

Research Article

Influence of Physical and Chemical Modification on the Optical Rotatory Dispersion and Biological Activity of Chitosan Films

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The optical and bactericidal properties of acetic and basic chitosan films were studied. By the ORD technique, we found that these films differed in the values of their specific optical rotation $[\alpha]$ and of their rotary and dispersive constants. A sign inversion of $[\alpha]$ was observed when the acetic chitosan films were heat-treated. The bactericidal activity of the initial and dehydrated acetic films was analyzed, and their moisture content and optical and biological activities were compared.

1. Introduction

The aminopolysaccharide chitosan is an optically active (chiral) biopolymer. The presence of asymmetrically substituted carbon atoms (chiral centers) in chitosan molecules leads to the emergence of optical activity in its link and, hence, in the macromolecule as a whole.

Optical rotatory dispersion (ORD) and circular dichroism (CD) spectroscopy techniques are commonly applied to studying optical activity. They are based on unequal changes in the phase and intensity (the polarization vector module) of the left and right circularly polarized waves (their factors of refraction and extinction) in optically active media.

CD spectroscopy is used to estimate the deacetylation degree of chitosan and its derivatives [1, 2], to characterize their physical and chemical properties [3, 4], to study liquid crystals (LC) of chitosan dispersions with nucleic acids [5] and lyotropic LC solutions of chitosan derivatives [6], and to establish the nativity degree of biologically active substances encapsulated into a polysaccharide matrix [7]. ORD is useful in estimating conformational properties of chitooligomers [8, 9], peculiarities of the spatial structure of chitosan and its salts with chiral organic acids [10], and so forth.

Comparison of the capabilities of the ORD and CD techniques shows that within the wavelength range outside of

the absorption bands of the chromophores (which, for the given aminopolysaccharide, falls within the far UV range), optical rotatory spectroscopy in some cases yields more information on optical activity and its correlation with other physical and chemical parameters of chitosan. For example, fairly recently, the ORD technique was used to reveal a correlation between chitosan's optical activity and its molecular weight and deacetylation degree [11, 12], as well as to clarify the character of its biological activity [13]. The influence of metal ions (Ca^{2+} , K^{+} , and Na^{+}) on macromolecular conformation was also shown [14].

It should be noted that all the previously-cited works have dealt with chitosan solutions and that no data on the optical activity of films made of this aminopolysaccharide have been found by us in the available literature. But such properties of chitosan films as their solubility in aqueous media, sorption-diffusion and physicochemical characteristics, and selectivity at separation of liquid mixtures are known to depend on the chemical form of the polymer [15, 16], the temperature and duration of heat treatment [16, 17], and other factors.

In view of these circumstances, the purpose of this work was to study the influence of physical and chemical modifications, namely, the polysalt-polybase transition and heat treatment, on the ORD and biological activity of chitosan films.

2. Experimental

Samples of powdered chitosan with different viscosity-averaged molecular weights (\overline{M}_η) and with similar deacetylation degrees were used (BioProgress Corp., Russian Federation). The physical and chemical characteristics of the samples are presented in Table 1. These chitosan samples were chosen for study because the optical activity of their 0.5% solutions in 2% acetic acid was almost the same (Figure 1(b)) [11]. Films made from chitosan in both salt (acetic chitosan) and basic (basic chitosan) forms and films of acetic chitosan modified by heat treatment were the objects of research.

The films were formed by a dry technique (casting of a polymer solution onto a polished poly(ethylene terephthalate) support). The chitosan concentrations in the film-forming solution were 1.5 and 2 wt.% for CTS-640 and CTS-87, respectively. For preparing initial films of the same thickness, these concentrations were varied depending on the polymer's \overline{M}_η . The volume of the film-forming solution to make films of fixed thickness was estimated experimentally.

The solvents were 2% aqueous acetic acid solutions prepared from glacial acetic acid in distilled water. Formation was carried out under normal atmospheric pressure at $22 \pm 2^\circ\text{C}$ for 3–4 days. The readiness of a film was fixed visually when the film sample separated from its support. The polymer in freshly formed films was acetic chitosan. To transfer it to basic chitosan, film samples were kept in a 1 M NaOH solution for 1 h. Then, the films were washed with distilled water to pH = 7 and were dried at $22 \pm 2^\circ\text{C}$ during 1 day. The moisture content (W) of the film samples was determined gravimetrically on an OHAUS Discovery DV 215 CD analytical balance (Switzerland) with an accuracy of weighing of ± 0.0001 g. The samples were dried at 95°C for approximately 6–8 h until a constant weight was achieved. The thickness of the films was $45\text{--}55\ \mu\text{m}$ (acetic chitosan) and $80\text{--}95\ \mu\text{m}$ (basic chitosan). The acetic chitosan films were heat-treated carried out at 95°C for 1 h and at 85, 110, 135, and 160°C for 1.5 h. Additionally, W and the weight loss of each sample, $\Delta W = (m_i - m_e)/m_i \times 100\%$, were calculated from the difference between the weights of the film before (m_i) and after heat treatment (m_e).

The optical activity was recorded on two automatic spectropolarimeters: PolAAr 3001 (Optical Activity Ltd, England), within the wavelength range of $\lambda = 300\text{--}600$ nm, and SPU-E (Moscow Pilot Plant, Russian Federation), within $\lambda = 280\text{--}710$ nm, $T = 22 \pm 2^\circ\text{C}$. The experimental conditions were standard, and the measurement error of the rotation angles did not exceed $\pm 0.002^\circ$. The specific optical rotation $[\alpha]$ ($\text{deg mL dm}^{-1} \text{ g}^{-1}$) was calculated by the following formula:

$$[\alpha]_{\lambda, \text{HM}}^{22 \pm 2^\circ\text{C}} = \frac{\alpha}{\ell \cdot \rho}, \quad (1)$$

where α is the measured angle of optical rotation of the film sample, in degrees; ℓ is the optical path length, dm; and ρ is the density of the film material, g cm^{-3} .

Because chitosan and its films are characterized by compositional and structural heterogeneity, α was measured over the whole surface of each film sample. For this purpose, we used a special cuvette with a cell rotating around its horizontal

TABLE 1: Characteristics of powdered chitosan samples.

Sample	Molecular weight \overline{M}_η (kDa)	Deacetylation degree DD (mol.%)	Bulk density ρ_b (g cm^{-3})	Moisture content W (wt.%)
CTS-87	87	83.6	0.34	6.5 ± 0.4
CTS-640	640	82.6	0.16	10.9 ± 0.6

axis and with a circular scale calibrated from 0 to 360° with a step of 5° relative to an arbitrarily chosen direction of reading. For measurements, a film was fixed vertically in the rotating cell of the spectropolarimetric cuvette—that is, perpendicularly to the incident beam of polarized light—and its optical rotation spectrum was recorded. Next, the sample was turned through a given angle θ in the plane perpendicular to the incident beam direction, and a new optical rotation spectrum was recorded. This procedure was repeated several times. For plotting ORD curves, the $[\alpha]$ values were averaged over all rotation angles from the range of $\theta = 0\text{--}360^\circ$.

The bactericidal properties of the chitosan films were assessed qualitatively by using a disk diffusion method and gram-negative and gram-positive microorganisms, namely, *Escherichia coli* (ATSS 25922) and *Staphylococcus aureus* (ATSS 25923). Both cultures were obtained from the collection of the Microbe Institute (Saratov, Russian Federation). Bacteria were lawn-plated onto a solid nutrient medium based on agarose gel. The incubation time was 24 h, and the incubation temperature was 37°C . A chitosan film of diameter 4 mm (d_{film}) was placed onto a lawn-plated bacterial culture. The exposure time was 18 h. The bactericidal activity was monitored by the inhibition zone diameter (D_i) for the microbial cultures at the site of application of the film sample being tested.

3. Results and Discussion

Our experiments showed that the ORD curves for the initial film samples of both acetic and basic chitosans (CTS-87 and CTS-640) had neither maxima, nor minima, nor points of inflection; that is, they were of the normal type (Figure 1(a)). In the wavelength range being examined, the dispersion curves for both forms of the polymer lay in the range of negative values of optical rotation. The films made of acetic chitosan were an exception, being characterized by both positive and negative $[\alpha]$ values (curves 1 and 2) at some orientation angles θ within the long-wave spectrum region ($\lambda > 540$ nm). As a result of transfer of the protonated amino groups ($-\text{NH}_3^+$) in the macromolecules to amine ones ($-\text{NH}_2$), the absolute value of specific optical rotation of basic chitosan films (curves 3 and 4) exceeded that for acetic chitosan (curve 1) by more than an order of magnitude.

It is noteworthy that the ORD curves of the acetic CTS-87 and CTS-640 films (Figure 1(a), curves 1 and 2) and solutions of the same polymer samples in acetic acid, where chitosan is also in acetic form (Figure 1(b)), are almost independent of the sample's \overline{M}_η . The ORD curves of the basic CTS-87 and CTS-640 films differ largely in their appearance (Figure 1(a), flatter curve 4) and absolute values of $[\alpha]$.

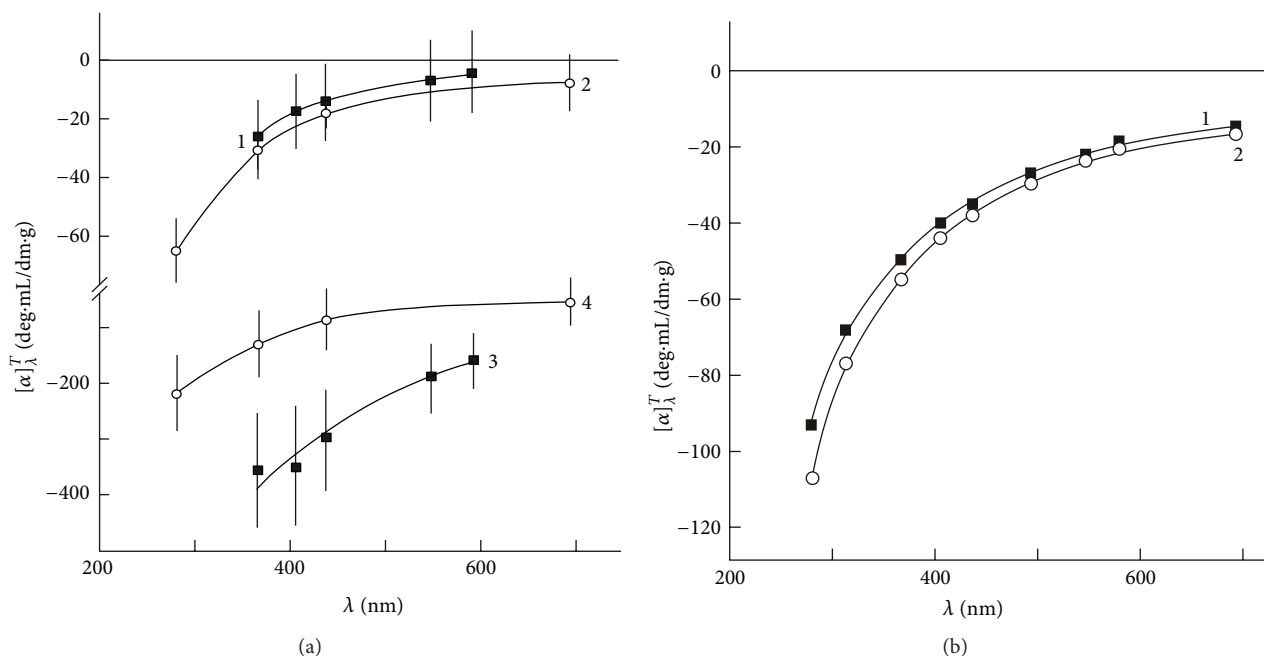


FIGURE 1: (a) ORD curves for CTS-87 (1, 3) and CTS-640 (2, 4) films in the acetic (1, 2) and basic (3, 4) forms, as recorded on PolAAR 3001 (1, 3) and SPU-E (2, 4) spectropolarimeters. The moisture content of the film samples was 22 ± 2 (1) and 12 ± 1 wt.% (2). Vertical bars show the variation range of $[\alpha]$ values for different orientation angles of the film sample. (b) ORD curves of 0.5% solutions of CTS-87 (1) and CTS-640 (2) in 2% acetic acid, as recorded on an SPU-E spectropolarimeter [11].

For processing the experimental ORD curves, the following single-term Drude equation was used:

$$[\alpha]_{\lambda}^T = \frac{K}{\lambda^2 - \lambda_0^2}, \quad (2)$$

where K and λ_0 are the rotation and dispersion constants, respectively. The Lowry ($1/[\alpha] - \lambda^2$), Young-Doty ($[\alpha]\lambda^2 - [\alpha]$), and Heller ($1/[\alpha]\lambda^2 - 1/\lambda^2$) graphic transformations were used to estimate these constants.

Analysis of the obtained K and λ_0 values of the chitosan films of both molecular weights (Table 2) showed that the optical rotation of the films made of chitosan as a polysalt or a polybase was caused by the contributions of optically active chromophores with different locations of their absorption bands: $\lambda_0 \approx 265$ nm (CTS-87) and 200 nm (CTS-640) for acetic chitosan and $\lambda_0 \approx 150$ nm (CTS-87) and 100 nm (CTS-640) for basic chitosan. The rotatory force of spatial-electronic transitions also differed. The absolute \bar{K} value for basic chitosan exceeded that for acetic chitosan by 5–30 times. The largest difference in the \bar{K} values was observed for the lower molecular weight chitosan films.

The obtained results suggest the existence of essential distinctions in the conformation and spatial arrangement of the macromolecules constituting supramolecular structures in film samples made of both chitosan forms. According to the current views [16], acetic chitosan films are characterized by level-by-level structural heterogeneity, the existence of a denser bottom layer adjoining to the support, and a less dense folded top layer. This feature of the morphological structure of films remains upon transfer of chitosan to its

basic form, but the accompanying relaxation processes lead to structure consolidation.

Heat treatment promoted still higher consolidation and reduction in the sizes of structural elements and still higher leveling of structural heterogeneity. Figure 2(a) presents the estimated results for the temperature influence on the ORD of acetic CTS-640 films as an example. The ORD curves of the initial and thermoprocessed films sharply differed not only in the type of the $[\alpha] = f(\lambda)$ dependence but also by the absolute value and in the sign of $[\alpha]$ at a fixed λ . During heat treatment, changes in the color of the films were visually observed: from light beige (curves 1–3) to yellow (curve 4) and orange (curve 5). Within the temperature range of ~ 20 – 160°C , the moisture content of the films (Figure 2, curve 2) almost linearly decreased (i.e., the water weight loss increased) and their $[\alpha]$ increased with inversion of the rotation angle sign (Figure 2, curve 1).

It is known that during heat treatment of acetic chitosan films, several processes may proceed, including amidation, crosslinking, or thermooxidizing destruction of the polymer [16, 17]. Similar conclusions were also made in an analysis of the dependences of the spin-spin relaxation times of sorbed water on the moisture content of the initial and thermoprocessed chitosan films [19]. It was also shown that thermomodification of chitosan film samples, accompanied by the formation of its dehydrated form, leads to the formation of a less crystalline but more homogeneous structure at a morphological level [16].

Conversion (as a result of annealing) of chitosan from hydrated to anhydrous form occurs in the solid-phase state of the polymer. In a paper by Okuyama et al. [18] the structure of

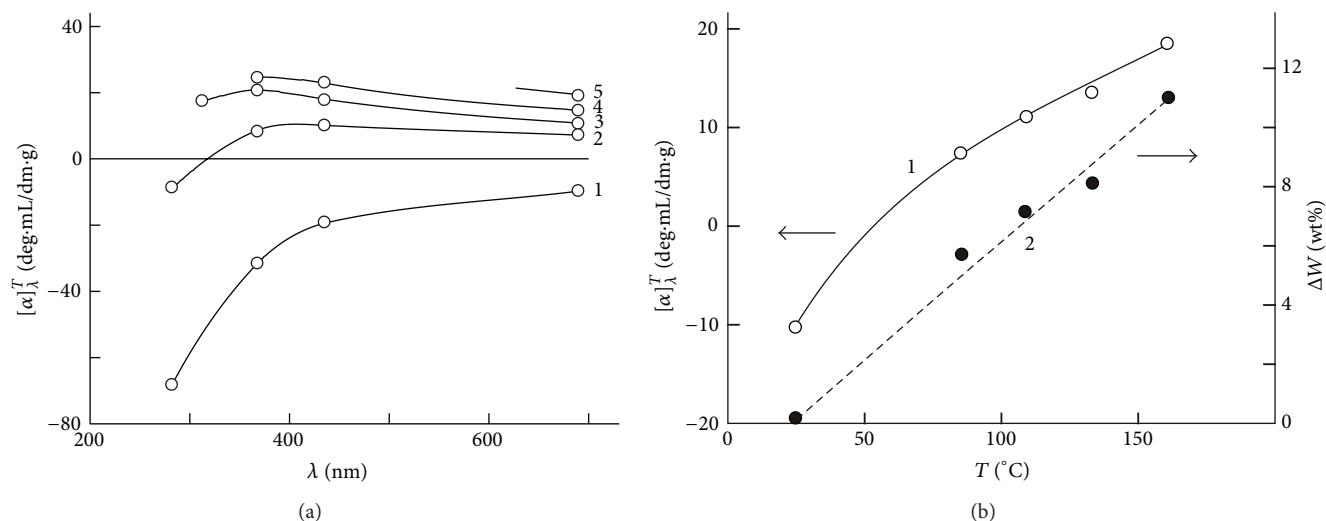


FIGURE 2: Change in the properties of an acetic CTS-640 film during thermal processing: (a) the ORD curves, recorded on an SPU-E, of the initial film with $W = 11.5 \pm 1$ wt.% (1) and of the film samples thermomodified for 1.5 h at 85 (2), 110 (3), 135 (4), and 160°C (5); (b) change in the specific optical rotation for $\lambda = 691$ nm (1) and the water weight loss (2) of the films as a function of the heat treatment temperature.

TABLE 2: Values of the rotation and dispersion constants of chitosan films in two chemical forms.

Sample	Chemical form of the polymer	Rotation constant $K \cdot 10^{-6}$				Dispersion constant λ_0 (nm)			
		Lowry	Young-Doty	Heller	\bar{K}	Lowry	Young-Doty	Heller	$\bar{\lambda}_0$
CTS-87	Acetic	-1.6 ± 0.2	-1.7 ± 0.2	-1.6 ± 0.1	-1.65	270 ± 7	260 ± 9	260 ± 8	265
	Basic	-54 ± 3	-56 ± 2	-55 ± 4	-55	140 ± 10	160 ± 5	160 ± 8	150
CTS-640	Acetic	-3.3 ± 0.4	-3.1 ± 0.3	-3.0 ± 0.4	-3.1	210 ± 6	190 ± 5	190 ± 5	200
	Basic	-14.6 ± 2	-15 ± 2	-15.4 ± 3	-15.0	110 ± 3	100 ± 2	100 ± 2	100

hydrated chitosan was studied by X-ray and thermal analysis, molecular models of chain packing were designed, and a probable scheme of solid-phase transformation of chitosan structure on water removal was offered (Figure 3).

The elementary cell of the crystal structure of both hydrated and anhydrous chitosan is orthorhombic $P2_12_12_1$, formed as a result of antiparallel packing of two helical chains. The stability of hydrated chitosan is provided by “water bridges” between adjacent polymeric chains (Figure 3(a)). When water is removed from the polymeric matrix, the intermolecular H-bonds break, the macromolecules shift along the a axis, and, as a result, the elementary cell shortens along the b axis and (somewhat) along the a axis [18].

Therefore, when acetic chitosan films are thermomodified, not only the polymer-analogous transformation chitosan \rightarrow chitin proceeds but also, as a result of water (plasticizer) evaporation, a different spatial structure of the polymer is formed. This is clearly shown by both literature data (Figure 3) and our experiments (Figure 2). We note in passing that a change in the chiral structure of the polymer with inversion of the sign of specific optical rotation $[\alpha]$ was observed by us for samples of cellulose diacetate with various moisture contents as well [20].

Because any change in the $[\alpha]$ value essentially influences the functional properties of the material [21, 22], we performed a comparative analysis of the bactericidal activity of

several chitosan films with both negative and positive $[\alpha]$ values: the initial, freshly formed films in both forms (hydrated) and a thermomodified film (anhydrous).

The distinction between the moisture content and the optical activity of chitosan films essentially reflected on their bacteriostatic action. The initial films of acetic CTS-87 and CTS-640 were bactericidal, inhibiting the growth of cultures of gram-negative and gram-positive microorganisms, while no such effect was observed for basic chitosan films. For example, the diameter of the inhibition zone of *S. aureus* and *E. coli* growth by the CTS-87 film exceeded by 3–5 times the diameter ($d_{\text{film}} = 4$ mm) of the chitosan film sample used for examination (Table 3, sample 1). Heat treatment of the acetic CTS-87 film at 95°C for 1 h essentially reduced its bactericidal activity (sample 2). The basic CTS-87 film sample under the accepted experimental conditions showed no inhibitory effect on *E. coli* and *S. aureus*, $D_i \leq d_{\text{film}}$ (sample 3). This is apparently due to complete loss of the water solubility of these films in the form of a polybase. It may be necessary to develop a special technique for estimating biological effects of water-soluble and insoluble samples under adequate conditions for a more accurate analysis of the bactericidal action of chitosan films in different chemical forms.

Thus, we have observed the dependence of the bactericidal properties of chitosan films on the dehydration degree and the chemical form of the polymer. These results

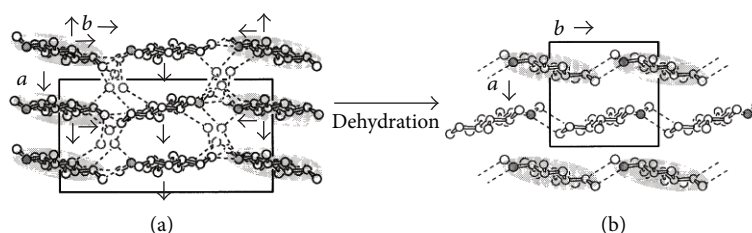


FIGURE 3: A model for the probable solid-phase transformation of the crystal structure of chitosan upon transition from hydrated (a) to anhydrous form [18].

TABLE 3: Bactericidal activity of the CTS-87 films.

No	Sample	Moisture content W (wt.%)	$[\alpha]_{691\text{ nm}}$ (deg.mL/dm.g)	Inhibition zone diameter D_i (mm)	
				<i>E. coli</i>	<i>S. aureus</i>
1	Initial acetic film	22 ± 2	-10 ± 7	12 ± 1	20 ± 5
2	Acetic film thermoprocessed at 95°C for 1 h	1.0 ± 0.5	$+9 \pm 5$	5 ± 0.7	7 ± 1
3	Initial basic film	12 ± 1	-190 ± 50	4 ± 0.5	3 ± 0.5

supplement the findings of a previous study [13], which showed the molecular-weight dependence of the bactericidal properties of chitosan solutions. These physical and chemical parameters determine the absolute value and sign of specific optical rotation of the preparations used, and an interrelation between the optical activity of chitosan and its biological action is now observed in two types of systems, namely, solutions and films.

We associate the further development of this direction of biophysical research with the need to estimate the influence of other characteristics of the biopolymeric system on the absolute value and sign of $[\alpha]$, including the deacetylation degree and the molecular heterogeneity of the aminopolysaccharide and the nature of the solvent used to prepare samples. All this may ensure reliable control over the structure and properties of chitosan in designing biomedical and biotechnological preparations on its basis.

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