-6297; fax: +54-11-4964-8274, int 8238.

*E-mail addresses:* gcalabe@ffyba.uba.ar (G.C. Calabrese), martarecondo@fibertel.com.ar (M.E. Fernández de Recondo), efrecondo@fibertel.com.ar (E.F. Recondo).

strength, and the presence of calcium ions. The sulfate content and the anticoagulant activity of the dermatan sulfate fraction isolated in the precipitate were three and four times greater respectively than the starting material. Our *in vivo* studies showed that this fraction has threefold higher thrombolytic activity than the DS. All these results suggest that this fraction could be used as a therapeutic agent for thrombi dissolution.

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## Introduction

In the last 25 years, thrombotic and vascular disorders have been recognized as a leading cause of mortality. The best treatment should allow a shortest therapy without recurrence.

Antithrombotic drugs represent a wide spectrum of natural, synthetic, semisynthetic, and biotechnology-derived agents that differ markedly in chemical composition, physico-chemical properties, biochemical actions, and pharmacological effects [1].

Our studies look for the development of new antithrombotic drugs or the modification of those in existence to an efficient control of the thrombotic process and the multifactorial thrombotic events.

Unfractionated heparin (UFH) is a major antithrombotic drug, widely used for prevention and treatment of venous thrombosis. The anticoagulant effect of heparin is mediated by the plasma protein antithrombin (AT), which inhibits most of the serine proteases involved in coagulation. Heparin increases dramatically the rate of formation of a complex of AT with each protease. However, its clinical use is limited by the risk of bleeding and thrombocytopenia. The last years, the employment of different low molecular weight heparins (LMWHs) has reduced the risk of haemorrhagic complications described for UFH; but higher doses used in treatments may induce some adverse side effects [2].

A second heparin dependent inhibitor of thrombin (T) was identified in 1974 by Briginshaw and Shanberge [3], but not until 1982 was purified and characterized from human plasma by Tollefsen et al. [4]. The new inhibitor, designed heparin cofactor II (HCII), is also a glycoprotein but different of AT and the concentration in normal human plasma is approximately one-half that of AT.

Dermatan sulfate (DS) is another glycosaminoglycan (GAG), incorporated in antithrombotic therapies when it was found [5] that this GAG also activate HCII for T inhibition. Unlike UFH and LMWH, DS is effective on both free and fibrin-bound T [6]. DS has been found effective with a low rate

of haemorrhagic complications, in the prevention of postoperative deep vein thrombosis [7,8].

Our research group has described previously the isolation of a fraction from UFH, LMWH and DS with very high anticoagulant activity, through the interaction with the lectin Concanavalin A (Con A) [9–12] under very specific conditions of low ionic strength and the presence of calcium ions. Similar results were obtained with UFH and LMWH in the interaction with the first protein of the complement system (C1) and its subunit C1q [13,14], and it was possible to select in the precipitate of the interaction a subpopulation that shows a great affinity for AT. Recently we demonstrated that AT and C1 recognize the same active heparin fraction [15].

The purpose of the present report is the study of the interaction between DS and human C1 based on the conclusions obtained with UFH, LMWH and employing the specific experimental conditions described previously. The sulfate concentration and the biological activity of the subpopulations isolated through the interactions were evaluated.

## Materials and methods

DS with maximum purity was a gift from Lic. Victor Díaz. IgG-agarose (A-6284) and GAGs molecular weight markers were purchased from Sigma (St. Louis, USA). Protein standard weight markers were from Bio-Rad Labs. (Richmond, Canada). The chromogenic substrate S-2238 was obtained from Chromogenix (Mölnådal, Sweden). General reagents were of analytical grade or higher.

### Protein complex C1 purification

Protein complex C1 isolation was carried out as described by Bing [16]. Human plasma (250 ml) was diluted with 750 ml of distilled water at 4 °C. The euglobin fraction was precipitated by adjusting the pH to 7.4–7.5. The suspension was stirred for 1 h at 4 °C and then centrifuged at 3000 rpm during 10

min. The small precipitate was resuspended in 250 ml of phosphate-buffered saline (PBS), the pH adjusted to 5.4 with 1 M  $H_3PO_4$  and keeping overnight at 4 °C. After centrifugation the precipitate was taken up in the same buffer.

C1 was purified by affinity chromatography with human IgG-agarose. The protein fraction was eluted with 1,4 diaminobutane solution at pH 11, collected and exhaustively dialyzed against 0.005 M TRIS (hydroxymethyl) aminomethane/acetate buffer solution, 0.09 M NaCl, pH 8.1 (TRIS/acetate, pH 8.1).

C1 protein concentration was determined by using an extinction coefficient  $E^{1\%} = 10$  [17]. Purified protein preparations were always verified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate 10% (SDS-PAGE). Samples and standard molecular weight markers were prepared and run under the same conditions. Coomassie blue was used for staining proteins.

### Conditions for DS and protein complex generation

Interaction assays between DS and C1 protein complex were run under strict and specific final conditions found (see Results).

DS (50, 250 or 500  $\mu$ g) in distilled water was added to 0.5 ml of 0.1 M phosphate or TRIS/HCl buffer, in the presence of 40  $\mu$ l of the required calcium solution. The interaction was started by addition of 1 ml of C1 (50  $\mu$ g). The final volume was 2 ml.

Assays were monitored by reading the turbidity developed at 420 nm, 1 h after incubation at 37 °C, by using a Hitachi U-2000 spectrophotometer. DS in the precipitated with C1 (pp DS), and DS free in the supernatant (free DS), were separated by centrifugation at 5000 rpm during 25 min. The pellet was dissolved by the addition of 0.5 ml of 300 mM NaCl; this increment in the ionic strength produced the release of DS from the ternary complex, and also the desnaturalization of C1 which precipitated; so DS in the solution and C1 could be separated by centrifugation at 5000 rpm during 25 min and desalting by Sephadex G-25.

In all cases, DS concentration was determined by the method of Rodríguez Montelongo et al. cited in Ref. [12], based on the reaction of carbohydrates with indole in HCl.

### In vitro studies

#### Biological activity

The in vitro potency of DS was standardized against the USP heparin reference standard (INAME), Argentine, using USP assay and human plasma.

Biological activity of DS and its subpopulations, obtained after interactions, were measured according to the technique described by Sie et al. [18] with some modifications.

Blood was collected in citrated tubes (Becton-Dickinson) from 10 healthy donors. The platelet-poor plasma was obtained by centrifugation at  $2000 \times g$  during 20 min, fractionated and stored at  $-70$  °C.

Plasma, before using, was diluted 1:25 in 150 mM NaCl, 20 mM TRIS/HCl, pH 7.4 buffer, containing 1% polyethyleneglycol 6000 (Merck, Germany).

A 0.5 UI/ml of human T (T6759, Sigma) and 1 mg/ml of the chromogenic T substrate S2238 (Chromogenix) were diluted in the buffer above described before using.

The different DS fractions to be measured were diluted in the same buffer in order to obtain concentrations lower than 20  $\mu$ g/ml. All samples were assayed in duplicate.

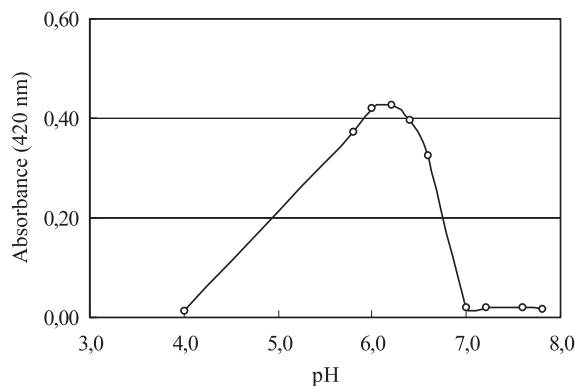
The plasma, DS fractions and T (50  $\mu$ l of each one) were put into a micro titer well plate and equilibrated at 37 °C for 10 min. Then 50  $\mu$ l of S2238 was added to each micro well plate. After 5 min of incubation, hydrolysis of S2238 was stopped by addition of 100  $\mu$ l of acetic acid 20% v/v and the absorbance at 405 nm was measured. A linear standard curve was obtained by plotting the absorbance against the dilutions of DS at 1, 2, 5, 10, 20  $\mu$ g/ml chemical concentration. Absorbance of a blanc containing all reagents except DS was assumed as no detectable DS activity (100% of T activity).

The concentration of each sample of DS was recalculated, according to the biological activity. A factor (*F*) that correlates DS concentration performed by its biological activity and the chemical method was calculated.

#### Chemical analysis

**Sulfate content.** The sulfate concentration was determined by the sodium rodizonate method [19].

**Polyacrylamide gel electrophoresis.** The molecular masses of the GAGs were estimated by polyacrylamide gel electrophoresis (PAGE) [20]. Samples (10  $\mu$ g) were applied to 1 mm thick 6% polyacrylamide slab gel, and after electrophoresis at 100 V for 30 min in 0.06 M sodium barbital (pH 8.6), the gel was stained with 0.1% toluidine blue in 1% acetic acid. After staining, the gel was washed overnight in 1% acetic acid. The molecular mass markers were: high molecular weight dextran sulfate (average molecular mass = 500 kDa), chondroitin 6-sulfate from shark cartilage (average molecular mass = 60 kDa), and low mo-



**Fig. 1** Influence of the pH on the interaction. The pH curves were performed under the following experimental conditions: relationship DS/C1 = 1, on a weight bases, 2 mM CaCl<sub>2</sub> and buffer concentration 25 mM. Each point of the curve was determined in triplicate.

lecular weight dextran sulfate (average molecular mass = 8 kDa).

## In vivo studies

### Animals

Male Wistar rats from the breeding laboratories of Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina) whose weights were 200–250 g, were used.

### Experimental model

Thrombolytic activity was studied using an experimental venous thrombosis model induced as was previously described by Doutremepuich et al. [2], but the subfractions of DS were administrated into the dorsal vein of the penis, employing physiological solution as vehicle. Two hours later the thoracic cavity was opened, the ligated segment was removed, opened longitudinally and the formed thrombus in each experiment was removed, rinsed, blotted on filter paper, dried at 37 °C till constant weight, and weighed.

### Statistical analysis

Statistical analysis of the distribution of dermatan sulfate subpopulations was carried out using ANOVA and Bonferroni's multiple comparison test.

Statistical analysis of the in vitro studies: biological activity and the sulfate concentration were performed using ANOVA and Dunnett's multiple comparison test.

Statistical analysis of the in vivo studies were performed using ANOVA and Tukey's test.

When *P* is lower or equal to 0.05 results are considered statistically significant.

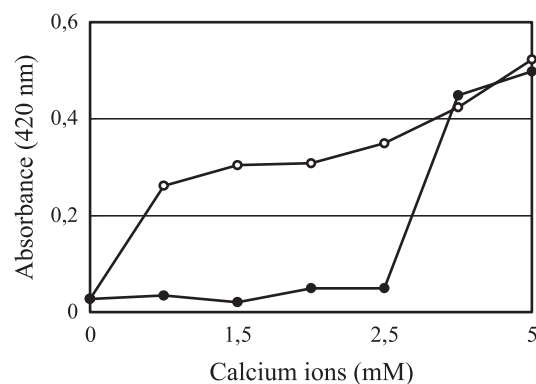
## Results

The C1 protein complex used in all experiments has an identical electrophoresis pattern as was described previously [15].

### Conditions for the dermatan sulfate and protein complex generation

Interactions between DS and C1 protein complex were carried out under specific final conditions previously found for DS/Con A protein and UFH/C1 protein complex [12,14]. These conditions are: 2 mM CaCl<sub>2</sub> a relationship GAG/protein = 1 (on a weight basis) and a very low ionic strength (25 mM phosphate or TRIS/HCl buffer). The influence of pH was evaluated carrying out a curve between 4.0 and 7.8 (Fig. 1). A maximum at pH 6.0 was observed.

The next step was the evaluation of the influence of calcium ion concentration on the interaction: a curve between 1 and 5 mM CaCl<sub>2</sub> concentrations was performed. Conditions are those of Fig. 1, but at the optimum pH obtained before (pH 6.0). A maximum at 3 mM CaCl<sub>2</sub> concentration was found (Fig. 2), but after 2.5 mM calcium concentration, the large C1 complex which consists of a single C1q and the calcium-dependent tetramer (C1r<sub>2</sub>-C1s<sub>2</sub>) probably sediments and the turbidity show up even in the absence of DS. Therefore, 2 mM CaCl<sub>2</sub> concentration was selected for all the experiments.



**Fig. 2** Influence of calcium ions on the interaction. The calcium ions curves were performed in the same conditions employed in the previous case, except for the use of different final calcium ion concentrations: from 1 to 5 mM. The figure shows the turbidity developed with the whole system (—○—) and in the absence of DS (—). A maximum at 3 mM calcium concentration was found, but after 2.5 mM turbidity was observed in the absence of DS. Each point of the curve was determined in triplicate.

**Table 1** Dermatan sulfate distribution

DS/C1 ratio	Dermatan sulfate (% $\pm$ S.E.)	
	Supernatant free DS	Precipitate pp DS
1 ( <i>n</i> =3)	85.67 $\pm$ 1.75	14.33 $\pm$ 1.70
5 ( <i>n</i> =6)	94.14 $\pm$ 0.29	5.85 $\pm$ 0.30
10 ( <i>n</i> =2)	94.11 $\pm$ 0.50	5.89 $\pm$ 0.59

The experiments were performed in the following conditions: 2 mM CaCl<sub>2</sub>, GAG/protein = 1.0, 5.0 or 10.0 (on a weight basis) and a very low ionic strength (25 mM phosphate buffer), at the optimum pH 6.0. *P* < 0.01, ANOVA, Bonferroni's Multiple Comparison Test (1:1 vs. 1:5 and 1:1 vs. 1:10).

Under these conditions no turbidity was developed in the absence of DS.

Another approach was then performed: DS/C1 protein complex ratio was changed and the GAG was measured in the precipitate and in the supernatant to determine the distribution of the DS. The results are shown in Table 1. The amount of DS ( $\mu$ g%) increased in the supernatant with a correlated decreased in the precipitate, when the DS/C1 increased from 1 to 5, but no differences could be observed between GAG/protein = 5 and 10 ratio. Turbidity was not detected in the absence of CaCl<sub>2</sub> at all DS/C1 ratio (data not shown). Therefore, conditions for the ternary complex generation are: 2 mM CaCl<sub>2</sub>, a relationship GAG/protein = 1.0, 5.0 or 10.0 (on a weight basis) and a very low ionic strength (25 mM phosphate buffer), at the optimum pH 6.0.

### In vitro studies of the dermatan sulfate fractions

#### Biological activity

The specific activity of the DS employed was 5 UI/mg as was described in Materials and methods.

The residual T activity in the presence of DS recovered from the precipitate or obtained in the supernatant was determined as described in Materials and methods. Table 2 shows the correlation of DS concentration determined by its biological activity and by the chemical method; as was expected *F* is near 1 in starting material. The ppDS has the highest *F*. The interactions were performed in the same conditions described previously at GAG/protein = 5.0.

Under the conditions employed, it was possible to isolate in the precipitate 5.85% of the total DS employed, and with a biological activity more than four times greater than the starting material.

#### Chemical Analysis

The sulfate content was compared among the original DS, the DS recovered from the precipitate, and

**Table 2** Anticoagulant activity and sulfate content of dermatan sulfate subpopulations

Dermatan sulfate	<i>F</i> $\pm$ S.E.	Sulfate content ( $\mu$ g% $\pm$ S.E.)
Starting material ( <i>n</i> =3)	1.10 $\pm$ 0.10	11.54 $\pm$ 0.83
Precipitate (pp DS) ( <i>n</i> =4)	4.26 $\pm$ 1.03 <sup>#</sup>	28.81 $\pm$ 1.19*
Supernatant (free DS) ( <i>n</i> =3)	0.65 $\pm$ 0.03 <sup>#</sup>	9.99 $\pm$ 1.16

The interactions were performed in the following conditions: 2 mM CaCl<sub>2</sub>, in a relationship DS/protein = 5.0 (on a weight basis) and a very low ionic strength (25 mM phosphate buffer), at the optimum pH 6.0.

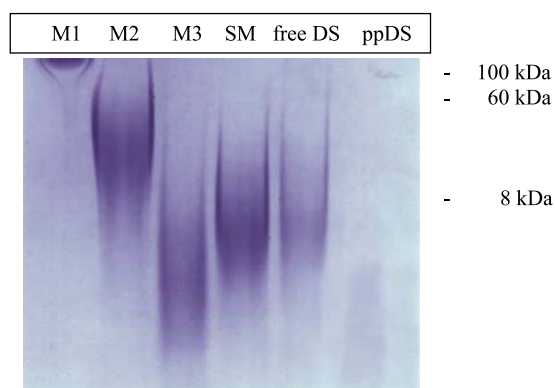
*F* = [DS] Anticoagulant activity / [DS] Chemical method.  
*n* = Number of determinations.

<sup>#</sup> *P* < 0.05, ANOVA, Dunnett's Multiple Comparison Test (starting material vs. precipitate and starting material vs. supernatant).

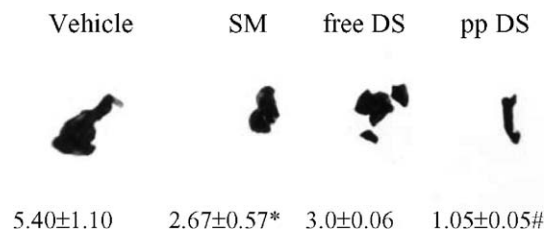
\* *P* < 0.05, ANOVA, Dunnett's Multiple Comparison Test (starting material vs. precipitate).

the DS from the supernatant of the interaction performed in the same conditions as before. As can be seen in Table 2, the activity of the different DS subpopulations obtained in the interaction correlates with the sulfate content.

The polyacrylamide gel electrophoresis of the DS starting material, free DS and DS isolated through



**Fig. 3** Polyacrylamide gel electrophoresis of dermatan sulfate subpopulations. Dermatan sulfate starting material and its subpopulations (10  $\mu$ g of each sample) were applied to 6%, 1 mm thick polyacrylamide gel slab in 0.06 M sodium barbital (pH 8.6) and run for 30 min at 100 V. After electrophoresis the dermatan sulfates and standards were stained with 0.1% toluidine blue in 1% acetic acid and then washed overnight in 1% acetic acid. The molecular mass (MM) markers were high molecular mass dextran sulfate (M1, ~ 500 kDa), chondroitin 6-sulfate from shark cartilage (M2, 60 kDa) and low molecular mass dextran sulfate (M3, 8 kDa). Samples are: SM, starting material; free DS, dermatan sulfate unbound; and pp DS, DS isolated through the specific interaction with C1.



**Fig. 4** Thrombolytic activity of the dermatan sulfate fractions. Dermatan sulfate starting material and its subpopulations were administered to animals with an induced thrombosis venous (0.3 mg/kg/h, i.v.). In each experiment, thrombus weights are expressed in milligrams (mg), as the average of six determinations  $\pm$  S.D. Samples are: vehicle, physiological solution; SM, starting material; free DS, dermatan sulfate unbound and pp DS, DS isolated through the specific interaction with C1. \* $P < 0.05$ , ANOVA, Tukey's test (vehicle vs. SM: starting material) # $P < 0.01$ , ANOVA, Tukey's test (vehicle vs. pp DS).

interaction with C1 is shown in Fig. 3. The DS fraction that is specifically recognized by C1 corresponds to a molecular weight below 8 kDa.

### In vivo studies of the dermatan sulfate fractions

The thrombolytic activity of the dermatan sulfate fractions is shown in the photograph of Fig. 4. Dermatan sulfate starting material and its subpopulations (0.3 mg/kg/h) [7] were administered to animals with an experimental venous thrombosis model induced as was previously described in Materials and methods. Significant thrombolytic activity is observed if control (physiological solution as vehicle) is compared with the starting material and with the DS fraction that is specifically recognized by C1.

The whole thrombolytic activity was isolated in the fraction recognized by C1 as no significant differences were found when supernatant (free DS) is compared with physiological solution (vehicle).

### Discussion

The main biological activity associated with DS is the acceleration of T inhibition by HCII. The discovery [3] and, years later, the purification [4] of this second circulating thrombin inhibitor has changed the attention from heparin, present in the mast cells, to heparan sulfate (HS) and DS present on the surface of cells or in the extracellular matrix of the vascular system.

Heparin and HS catalyze the inhibition of factor Xa and T by AT. In contrast, DS has no effect on the

rate of protease inhibition by AT. When Tollefsen et al. [21] started to use the purified HCII, they demonstrated that the anticoagulant properties of DS observed in vitro are explained by the activation of HCII. DS increased more than 1000 times the constant rate for inhibition of T by the new cofactor. Almost simultaneously Griffith and Marbet [22] reported that a subpopulation of DS molecules, bound to a column of immobilized HCII and eluted with NaCl between 0.25 and 0.50 M, was four to five times more active than the unfractionated material. Subsequently, Tollefsen et al. [23] prepared fragments of DS by partial chemical depolymerization and found that the smallest one that stimulates T inhibition by HCII were dodesaccharides. They determined the chemical structure of the binding site of DS to HCII. By a combination of several methods they isolated a mixture of hexasaccharides as the smallest HCII binding fragments. Analysis of this mixture showed that the hexasaccharide with high affinity to HCII was constituted by the repetition of the same disaccharide with two sulfates groups, one localized in the position 2 of iduronic acid and the other in the position 4 of the acetyl galactosamine, respectively [24].

We are studying the structure–activity relationships of DS's anticoagulant and thrombolytic activities, by using a very simple system based in its interaction with the C1. The results of the experiments we have done, which are presented in Tables 1 and 2, show the existence of a very small fraction of the DS molecule, which can be isolated by precipitation with C1. Very strict conditions of low ionic strength, and the presence of calcium ions, are absolutely necessary as we have found previously for the heparin proteins interactions.

Two comments deserve the results described above as compared with ours. As Table 2 shows, the subpopulation of DS that we isolated by precipitation with C1 has more than four times higher biological activity than the starting material. This means that we have concentrated the fraction of the DS molecule active for T inhibition. The hexasaccharide with high affinity for HCII has two sulfate groups per disaccharide unit. The figures of the same table show that the fraction isolated has almost three times more sulfate content than the starting material. This increment agrees with several previous reports from other laboratories in regard to the requirement of the presence of highly sulfate sequences in DS to stimulate HCII.

In heparin, AT binds very strongly to a specific pentasaccharide that contains an unique 3–O–sulfate glucosamine residue [25]. This sulfate group and three more are essentials for the binding to AT,

and about 30% of the heparin molecules contain the AT binding pentasaccharide [26], but in order to show up stimulation for the T–AT reaction, at least a minimum chain length of 18 monosaccharides is required in agreement with the template model of heparin action [27].

In the case of DS, the high affinity hexasaccharide is at least 20 times less active than the intact DS for inhibiting T in the presence of HCII, which implies the requirement of longer chains for the most efficient stimulation of the reaction. The high activity observed in vitro and in vivo and the molecular weight (Fig. 3) of our DS subpopulation suggest that it fulfils the necessary requirement for its biological functions.

As was mentioned previously, the degree of sulfation is another important property that contributes significantly to the thrombolytic and anticoagulant activities of DS and HS. In 1987, Ofosu et al. [28] have published significative results on this respect. They have compared the anticoagulant activities of HS and DS with those of their derivatives resulfated “in vitro”. They found that increasing the degree of sulfation improved the catalytic effects of these two GAGs on the inhibition of T by HCII in plasma. Almost simultaneously, Scully et al. [29] reported that the ability of DS to accelerate AT–factor Xa interaction, or HCII–T interaction is considerably increased in over-sulfated naturally occurring forms, and concluded that the possession of high charge density is not the only requirement for the interactions, and the spatial position of the sulfates are also of importance. On this respect, Pavao et al. [20] have demonstrated that 4-O-sulfation of the galactosamine unit is essential for the anticoagulant activity of natural DSs isolated from marine invertebrates. Finally, we have demonstrated recently that the specific activity of the DS of high purity increased three times after a resulfation under mild conditions [30].

All these results suggest that alterations in the sulfation pattern of the GAGs, and particularly DS, could be an important factor for the efficient control of the thrombogenic process and the multifactorial thrombotic events.

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