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Article

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Structural Characteristics, Stability, and Anticoagulant Activity of *Bombyx mori* Chitosan Sulfate

Chitosan sulfate (CS) is a promising polyelectrolyte for biomedical applications due to its anticoagulant properties. However, the relationship between the synthesis conditions of CS derived from *Bombyx mori* silkworm chitosan, its structural characteristics, and its biological activity remains insufficiently studied. CS samples with varying degrees of substitution (DS) were synthesized via sulfation with chlorosulfonic acid at different temperatures (50, 60, and 80 °C). The structure was characterized using FTIR spectroscopy and X-ray diffraction analysis. The physicochemical properties were evaluated through water vapor sorption, rheology, and solution stability tests conducted over 30 days. The anticoagulant activity of CS was assessed *in vivo* using a rabbit model of hypercholesterolemia. Successful sulfation resulted in DS values ranging from 0.96 to 1.21, leading to significant amorphization of the polymer structure. All CS samples exhibited high hydrophilicity with sigmoid-shaped sorption isotherms and demonstrated typical polyelectrolyte rheological behavior. Aqueous solutions of CS remained stable throughout the observation period. Importantly, the CS sample with the highest DS (1.21) showed the most pronounced anticoagulant effect, reducing platelet aggregation by 20 % compared with the heparin control group. The sulfation temperature is a key parameter determining the DS and, consequently, the properties of *Bombyx mori* chitosan sulfate. The derivative with DS = 1.21 demonstrates anticoagulant activity comparable to heparin, highlighting its potential as a bioactive material. Further research should focus on elucidating the precise molecular mechanisms of its anticoagulant action and evaluating its long-term biocompatibility and efficacy *in vivo*.

Keywords: chitosan sulfate, *Bombyx mori*, degree of substitution, IR spectroscopy, X-ray structural analysis, water sorption, rheology, anticoagulant activity

Introduction

Chitosan and its modified derivatives are of significant interest in the field of biomaterials due to their biocompatibility, low toxicity, and ability to biodegrade [1–5]. Special attention is paid to sulfated chitosan derivatives, which combine polyanionic properties with high hydrophilicity, expanding their potential applications [6–16]. These derivatives exhibit a range of biological activities, including antimicrobial, anticoagulant, and immunomodulatory properties, making them promising candidates for the development of new drugs [17–27].

A promising source for obtaining chitosan is the chitin of *Bombyx mori* silkworm pupae [28–32]. Sulfation of chitosan allows for the production of a polyampholyte that contains both basic (amino) and highly acidic (sulfate) groups in its structure. This gives the derivative unique properties: water solubility over a wide pH range, high charge density, and the ability to interact electrostatically, which determines its biological activity and low toxicity [33–40].

Despite active research in this area, the relationship between the synthesis conditions (such as the sulfation temperature), the degree of substitution, the supramolecular structure, the physical and chemical properties, and the biological activity of *Bombyx mori* chitosan sulfate has not been sufficiently studied. In particular, the effect of the degree of substitution on structural and rheological characteristics, sorption behavior with respect to water, and solution stability requires detailed analysis. In addition, it is necessary to assess the anticoagulant potential of such derivatives under conditions as close to physiological as possible.

This work aimed to establish a relationship between the synthesis conditions, structure, properties, and biological activity of *Bombyx mori* chitosan sulfate.

The work was aimed at synthesizing a series of chitosan sulfate samples with varying degrees of substitution by changing the reaction temperature, studying their chemical and supramolecular structure using IR-Fourier spectroscopy and X-ray diffraction analysis, and investigating the dependence of their sorption characteristics on water vapor and the rheological properties of their solutions. In addition, it was planned to evaluate the stability of aqueous solutions of the obtained derivatives and study their anticoagulant activity in an animal experiment with a model of hypercholesterolemia.

Experimental

Chemicals and Materials

Objects and Methods of Research

Chitosan obtained from chitin isolated from the pupae of the silkworm *Bombyx mori* was used as the main object for sulfation according to the methods [13, 40]. Its characteristics include degree of deacetylation, solubility, and molecular weight. Chlorosulfonic acid (CAS 7790-94-5), dimethyl sulfoxide (CAS 67-68-5), sodium acetate (CAS 127-09-3), sodium chloride (CAS 7647-14-5), sodium hydroxide (CAS 1310-73-2).

Synthesis of Chitosan Sulfate (CS)

Sulfation was carried out with chlorosulfonic acid in dimethyl sulfoxide medium by a modified technique [13] at temperatures of 50, 60, and 80 °C. The obtained samples are designated as CS (I), CS (II), and CS (III), respectively.

Determination of the Degree of Substitution (DS)

The degree of substitution by sulfonic groups was determined by titration on an EC 215 conductivity meter (Hanna Instruments, Germany) according to the method [35, 41]. The calculated values of the CX were: 1.12 (CX I), 1.21 (CX II), 0.96 (CX III). The non-monotonic dependence of the *DS* on temperature (maximum at 60 °C) can be explained by the competition between the sulfation processes and the possible degradation of the polymer chain at elevated temperatures (80 °C).

IR Spectroscopic Studies (FTIR)

Spectra were recorded on the Invenio-S Fourier spectrometer (Bruker, Germany) in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The samples were prepared as tablets with KBr [42]. The spectrum of native *Bombyx mori* chitosan was used for comparison [43, 44].

X-Ray Diffraction Analysis (XRD)

Diffraction measurements were performed on a Miniflex 600 diffractometer (Rigaku, Japan) with CuKα-radiation (λ = 1.5418 Å, 40 kV, 15 mA) in the Bragg-Brentano mode in the range of angles 2θ = 2–40° [45].

Investigation of Water Vapor Sorption

Sorption-desorption isotherms of water vapor were obtained on a high-vacuum unit with a McBain quartz balance (similar to the gravimetric method) at temperatures of 20, 25, and 30 °C (±0.1 °C) and residual pressure 10^{-3}–10^{-4} Pa [46]. The samples (~100 mg) were pre-dried to a constant mass at 50 °C. The equilibrium moisture content was determined by stepwise changes in relative humidity (p/p_0 from 0.05 to 0.95) [47]. The criterion for achieving equilibrium at each stage was the constant mass of the sample over a period of 2 hours [48]. The classical BET (Brunauer–Emmett–Teller) analysis, which is used to process data in the range of p/p_0 = 0.05–0.35 and is represented by formulas (1–4), has limitations in its applicability to hydrophilic polymer systems where swelling and bulk dissolution of the sorbate are possible [49]. Therefore, the obtained parameters (monolayer capacity, specific surface area, etc.) should be considered as effective, comparative characteristics.

Rheological Studies

The measurements were carried out on an MCR 92 rheometer (Anton Paar, Austria) in a shear mode in a system of coaxial cylinders for NaCl 0.1 N solutions and in an oscillation mode with parallel plates. The temperature range is 20–45 °C [50].

Conductometric Titration and Calculation of the Degree of Substitution

The quantitative characteristics of sulfogroup substitution were determined by titration on an EC 215 conductometer (Hanna Instruments, Germany) [51]. The change in electrical conductivity (*G*) was controlled

as a function of the volume (V) of the added titrant NaOH 0.5 N to the solutions of CS in HCl 0.1 N. The content of sulfur (ω_S) and nitrogen (ω_N) was calculated using the formulas [41, 35]:

$$\omega_S = \frac{km_S\Delta V_S}{m_{CS}}, \quad \omega_N = \frac{km_N\Delta V_N}{m_{CS}}, \tag{1}$$

where k is the normality of the titrant; m_S and m_N are molecular weights of sulfur and nitrogen; ΔV_S and ΔV_N are titrant volumes used for titration of sulfo- and amino groups, respectively; m_{CS} is the weight of the CS suspension.

The degree of substitution of sulfogroups (γ_S) was calculated using the formula:

$$\gamma_S = \frac{168.4 * \omega_S}{3200 - 81\omega_S}. \tag{2}$$

Determination of the Molecular Weight and Stability of Solutions

The characteristic viscosity (η) and molecular weight (M_η) were determined viscometrically using a Ubbelode viscometer in a water + 2 % NaCl system at 25 °C using the Mark-Kuhn-Hauvink equation [52–55]:

$$\eta = 4.97 \times 10^{-5} M_\eta^{0.77} \text{ dl/g}. \tag{3}$$

The stability of the solutions was assessed by changes in relative viscosity:

$$\eta_{отн} = \frac{\tau_i}{\tau_0}, \tag{4}$$

where τ_i is the expiration time of the solution, $\tau_0 \approx 92.8$ s is the expiration time of water for 30 days of storage at room temperature.

Investigation of Anticoagulant Activity In Vivo

Experiments were performed on male Chinchilla rabbits (n = 10 per group) with a model of atherosclerosis induced by an atherogenic diet [56]. CS samples were administered intragastrically at a body weight 5 mg/kg dose daily for 30 days. The control drug was heparin. At the end of the course, platelet aggregation, thrombin time, and the level of soluble fibrin-monomer complexes (RFMC) were determined on a Human analyzer (Germany). All procedures are performed in accordance with ethical standards [57].

Statistical Analysis

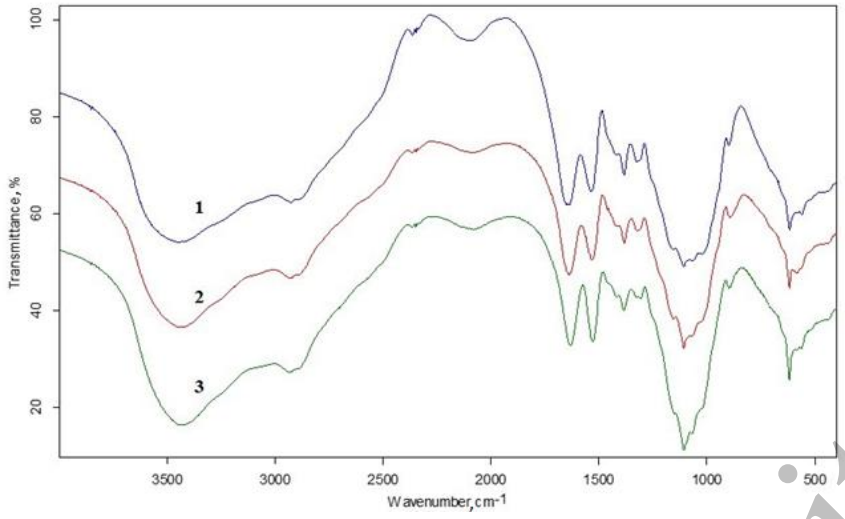
All experimental data were collected in triplicates and the data were expressed as average ± standard deviation. Data were compared using a one-way ANOVA with post-Bonferroni test using GraphPad Prism 5.04 (GraphPad Software Inc.)

Results and Discussion

IR Spectroscopic Analysis

The IR spectrum of the native *Bombyx mori* chitosan [40, 48] shows a wide absorption band in the 3000–3600 range with a maximum of about 3444, characteristic of valence vibrations and groups involved in hydrogen bonds. The bands at 2920 and 2980 correspond to the valence vibrations of the bonds. The characteristic bands of amide-I and amide-II are located at 1655 and 1591, respectively.

New intense absorption bands are observed on the IR spectra of all sulfated samples (Fig. 1). The appearance of bands in the region of 898, 1106, and 1156 corresponds to valence fluctuations of bonds and sulfate groups, which is direct evidence of successful sulfation. The key change is the shift of the amide-II band from 1591 (native chitosan) to the region of 1530–1533 for all samples of CS. This shift indicates substitution mainly by the amino group at the C-2 atom of the glucosamine unit, which is consistent with the literature data.



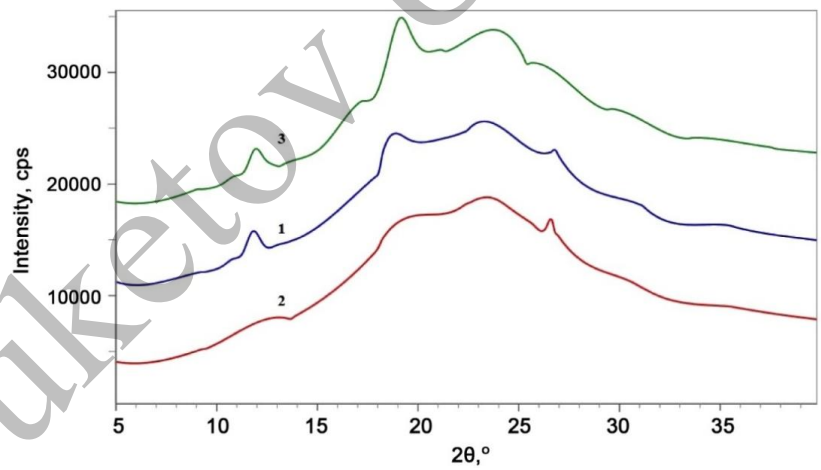
1 — CS (I-50 °C); *2* — CS (II-60 °C); *3* — CS (III-80 °C)

Figure 1. The IR Fourier spectra of the samples

The intensity of the new band in the 1530 region correlates with the degree of substitution. The highest intensity is observed for the CS (II) sample (DS = 1.21), which confirms the maximum content of sulfogroups. For the CS (III) sample (DS = 0.96), the intensity of this band is lower, which is consistent with the results of conductometric titration.

X-Ray Diffraction Analysis (XRD)

Diffractograms of the CS samples (Fig. 2) show significant changes in the supramolecular structure compared with native chitosan. Native chitosan is characterized by intense reflexes at 2θ ≈ 10° and 20° (corresponding to interplane distances and *d* ≈ 8.8Å and 4.4Å), indicating its high crystallinity [41].



1 — CS (I-50 °C); *2* — CS (II-60 °C); *3* — CS (III-80 °C)

Figure 2. Diffractograms of samples

As a result of sulfation, significant amorphization of the structure occurs. Weak and blurred reflexes are observed for the sample of CS (I) (DS = 1.12). The most intense maximum at 2θ = 21.40° (*d* = 4.15Å) can be attributed to the reflex (130) of the hydrated form of chitosan. This indicates the preservation of residual crystallinity due to the ordered packing of polymer chains on a nanometer scale (inter-chain distances). The CS (II) sample (DS = 1.21) is characterized by the most diffuse diffraction pattern with a wide amorphous halo (maximum about 2θ = 20.5°, *d* ≈ 4.3Å). This indicates the maximum destructurization of crystalline regions caused by a high degree of substitution, which disrupts the regularity of macromolecule packaging. The diffractogram of the CS (III) sample (DS = 0.96) shows a greater number and higher intensity of reflexes at 2θ = 11.96° (*d* = 7.39Å), 16.75° (*d* = 5.29Å), 19.04° (*d* = 4.66Å), etc. The increase in the degree of

crystallinity at a lower DS is explained by the fact that the replaced chains retain a greater similarity to the original structure of chitosan and are capable of more orderly packing.

The data obtained allow us to conclude that the sulfation process leads to significant amorphization of chitosan, and the degree of structurization of the crystalline phase directly correlates with the degree of substitution by sulfate groups.

Investigation of Sorption Characteristics

The isotherms of water vapor sorption by samples of CS obtained at temperatures 20, 25, and 30 °C are shown in Figures 3–5. All isotherms have the classic sigmoid shape typical of hydrophilic polymer materials. This shape indicates a complex multi-stage process, including initial monolayer adsorption on active centers, subsequent multilayer coating, and, at high relative humidity ($p/p_0 > 0.7–0.8$), capillary condensation in mesopores and the process of volumetric swelling of the polymer matrix.

The influence of the degree of substitution (DS). Samples with different DS demonstrate similar qualitative behavior, but differ in absolute sorption capacity. It is interesting to note that the samples with the most different DS — CS (II) (1.21) and CS (III) (0.96) — show similar sorption values, especially in the region of high humidity ($p/p_0 > 0.7$). This can be explained by the balance of two competing factors: 1) a higher concentration of hydrophilic sulfogroups in CS(II) increases water resistance; 2) the more amorphous structure of the same sample (according to XRD, Section 3.2) facilitates penetration and swelling, while the more ordered structure of CS(III) may limit this process. The CS (I) sample with an average CS = 1.12 occupies an intermediate position.

Effect of Temperature

In the entire studied range (20–30 °C), an increase in temperature leads to a decrease in the equilibrium water content at a fixed relative humidity for all samples. This dependence is characteristic of a process dominated by exothermic interactions, such as the hydration of ionic sulfonic groups and the formation of hydrogen bonds [58]. The observed minimum sorption capacity at 25 °C for a number of samples (Table 1) may be related to changes in the flexibility of polymer chains and the availability of sorption sites in this temperature range, but data obtained at only three temperatures are insufficient for a definitive conclusion about thermodynamic parameters (sorption enthalpy).

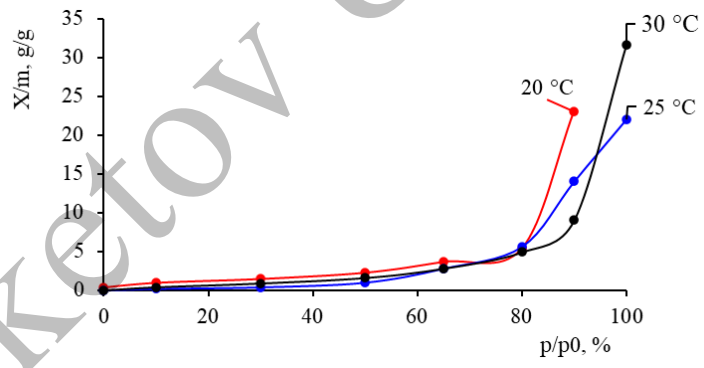


Figure 3. Sorption isotherms of water vapor by the CS (I) sample at 20, 25, and 30 °C

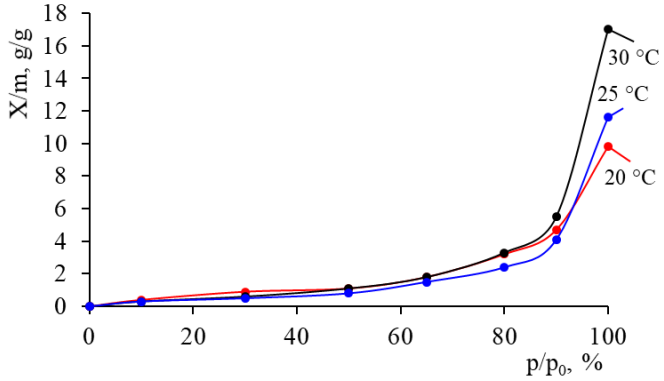


Figure 4. Sorption isotherms of water vapor by the CS (II) sample at 20, 25, and 30 °C

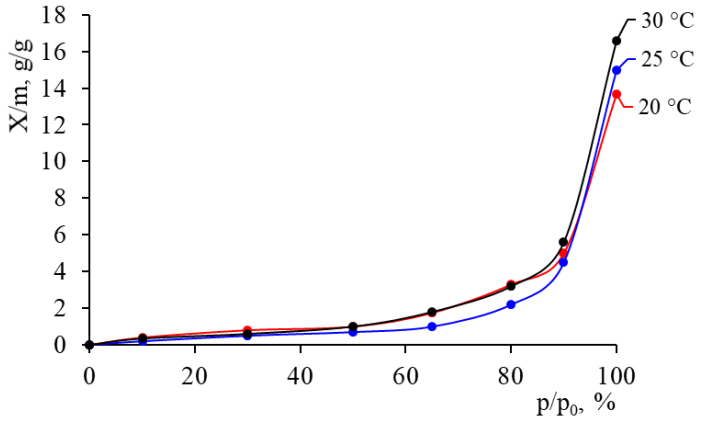


Figure 5. Sorption isotherms of water vapor by the CS (III) sample at 20, 25, and 30 °C

Notes on Data Analysis

In the original version, the BET equation was used to process isotherms in the range of relative pressures 0.05–0.35, and effective parameters (monolayer capacity, specific surface area, etc.) were calculated based on this equation. These parameters are presented in Table 1.

Table 1

Effective parameters of water vapor sorption for CS samples calculated using the BET model in the range p/p_0 = 0.05–0.35

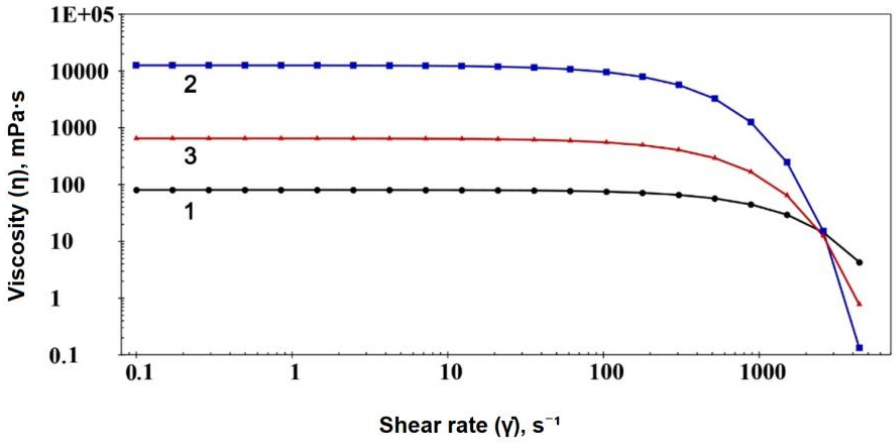
Sample	Temperature, °C	X_m, g/g	SSA, m^2/g	W_o, cm^3/g	r_k, Å
CS (I)	20	0.0056	19.81	0.23	232.2
	25	0.0036	12.67	0.22	347.4
	30	0.0058	20.54	0.32	307.7
CS (II)	20	0.0051	17.84	0.10	109.9
	25	0.0041	14.28	0.17	238.1
	30	0.0035	12.32	0.12	188.4
CS (III)	20	0.0048	16.76	0.14	163.5
	25	0.0028	9.79	0.15	306.4
	30	0.0042	14.80	0.17	224.3

It should be emphasized that the classical BET theory is designed for adsorption on impermeable surfaces and does not take into account the possibility of sorbate penetration into the polymer volume (swelling), which is typical for hydrophilic systems [59, 60]. Therefore, the obtained numerical values should be interpreted with caution, considering them not as absolute characteristics of the porous structure, but as comparative parameters reflecting changes in the availability of sorption centers and affinity for water for different samples of CS. To correctly determine the specific surface area and porosity parameters of dry samples, nitrogen sorption is required, and small-angle X-ray scattering (SAXS) is required to study the structure in the swollen state.

Thus, the conducted studies confirm the high hydrophilicity of all synthesized chitosan sulfate samples due to the presence of ionic sulfonic groups. The sorption capacity is complexly dependent on the degree of substitution, which determines the chemical nature of the polymer, and the supramolecular structure, which affects the kinetics of swelling.

Rheological Properties of CS Solutions

A study of the rheological properties of CS solutions (0.1 N NaCl) showed their pronounced non-Newtonian behavior. The dependence of the effective viscosity (η) on the shear rate gradient (γ) for all samples (Fig. 6) demonstrates the shear (pseudoplasticity: viscosity decreases with increasing strain rate) characteristic of dilute electrolyte solutions. This is due to the destruction of the temporary grid of hydrogen and electrostatic bonds and the deformational ordering of macromolecules in the flow.



1 — CS (I, DS = 1.12); *2* — CS (II, DS = 1.21); *3* — CS (III, DS = 0.96)

Figure 6. Dependence of the effective viscosity (η) on the shear rate gradient (γ) for chitosan sulfate solutions in 0.1 N NaCl at 25 °C

The effect of the degree of substitution is clearly visible: as the degree of substitution increases, the viscosity of the solution increases in the low shear gradient region. The highest viscosity values were shown by the CS (II) sample with a maximum DS of 1.21, while the CS (III) sample with a minimum DS of 0.96 had the lowest viscosity. The increase in viscosity with increasing SC is due to an increase in the charge density on the macromolecule, which leads to increased electrostatic repulsion between the chains, resulting in a more extended conformation in solution and, consequently, more intense intermolecular interactions.

Solubility and Stability of Solutions

The substitution of amino and hydroxyl groups with sulfate groups resulted in the production of water-soluble derivatives. As can be seen from Figure 7, the solubility (S) of the samples in water at 25 °C increases sharply when the degree of substitution ($\gamma_s \approx 0.85$) reaches the threshold value and exceeds 95 % for samples with $\gamma_s > 0.85$. This confirms the successful synthesis of hydrophilic derivatives and is consistent with the well-known principle that the introduction of highly hydrated ionogenic groups dramatically improves the water solubility of polysaccharides.

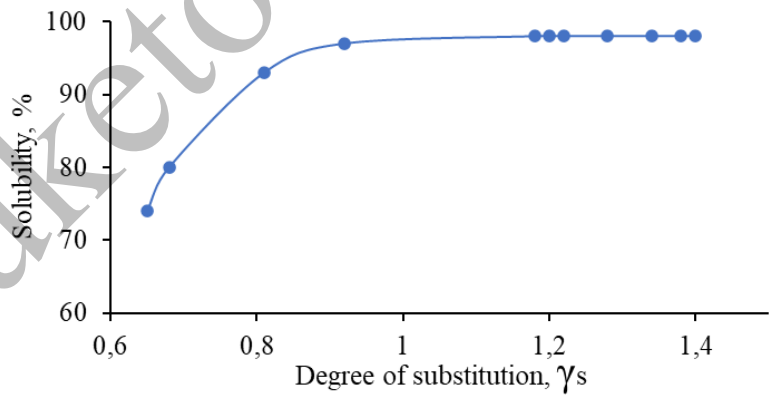
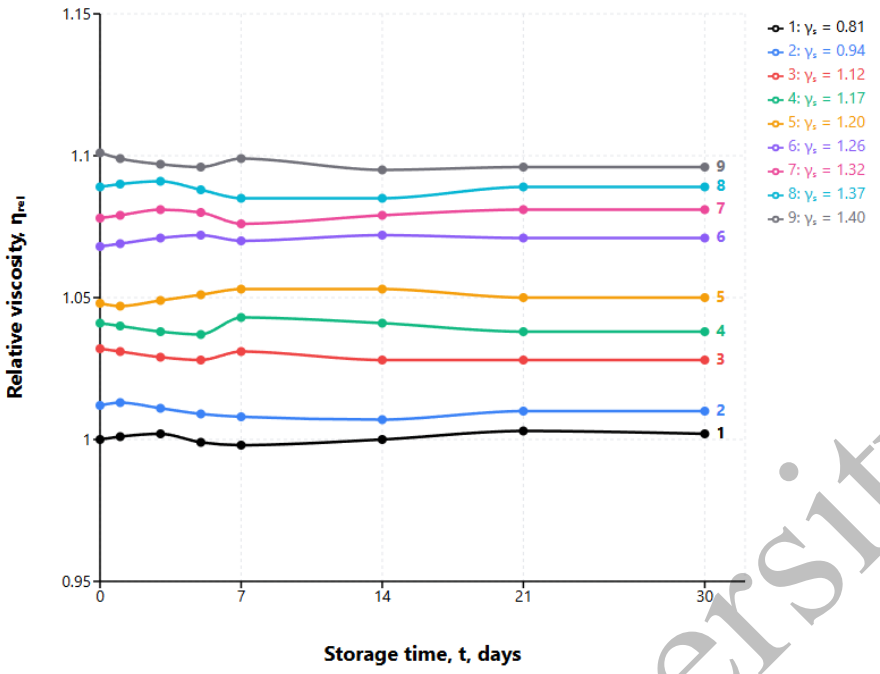


Figure 7. Dependence of the solubility (S) on the degree of substitution of sulfonic groups (γ_s) of chitosan sulfate samples in water at 25 °C

The stability of diluted aqueous solutions (Debye criterion $C[\eta] \leq 0.5$) of CS with different γ_s (from 0.81 to 1.40) was monitored for 30 days by measuring the relative viscosity (η_{rel}). As shown in Figure 8, the initial values η_{rel} correlate with the degree of substitution. During the entire observation period, the values for η_{rel} in all nine samples remained almost constant.



γ_s: *1* — 0.81; *2* — 0.94; *3* — 1.12; *4* — 1.17; *5* — 1.20; *6* — 1.26; *7* — 1.32; *8* — 1.37; *9* — 1.40

Figure 8. Dependence of the relative viscosity (η_{rel}) on the storage time (t) for aqueous solutions of chitosan sulfate samples with different γ_s

The absence of a decrease in viscosity indicates that there is no noticeable destruction or aggregation of the CS macromolecules in aqueous solutions at room temperature, which confirms their high colloidal stability.

Anticoagulant Activity in Animal Experiments

The anticoagulant potential of the CS samples was evaluated in an animal experiment (*in vivo*) using a hypercholesterolemia model in rabbits. The results of the effect of 30-day oral administration of CS on key hemostasis indicators are presented in Table 3.

Table 3

Effect of chitosan sulfate samples on hemostasis parameters in rabbits with experimental atherosclerosis (M±SD, *n* = 10)

Indicator	Heparin	CS-I (DS = 1.12)	CS-II (DS = 1.21)	CS-III (DS = 0.96)
Platelet aggregation, %	30.0 ± 0.58	31.33 ± 2.74	24.0 ± 1.15	36.33 ± 1.96
Thrombin time, with s	14.02 ± 0.00	14.08 ± 0.00	13.83 ± 0.17	14.11 ± 0.00
Soluble fibrin-monomer complex, mg %	3.04 ± 0.09	3.65 ± 0.93	3.02 ± 0.06	3.62 ± 0.14
Note: $p < 0.05$ compared to the group that received heparin				

The most pronounced effect was demonstrated by the CS (II) sample with a maximum DS of 1.21. Its administration led to a statistically significant 20 % reduction in platelet aggregation compared to the group that received heparin ($p < 0.05$). The samples of CS (I) and CS (III) also exhibited anticoagulant activity, which did not differ significantly from heparin in terms of the parameters studied. Thus, the anticoagulant effect of *Bombyx mori* chitosan sulfate depends on the degree of substitution, reaching a maximum at DS = 1.21.

Conclusions

As a result of the study, a relationship was established between the synthesis conditions, the degree of substitution (DS), the structural features, the physical and chemical properties, and the biological activity of

chitosan sulfate (CS) obtained from the chitin of the *Bombyx mori* silkworm. It has been shown that the sulfation temperature (50, 60, 80 °C) is a key parameter that determines the DS (0.96–1.21), which in turn has a significant impact on the properties of the polymer.

The successful introduction of sulfate groups and the amorphization of the polymer structure as a result of the sulfation reaction were confirmed using IR-Fourier spectroscopy and X-ray diffraction analysis. The study of water vapor sorption revealed the high hydrophilicity of all CS samples and the typical sigmoid shape of the isotherms, which is characteristic of hydrophilic polymers. Rheological measurements confirmed the poly-electrolyte behavior of aqueous solutions of CS. Good stability of the studied solutions during 30 days was established. The most significant result of the work is the proof of the pronounced anticoagulant activity of the synthesized chitosan sulfate with a high degree of substitution (DS = 1.21). In an experiment using an animal model of hypercholesterolemia, it was shown that this sample is comparable to heparin in terms of its effect on reducing platelet aggregation.

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Vazira Norqulovna Rakhmanova** investigation, validation, visualization, writing-original draft, **Svetlana Mihaylovna Yugay** investigation, methodology, formal analysis; **Rakiya Yunusovna Milusheva** conceptualization, data curation, investigation, writing-review & editing, **Sirojiddin Shamsutdinovich Shakhabutdinov** investigation, methodology, formal analysis, **Nurbek Shodievich Ashurov** conceptualization, data curation, investigation, methodology, visualization, writing-original draft, writing-review & editing; **Khumoyunmirzo Adakhamjon o'g'li Gulomjonov** investigation, methodology, formal analysis, **Abdumutolib Abdupatto o'g'li Atakhanov** conceptualization, data curation, formal analysis, validation, writing-review & editing; **Sayyora Sharafovna Rashidova** conceptualization, supervision, editing.

Conflict of Interest

The authors declare no conflict of interest.

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