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# Preparation and anticoagulant activity of a low-molecular-weight sulfated chitosan

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**Abstract**

Synthesis of chitosan sulfates with low molecular weight ($M_v$ 9000–35,000 Da) was carried out by sulfation of low molecular weight chitosan ($M_v$ 10,000–50,000 Da). The oleum was used as sulfating agent and dimethylfornamide as medium. The chitosans were prepared by enzymatic and acidic hydrolysis of initial high molecular weight chitosan as well as by extrusion solid-state deacetylation of chitin. As was shown by FT-IR and NMR-methods and elemental analysis, the sulfation occurred at C-6 and C-3 positions and substitution degree is 1.10–1.63. The molecular weight sulfated chitosan was determined by viscometric method and the Mark–Houwink equation $[\eta] = 10^{-5}\ 4.97\ M^{0.77}$. Study of anticoagulant activity showed that chitosan sulfates with lowered molecular weight demonstrated a regular increase of anti-Xa activity like heparins.


*Keywords:* Chitosan; Sulfation; Anticoagulant activity

## 1. Introduction

Chitin is a natural cationic polysaccharide found particularly in the shell of crustacean, in the cell walls of fungi and the cuticles of insects. Chitosan, the fully or partially deacetylated form of chitin, consist of linear 1→4 linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Chitin and chitosan can be depolymerized by enzymes, including certain human enzymes, especially lyzozyme and are considered as biodegradable (Muzzarelli, 1997; Sashiwa et al., 2003). Chitosan also is known as biocompatible polymer. It has been proved to be invaluable material in the field of biomedical engineering and biotechnology (Arvanitoyannis, Kolokuris, Nakayama, Yamamoto, & Aiba, 1997; Arvanitoyannis, Nakayama, & Aiba, 1998; Berger et al., 2004; Kweon, Song, & Park, 2003). The chemical modification of the amino and hydroxyl groups can generate products for pharmaceutical applications, for example: sulfated chitosans possess a wide range of biological activities. Thus, chitosan sulfates (SCh) as the nearest structural analogues of the natural blood anticoagulant heparin, demonstrate anticoagulant, antisclerotic and antiviral activities (Desai, 2004; Drozd et al., 2001; Hirano, Tanaka, Hasegawa, Tobetto, & Nishioka, 1985; Nishimura et al., 1998; Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002).

As known, non-fractionated heparin with an average molecular weight ($M_v$) 5000–25,000 equally inhibits key enzymes of the blood coagulation cascade, namely serine proteases, such as thrombin (IIa factor) and Xa factor, while preparations of low molecular weight heparin ($M_v$ 5000–7000) exhibit a higher anti-factor Xa activity (aXa), owing to what their pharmacokinetic properties are improved compared to non-fractionated heparin (Rasine, 2001; White & Grinberg, 2003). The correlation between aXa and

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antithrombin (aIIa) activities is 4.0 (for dalteparin), 3.9 (for enoxiparin) and 1.5 (for logiparin) correspondingly, whereas for non-fractionated heparin this ratio is close to 1.

It is interesting to study the influence of a chitosan sulfate molecular weight onto its anticoagulant activity and to establish whether the correlation of a SCh molecular weight and the ratio of aXa/aIIa activity is similar for heparin and for its SCh analogues. As can be seen from the paper cited above (Hirano et al., 1985), N, O-chitosan sulfate with $M_v$ 12,000 has no the anti-factor Xa activity, while N, O-chitosan sulfates with a little bit larger molecular weight (19,000–71,000) do have this activity (Vongchan et al., 2002). Varying $M_v$ within this range does not influence on its level. A limited number and an inconsistency of data available in the literature require an additional study on an influence of a molecular weight of chitosan sulfate on its anticoagulant activity determined in the different tests.

Earlier we prepared low molecular chitosan sulfate using immobilized chitinolytic enzyme complex from *Streptomyces kurssanovii* by enzymatic hydrolysis of high molecular weight chitosan sulfate (Bannikova, Sukhanova, Vikhoreva, Varlamov, & Gal'braikh, 2002). However, its anticoagulant activity was rather low because of the low content of sulfated groups. In the present paper, low molecular weight SCh samples with higher degree sulfation (DS) have been synthesized by sulfation of low molecular weight ($M_v$ 10,000–50,000) chitosans, prepared by enzymatic (ChE) (Ilyina, Tatarinova, & Varlamov, 1999) and acidic (ChA) hydrolysis of initial high molecular weight chitosan, as well as by extrusion solid-state deacetylation of chitin (ChM) (Rogovina, Akopova, & Vikhoreva, 1998).

## 2. Materials and methods

### 2.1. Materials

Samples of low molecular weight ChE were prepared from commercial crab shell chitosan (Bioprogress, Russia), by depolymerization with *S. kurssanovii* (Ilyina et al., 1999). ChM were prepared from chitin of crabs by extrusion solid state deacetylation in conditions of the mechanical destruction (Rogovina et al., 1998). ChA samples were got from 'Echo' Co. (Russia). The deacetylation degree (DD) of chitosan samples was determined by potentiometric titration (Lim, Hattori, & Hadson, 2000), and an average molecular weight $M_v$ was measured by a viscometric method (Wang, Bo, Li, & Qin, 1991).

All commercial reagents were of analytical grade and were used without further purification. The following reagents were used for anticoagulant activity determination: Human Thrombin and Human Antithrombin III (Dade Behring), Heparin AccucolorTM (CRS 106), Berichrom Heparin (OWLD 11) Human antithrombin III, Chromogenic substrates 2238 (Unichrom Unttest ATIII) and 2222 (Behring), Heparin (Sigma).

### 2.2. Sulfation of chitosan

Sulfating complex was prepared in a reaction vessel by adding of small portions of oleum to a DMF excess (3–4 mol oleum and 29 mol DMF per 1 mono-mol of chitosan). The mixture was stirred vigorously at 0–5 °C. Then activated chitosan was added and sulfation was run at 60 °C and stirring for 1–3 h. The activation of chitosan was conducted by includation in DMF or re-precipitation and includation. The reaction mixture was cooled to 20–25 °C and a solid fraction was separated by centrifugation or by filtration through a glass filter. Crude chitosan sulfate was precipitated from solution in 5-fold acetone volume and washed with acetone twice, then dissolved in distilled water with simultaneous neutralization with a 20% NaOH solution. Then the chitosan sulfate Na-salt solution was purified and fractioned by size exclusion chromatography on Sephadex G-10, equilibrated in distilled water (a column $1.85 \times 25$ cm, flow rate 80 ml/h). Fractions of 2 ml were collected, assayed by conductometry and assayed by the 1,9-Dimethylmethylene Blue binding assay (Farndale, Buttle, & Barret, 1986) and then were freeze-dried.

### 2.3. General methods

A sulfur content was determined using the automatic analyzer CHNS EA1108 'Carlo-Erba'. FT-IR spectra were recorded on Spectrometer 'Nicolet' with a KBr disk. $^{13}C$ NMR spectra were recorded on a 'Bruker WP-200' spectrometer at 200 MHz in $D_2O$ with concentration of 0.2 g/ml and pH 5 for chitosan solution and 7.5 for chitosan sulfates, respectively. X-ray diffraction patterns were recorded using URS-55 with Cu Kα-radiation and a Ni-filter.

The average molecular weight of sulfated chitosans was determined by viscometric method using the Ubbelohde viscosimeter (with a diameter of 0.54 mm) and the Mark–Houwink equation: $[\eta] = 10^{-5}\, 4.97\, M^{0.77}$, (0.5 M NaCl, 25 °C) (Noreyka, Kolodzeykis, & Dugenas, 1985). Values of coefficients were determined for sulfated chitosan with 14–15% sulfur.

### 2.4. Anticoagulant assays

The anticoagulant activity of chitosan sulfate derivatives was determined in vitro by two assays, which characterized their ability to accelerate the inhibition of Xa factor and of IIa factor at the European Pharmacopoeia base. To determine chitosan sulfate aIIa and aXa activities, we prepared a series of solutions with various SCh concentrations, the first International standard for low molecular heparin NIBS code 85/600 and the fifth International standard for non-fractionated heparin NIBS code: 97/578. Then we selected the dependence having a linear correlation to the standards and determined the anticoagulant

Table 1
Characteristics of chitosan and sulfated chitosan in Na-salt form

| Number of SCh | Chitosan | | | Sulfated chitosan | | | |
|---|---|---|---|---|---|---|---|
| | Code | $M\times10^{-3}$/DP | DD | Yield (g/g) | $S$ (%)/DS | $m$[a] | $M\times10^{-3}$/DP |
| 1 | ChE | 10/60 | 0.90 | 0.40 | 13.9/1.30 | 297 | 10/34 |
| 2 | ChE[b] | 30/185 | 0.90 | 1.10 | 15.7/1.63 | 331 | 17/52 |
| 3 | ChE[b] | 50/300 | 0.90 | 0.86 | 15.6/1.60 | 328 | 25/75 |
| 4 | ChA[b] | 10/60 | 0.88 | 1.07 | 15.7/1.63 | 331 | 10/30 |
| 5 | ChA[b] | 10/60 | 0.88 | 1.00 | 14.6/1.42 | 310 | 9/30 |
| 6 | ChA[b] | 40/242 | 0.90 | 1.09 | 10.6/0.83 | 250 | 20/80 |
| 7 | ChM | 40/243 | 0.91 | 0.95 | 14.3/1.35 | 303 | 22/72 |
| 8 | ChM | 45/274 | 0.91 | 1.10 | 15.9/1.66 | 334 | 25/75 |
| 9 | ChM | 50/300 | 0.92 | 1.20 | 12.7/1.10 | 277 | 35/126 |

[a] $m$—weight of 1 mono-mol SCh calculated by $m=165+102$ DS.
[b] Initial chitosan before includation was re-precipitated.

concentration, which was appropriate one to international units, using standard statistical analysis methods.

Anti-factor Xa activities were determined by a modification of the Teien and Lie procedure (Teien, Lie, & Abildgaard, 1976). Human factor Xa (2.4 nkat/ml) was incubated with human antithrombin III (0.17 U/ml) and SCh or standard heparin (various concentrations) in Tris–HCl buffer (50 mmol/l) plus NaCl (175 mmol/l) at pH 7.4 and 37 °C for 2 min. To measure the residual factor Xa activity the S-2222, dissolved in $H_2O$ (4 mmol/l) was added. The increase of absorbance at 405 nm per minute was recorded.

Anti-factor IIa activities were determined according to Walenga (Hoppensteadt, Walenga, & Fareed, 1985). Human thrombin (0.5 U/ml) was incubated with human antithrombin III (1.0 U/ml) and SCh or standard (various concentrations) in Tris–HCl buffer (50 mmol/l) plus NaCl (175 mmol/l) at pH 7.4 and 37 °C for 2 min. To measure the residual factor IIa activity, the S-2238, dissolved in $H_2O$ (2 mmol/l) was added. The increase of absorbance at 405 nm per minute was recorded. All assays were performed in duplicate and repeated at least 3 times on different days.

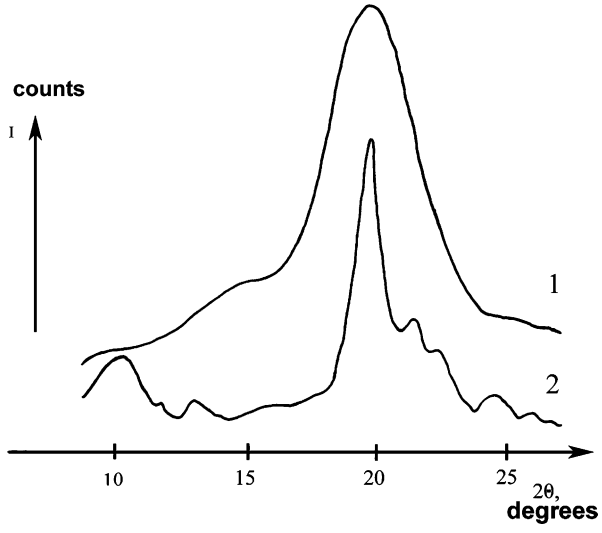


Fig. 1. X-ray diffraction of chitosans, obtained by extrusion method (1) and enzymatic hydrolysis (2).

## 3. Results and discussion

Characteristics of chitosans and chitosan sulfates in a Na-salt form are listed in the Table 1. It should be noted that the samples of low molecular weight chitosans differ in their reaction ability, which can be characterized by output of the soluble fraction of sulfated chitosans, and by the sulfation degree. Low molecular weight chitosan from a ChM batch did not require re-precipitation. High reaction ability of ChM associated with their highly amorphous structure, formed in conditions of extrusion deacetylation, and isolated by extraction with an organic solvent in the absence of water, which could cause polymer re-crystallization (Fig. 1). Chitosans ChE and ChA prepared by enzymatic and acidic hydrolysis had lower reaction abilities. For these samples the additional re-precipitation in ethanol was carried out, with following including in dimethylformamide, that enabled to obtain SCh samples number 2–6, with the output approximately 1 g of SCh per 1 g of chitosan, and a high degree of sulfation.

Sulfation of low molecular weight chitosans enabled to obtain chitosan sulfates with $M_v$ within the range of 9000–35,000. The equality $M_v$ of initial chitosan and obtained SCh did not mean the absence of polymer chain degradation during sulfation. For the evaluation of lowering polysaccharide $M_v$ resulted from degradation, the most proper

Table 2
Characteristics of SCh-8 fractions

| SCh-8 and fractions | $[\eta]$ (dl/g) | $M_v$ ($10^{-3}$) | DP | $S$ (%) | DS | Yield (%) |
|---|---|---|---|---|---|---|
| Initial | 0.12 | 25 | 75 | 15.9 | 1.65 | – |
| 1 | 0.18 | 42 | 122 | 16.2 | 1.72 | 12 |
| 2 | 0.14 | 30 | 80 | 16.0 | 1.70 | 35 |
| 3 | 0.12 | 25 | 75 | 15.8 | 1.65 | 21 |
| 4 | 0.07 | 15 | 45 | 15.7 | 1.63 | 16 |

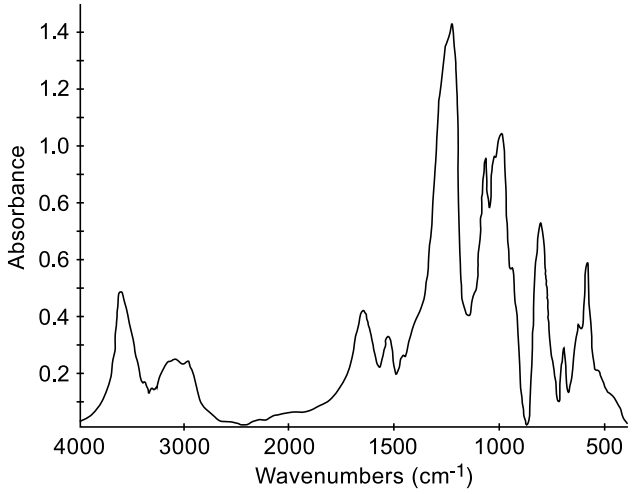


Fig. 2. FT-IR spectra of SCh-8.

way is the comparison of polymerization degree (DP) that falls 2–4 times (Table 1).

Despite the high activity of the ChM in sulfation, the attempts to obtain SCh with $M_v$ lower than 22,000 were unsuccessful. As for carrying out the biological tests samples with lower $M_v$ are needed, the sample SCh-8 (which was the higher sulfated one among all others obtain from ChM) was separated into fractions, and the fraction with $M_v$ 15,000 (DP = 45) was obtained with 16% output (Table 2). These results were obtained using the Sephadex G-10 column.

Additionally to elemental analysis the structure of chitosan sulfates was investigated with FT-IR-spectroscopy. There are specific absorption bands of sulfate groups in the area of 1200–1260 $cm^{-1}$, representing asymmetric valence fluctuations of $SO_2$, and the band of symmetric valence fluctuations at 1060 $cm^{-1}$; as well as there are bands of S–O bonds in the area of 580–625 $cm^{-1}$, and also valence fluctuations of C–OS in the area of 800 $cm^{-1}$ (Fig. 2).

The study of the polysaccharide structure by a NMR-spectroscopy has shown that all initial chitosans (as well as chitosan sulfates obtained from them) had practically identical spectra irrespective of the preparation method. The reference of C-atoms signals in NMR-spectra of polysaccharides is made in the correspondence with the data reported earlier (Gamzazade et al., 1997; Naggi, Torri, Compagnoni, & Casu, 1986). The magnitudes of chemical shifts in SCh-8 and SCh (Fig. 3), described in these papers

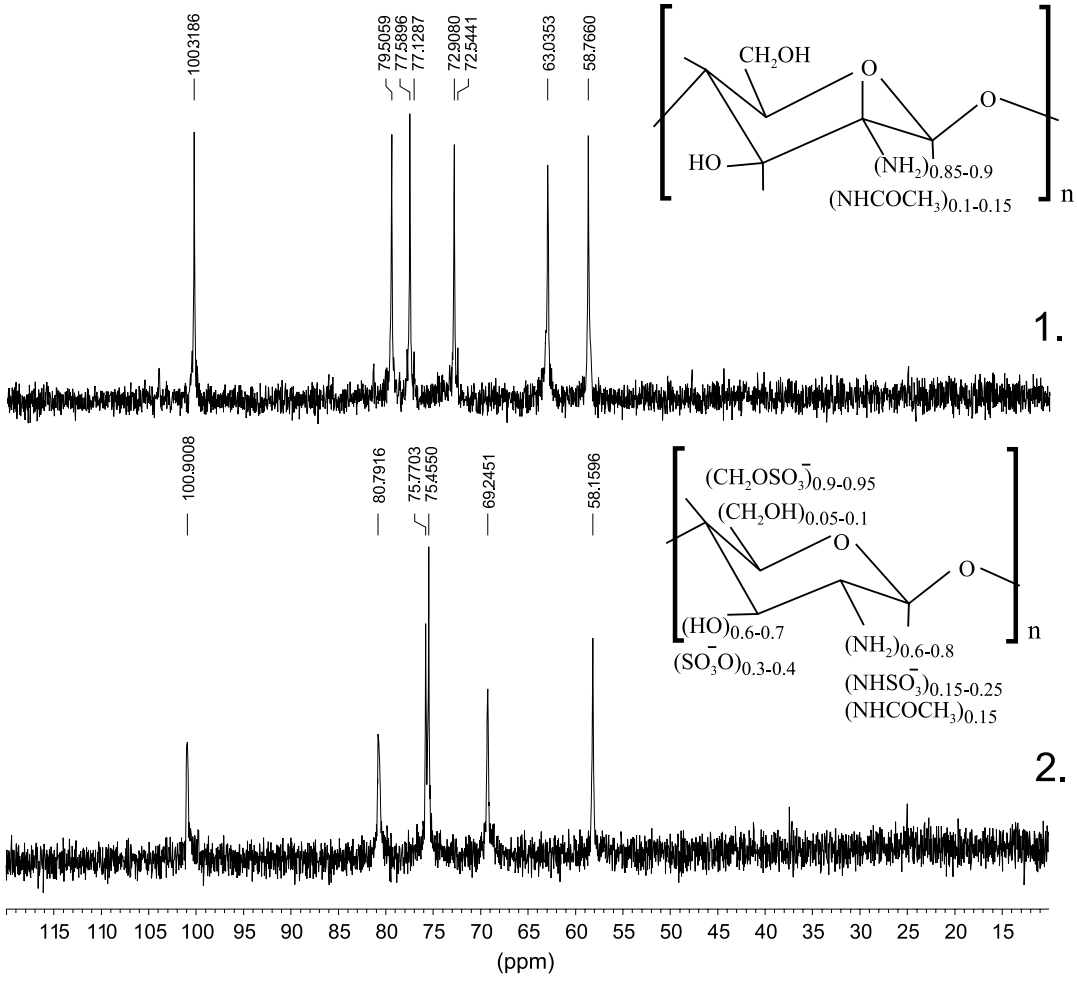


Fig. 3. $^{13}C$ NMR of chitosan (1) and sulfated chitosan (2).

Table 3
$^{13}C$ NMR data for sulfated derivatives of chitosan

| Compound | pD | Chemical shifts (ppm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | C5 | C6 |
| 6-*O*-mono sulfate (Naggi et al., 1986) | 5.0 | 99.0 | 57.8 | 75.3 | 78.5 | 75.8 | 69.2 |
| 3,6-di-*O*-sulfate (Gamzazade et al., 1997) | – | 98.0 | 56.7 | 77.2 | 74.9 | 74.4 | 68.4 |
| SCh-8 | 7.5 | 100.6 | 58.2 | 76.8 | 80.8 | 75.5 | 68.3 |

(Table 3), allow to draw a conclusion that obtained SCh contain 6-*O*-mono, and 3,6-di-*O*-sulfated links, and therefore are non-selectively but mainly substituted at the C-6 primary OH-groups. The stability of the signals from C-2 confirms the low sulfation of aminogroups in synthesized chitosan sulfates samples. Some displacement of C-1 and C-4 atoms indicate the conformation change of elementary chain links and the whole chain, most likely as a result of transition from protonated form of amino groups to non-protonated one, as well as a result of appearance of sulfated groups.

Heparin and chitosan sulfate are anticoagulants of direct action and prevent from activation of blood coagulation both in vivo, and in vitro. Earlier we demonstrated that sulfated chitosan derivatives, as well as non-fractionated heparin, accelerate thrombin inactivation, forming an equimolar complex with antithrombin III (Drozd, Bashkov, Makarov, Kheilomskii, & Gorbachiova, 1992; Drozd et al., 2001). Serine proteases play an important role in thrombogenesis, a process leading to thrombus formation. In the blood coagulation cascade, the consecutive activation of various serine proteases favors synthesis of serine protease and Xa factor playing the central role in the coagulation process. Xa factor is an important ingredient of prothrombinase complex, from which thrombin is formed; the latter further directly affects the thrombin formation process. Therefore both synthesis and inhibition of Xa factor are important for the strategy of development of new antithrombotic drugs (Hoppensteadt et al., 1985; Walenga, Jeske, Hoppensteadt, & Fareed, 2003).

Anticoagulant activity of chitosan sulfates was determined according to their ability to inhibit IIa and Xa factors. According to the data presented in Table 4, the SCh fraction with $M_v$ 30,000, produced from initial high molecular weight chitosan, as well as SCh-8 and its fractions with molecular weight 25,000 and 35,000, possess a very small ratio of aXa/aIIa activities. It may be correlated to relatively high average molecular weight of preparations and a low content of low molecular weight fractions.

Table 4
Anticoagulant activity of sulfated chitosans

| Number SCh and fraction | $M_v$ ($10^{-3}$) | *S* (%) | aXa (U/mg) | aIIa (U/mg) | aXa/aIIa |
|---|---|---|---|---|---|
| SCh-30[a] | 30 | 16.7 | 2.3 | 37 | 0.06 |
| SCh-8 | 25 | 15.8 | 2.2 | 13 | 0.17 |
| Fraction-4 | 15 | 15.7 | 7.6 | 10 | 0.76 |
| SCh-1 | 10 | 13.9 | 4.3 | 10 | 0.43 |
| SCh-4 | 10 | 15.7 | 64.0 | 13 | 4.9 |
| SCh-5 | 9 | 14.6 | 57.0 | 14 | 4.0 |

[a] Fraction of SCh prepared from initial high molecular weight chitosan.

However, even decreasing molecular weight to 10,000 of SCh-1, does not allow to get a high level of aXa activity. This could be explained by its rather low degree of sulfation. And only SCh with average molecular mass 8000–10,000 and DS 1.4–1.6 obtained from chitosans ChA, and obviously, more enriched with low molecular weight fractions, demonstrate rather high aXa activity, which exceeded antithrombin activity 4–5 times. It cannot be excluded that for manifestation of overall high and prevailing aXa activity, the preparations at given average $M_v$ should contain fractions with various molecular weight in a certain proportion.

Sulfated chitosans derivatives with such high aXa activity are not described in literature.

A comparative investigation of the SCh ($M_v$ 19,000–71,000) and pentosan polysulfate ($M_v$ 1500–2000), conducted by Vongchan et al. (2002) with using a non-fractionated heparin ($M_v$ 5000–25,000) as standard has allowed to reach a conclusion about identity of the mechanism of an anticoagulant action of all these preparations, despite of essential distinction of their molecular weights. From this paper it is not clear also what ratio of aXa/aIIa activities, so important for practical using have the prepared SCh.

In general, for heparin and chitosan sulfates samples the increase of aXa activity at decreasing the molecular mass is trend. Thus, the correlation coefficient between aXa activity and molecular weight is $r = -0.84$, and the alteration in the sulfur content. For aIIa activity a positive correlation was found with the molecular mass ($r = 0.74$).

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