

## Anticoagulant activities of $\beta$ -1,3-glucansulfates in dependence on their molecular weight

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**Abstract:** In order to examine the molecular weight (MW) dependence of the anticoagulant activity in various test systems, sulfated polysaccharides only differing in their MW were synthesized and tested. They were obtained by thermal degradation of the starting polymer curdlan before the sulfation or by the GPC fractionation of polydisperse curdlansulfates. It could be shown that besides the degree of sulfation also the MW influences the activity. An increase in MW results in a bell shaped curve and a continuous decrease of the ratio of the anti-thrombin- to the anti-Factor Xa-activity representing a change of the activity profile.

### INTRODUCTION

For about 50 years heparin is the drug of choice in the clinical pre- and postsurgical prophylaxis of thrombotic events. However, due to its side effects like bleeding and other disadvantages like its chemical inhomogeneity and the variability of its physiological activities, an important field of research are alternatives to genuine heparin. After fractionating heparin one had to realize that the antithrombotic effects were strongly depending on the molecular weight (MW) (1). This knowledge opened a new area of antithrombosis research and led to the discovery of the low molecular weight heparins. The anticoagulant activities of heparin are mostly due to its promoting effect on the in plasma occurring serine protease inhibitor antithrombin III (ATIII) (2). In order to accelerate the neutralization of Factor Xa (FXa) by the interaction of ATIII only the presence of the 'antithrombin binding site', a specific pentasaccharide, is essential. For an effect on the thrombin interaction a chainlength of 18 sugar units is needed (3). In a similar manner as for the ATIII dependent thrombin inactivation also for the heparin cofactor II (HCII) induced neutralization of thrombin by heparin the length of the sugar chain is a critical factor (4). The same dependence was found for other heparinoids which as well interfere with HCII like dermatansulfate, fucansulfate (5) and others. Furthermore, it could be demonstrated that, as in the case of the fucansulfate acting on the intrinsic and extrinsic system of the coagulation cascade and on thrombin, some anticoagulant active substances interact at several levels of the coagulation reaction. These interactions again are dependent upon the MW and show their optimal activity in different MW regions.

In consequence, the respective results when testing the MW dependence of an anticoagulant compound is strongly dependent upon the test system. This might explain the contradictory results in the literature (6-10). Based on these rather uncertain data the aim of this work was to examine the MW / anticoagulant activity relationship by utilizing several test systems. For this purpose, distinct sulfated polysaccharides only differing in their respective chain length were synthesized and submitted to the various test systems.

### THERMAL DEGRADATION AND SULFATION OF CURDLAN

#### Thermal degradation of genuine curdlan:

As a basic polysaccharide structure  $\beta$ -1,3-glucans such as curdlan were utilized. This extracellular polysaccharide with a DP of > 500 is produced by the agrobacterium *Alcaligenes faecalis var. myxogenes* (11). In contrast to other  $\beta$ -1,3-glucans curdlan is strictly linear.

One possibility to obtain distinct glucansulfates with defined chain length is the sulfation of glucans of different MW. For this purpose, the high MW curdlan was partially degraded by the treatment of a 0.33 % suspension of the glucan for 1-3 h at an elevated temperature > 130°C (12). The weight average MW ( $M_w$ ) of the products was determined by GPC on a Superose<sup>TM</sup>6 in a Pharmacia-FPLC-system using 0.2 N NaOH as eluent for the water-insoluble glucans (12). The column was calibrated with defined pullulan fractions. Treatment for 3 h at 174°C completely degraded the glucan to glucose. The solubility of the different thermal degraded curdlans (**HdC**) in 0.2 N NaOH increased with decreasing MW (Table 1).

TABLE 1. Thermal degradation of curdlan

	t	T	M <sub>W</sub>	Sol. <sup>1</sup>
<b>Curdlan</b>	-	-	>500 kd	2
<b>HdC I</b>	1.0 h	148°C	26 kd	5
<b>HdC II</b>	2.5 h	150°C	20 kd	4
<b>HdC III</b>	2.0 h	162°C	15 kd	3
<b>HdC IV</b>	3.0 h	174°C	Glucose	1

<sup>1</sup> Series of solubility in 0.2 N NaOH

TABLE 2. DS, MW and sulfation pattern of **HdCS I/II/III**

	DS	M <sub>W</sub>	M <sub>N</sub>	M <sub>W</sub> /M <sub>N</sub>	C2 : C4 : C6 <sup>1</sup>
<b>HdCS I</b>	0.85	30 kd	18 kd	1.67	0.04 : 0.03 : 1
<b>HdCS II</b>	0.85	18 kd	11 kd	1.64	0.05 : 0.10 : 1
<b>HdCS III</b>	0.85	14 kd	9 kd	1.56	0.04 : 0.02 : 1

<sup>1</sup> Relative ratio of the three glucose-monosulfates

### Sulfation of the degraded curdlans **HdC I/II/III**

After the thermal degradation the breakdown products were sulfated by a modified SO<sub>3</sub>-pyridine method in DMF (13), which was shown not to further degrade the glucan chains and resulted in the homogeneously substituted products **HdCS I/II/III** (Fig. 1). Prior to derivatization the polysaccharide was submitted to ultrasonication and DMF-treatment (14). Hereby hydrogen bonds are desintegrated and as a consequence the substitution reaction is facilitated.

### Characterization of the curdlansulfates **HdCS I/II/III**

A degree of sulfation value (DS = number of SO<sub>4</sub>-groups/glucose unit) of 0.85 was calculated for all three products after conductimetric titration (15). The M<sub>W</sub> was established by GPC on Superose™12 (13), the number average MW (M<sub>N</sub>) after a determination of the reducing end groups according to Somogyi (16) (Table 2). The M<sub>W</sub>/M<sub>N</sub> ratio of about 1.6 is an indication for the polydispersity of the respective products. The substitution pattern (Fig.1) was obtained after a modified methylation of the products followed by GC/MS-analysis (12). The reactivity of the primary OH at C6 is about ten times higher compared to the secondary OH at C2 and C4. Consequently, the monomers of products with a low DS are either C6-mono- or non sulfated. An increase of the DS results in a loss of homogeneity and selectivity. Derivatives with a DS of 0.85 showed that more than 50 % of the glucose monomers were C6-monosulfated. Besides 13 % disubstituted and 30 % non sulfated glucose units only a very small amount of trisulfated monomers is present.

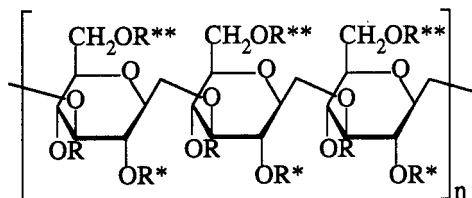
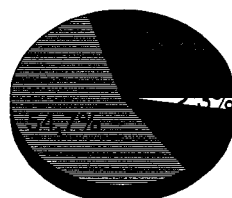


Fig. 1 Repeating unit of **HDCS I/II/III** with R = R\* = H >> SO<sub>3</sub>, R\*\* = SO<sub>3</sub> >> H



■ non sulfated  
 ▨ monosulfated  
 ■ disulfated  
 □ trisulfated

Fig. 2 Homogeneity of the sulfate-distribution of **HDCS II** (100 % = all the glucose units of the molecule)

## FRACTIONATION OF A LOW-DS AND HIGH-DS CURDLANSULFATE

### Fractionation of the low sulfated **FraCS L** (DS = 0.60)

Since it could be shown that thermal degraded curdlansulfates showed different MW dependent anticoagulant activities (see below), the influence of the chain length had to be determined with a broader MW spectrum and with compounds of reduced polydispersity. Therefore, the polydisperse curdlansulfates **FraCS L** (DS = 0.60) and **FraCS H** (DS = 1.20) were separated by GPC. (12)

The M<sub>W</sub> of **FraCS L** was shown in the range of 2 - 380 kd with a peak maximum at 18 kd. The separation on a HiLoad™16/50 Superdex™200, prep. grade produced 10 distinct fractions out of which **L2-L8** were tested (Fig. 3). One has to realize though, that by GPC not the real but the hydrodynamic volume is determined, which increases in parallel to the charge density (17). Consequently, the separation of the curdlansulfates could be due to differences in the respective charge densities. However, an examination of the DS values in the different fractions revealed a uniform sulfate distribution. It can be concluded that the above separation was only due to the differences in MW.

### Fractionation of the high sulfated **FraCS H** (DS = 1.20)

In order to examine if the found MW/activity relationship was identical for compounds with higher DS values, **FraCS H** with a DS of 1.20, a M<sub>W</sub> range of 2 - 230 kd and a maximum of 11 kd was fractionated (Table 3).

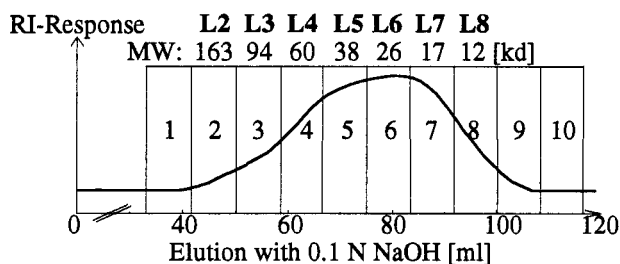


Fig. 3 Separation of **FraCS L** on a HiLoad™16/50 Superdex™200

TABLE 3.  $M_w$  of the tested **FraCS H**-fractions

No.	$M_w$	No.	$M_w$	No.	$M_w$
<b>H1</b>	190 kd	<b>H6</b>	92 kd	<b>H13</b>	15 kd
<b>H2</b>	160 kd	<b>H7</b>	78 kd	<b>H18</b>	11 kd
<b>H3</b>	145 kd	<b>H8</b>	67 kd	<b>H23</b>	9 kd
<b>H4</b>	115 kd	<b>H9</b>	63 kd	<b>H27</b>	8 kd
<b>H5</b>	105 kd	<b>H10</b>	19 kd	<b>H30</b>	5 kd

ANTICOAGULANT ACTIVITY OF CURDLANSULFATES WITH DISTINCT MW

Anticoagulant activity of HdCS I/II/III

First of all the thermal degraded curdlansulfates were tested in the three classical clotting systems, which should give an indication for a possible interaction of these substances in the different reaction sequences of the coagulation cascade. Thus, the thrombintime (TT, aIIa) records a disturbance of the thrombin mediated fibrine formation, the Heptest® (aXa) determines the anti-FXa-activity. By means of the activated partially thromboplastintime (aPTT) an intervention in the intrinsic pathway can be ascertained.

As it can be seen in Fig. 4, the curdlan derivatives, whose specific activity was calculated by means of the 4. Internat. Standard for Heparin, had an optimal effect in the aPTT test system and a minimal one in the TT. By lowering the MW the specific activity in all three test systems decreased.

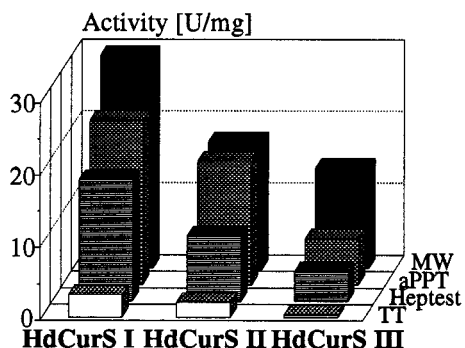


Fig. 4 Anticoagulant activity of **HdCS I/II/III** in aPTT, Heptest® and Thrombintime

Anticoagulant activity of FraCS L (DS = 0.60)

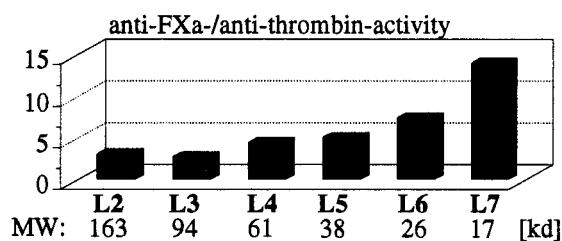


Fig. 5 aXa/aIIa ratios of the **FraCS L**-fractions

length and not of the individual charge differences. When calculating the activity ratio of the three test systems, the aIIa/aPTT ratio decreased when lowering the MW of the individual tested fractions; the aXa/aIIa ratio, however, increased. As a consequence the aXa/aIIa ratio increased from 3.2 for L2 up to 76.3 for L7 (Fig. 5).

By the testing of **FraCS L2-L8** a MW-range of 12-163 kd was covered. After an initial increase in activity in all the tests which was obtained with fractions with decreasing MW, above fraction **L5** (38 kd) a loss in anticoagulant efficacy could be observed (Fig. 6). The results of fractions **L6-L8** are in good correlation with the thermal degraded curdlansulfates and again showed the loss in activity comparing the three systems aPTT > Heptest® > TT. Since the individual fractions matched in their respective DS, the differences in activity must be the result of varying chain

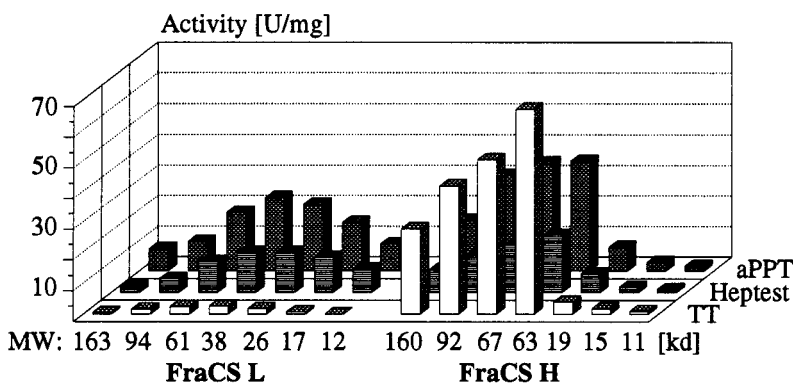


Fig. 6 Anticoagulant activity of **FraCS L-** (DS = 0.60) and **FraCS L**-fractions (DS = 1.20) with comparable MW in Thrombintime, Heptest® and aPTT

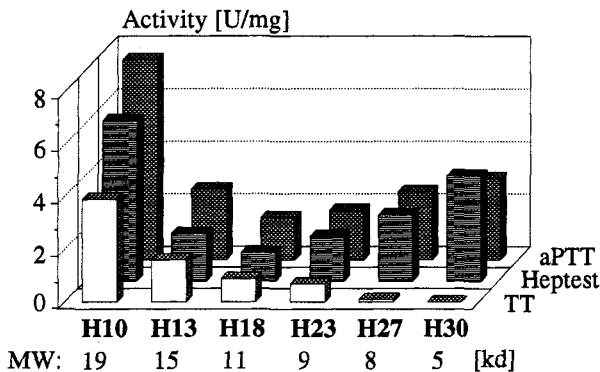
Anticoagulant activity of FraCS H (DS = 1.20)

Fig. 7 Anticoagulant activity of FraCS H-fractions with MW < 20 kDa in aPPT, Heptest® and Thrombintime

mainly in the Heptest® a rise in the specific activity was obvious. While the aXa/aIIa ratios for the MW range > 10 kd with values < 2 were considerably smaller than that of low sulfated FraCS L with very low TT-activity, a strong rise could be shown for MW < 10kd (H30: aXa / aIIa = 40).

## DISCUSSION

The physiological testing of the anticoagulant activities of these three curdlansulfates with DS of 0.60, 0.85 and 1.20 demonstrates that the increase in activity parallels with increasing DS values. When comparing compounds with similar DS it becomes obvious that an increase in MW results in bell shaped curve and a continuous decrease of the aXa/aIIa ratio. This demonstrates the direct influence of the chain length on the activity profile. The coagulation mechanism, which is responsible for the prolongation of the clotting time in the Heptest®, is favoured by short chain molecules. The TT anticoagulating effect, which is due to an interaction with the fibrin formation, needs a minimum chain length as well as a higher DS value. By the aPTT representing the complete intrinsic system, the sum of all the effects is covered which makes it clear that in this one the highest activities are being found. This was again shown with the non fractionated curdlansulfates where always aIIa/aPTT- and aXa/aPTT ratios of < 1 were found (12). It is possible that for the effects in the aPTT test system still other reaction mechanisms are responsible which can not be shown with the TT nor the Heptest®.

In conclusion one can postulate that  $\beta$ -1,3-glucansulfates interfere at several sites with the blood coagulation cascade. The individual activities are influenced by the MW of the tested compounds in a different way. For an inhibition of the thrombin dependent fibrin formation a minimal MW (> 30 kd) is essential.

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Comparing FraCS L- with the higher charged FraCS H-fractions, the latter were more active in general. Otherwise, they produced a similar picture with a maximal effect in the intermediate MW area between 40 and 60 kd and up to the fraction H10 (19 kd) the similar profile for the three test systems (Fig. 6). Starting from fraction H9 (63 kd) one could observe a rapid increase in activity of TT in parallel with aXa/aIIa values < 1. With other glucansulfates similar results could be obtained whereby (12) for a pronounced TT-activity (> 5 U/mg) first a DS > 0.75 is necessary and second a minimal chain length is essential.

With decreasing MW values the activity in all three test systems went down. In the case of curdlansulfates < 10 kd (Fig. 7), this tendency in the TT continued, whereas in the aPTT and