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STRUCTURE OF A MATRIX BASED ON POLYSACCHARIDE DERIVATIVES FOR THE IMMOBILIZATION OF BIOLOGICALLY ACTIVE SUBSTANCES

N. D. Burkhanova, S. M. Yugai, M. Yu. Yunusov, G. V. Nikonovich, and T. I. Usmanov

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The supermolecular, molecular, and physicochemical characteristics of natural and modified natural polysaccharides (cotton cellulose, amylose of the U-type) used as supports for obtaining enzyme conjugates have been studied. It has been shown that by varying the process conditions it is possible to obtain cellulose matrices with different amounts of CHO groups and correspondingly different capacities for immobilizing an enzyme and modifying its activity.

Using a combination of methods (x-radiography, sorption, optics, electron microscopy in the scanning and transmission regimes (SEM and EM, respectively), and chemical analysis), we have investigated the supermolecular, molecular, and physicochemical characteristics of natural and modified natural polysaccharides (cotton cellulose, amylose of the U-type) used as supports for obtaining enzyme conjugates [1-3].

As examples we took microcrystalline cellulose (MCC), obtained by acid hydrolysis from cotton cellulose, and U-type amylose. Chemical modification of the selected material was performed by specific oxidation with periodic acid and its salts.

The purity of the preparations was evaluated by high-resolution ^{13}C NMR spectroscopy in DMSO-d_6 solution (Table 1). It was established that the formation of functional groups took place predominantly at the C_2 and C_3 OH groups. For every 100 elementary units there were 5 reactive groups — such substitution determines the optimum conditions for immobilization. The CHO groups formed in cellulose and amylose possess the capacity for interacting with suitable functional groups of the enzymes to be immobilized.

An investigation of the structures of the samples showed that, in the optical microscope, the MCC preparations were represented mainly by particles from 5-10 to 200 μm long and, on average, 10-20 μm wide, which corresponds approximately to the diameter of the initial cellulose fiber from which the sample was obtained. The majority of the MCC samples investigated possessed a high birefringence and luminesced brightly in polarized light, which showed considerable anisotropy (Fig. 1).

The amylose consisted of smooth structureless samples. At low and moderate degrees of oxidation (from 2 to 5-6 mass-% of CHO groups), no appreciable changes in the macrostructure of oxidized MCC were observed in comparison with the untreated sample. The dimensions, shape, and external form of the particles changed little, but the brightness of their luminescence became less intense. An increase in the degree of oxidation led to a rise in the number of small particles in the samples through their transverse cleavage.

In SEM investigations (Fig. 2) it was established that, before being ground, the MCC obtained from cotton cellulose (CC) included particles with a fairly smooth surface in which it was possible to see wrinkles or sometimes fissures arranged mainly along the fiber. Details of their internal structure (lamination) could be detected at the ends of the particles. The twisting and wrinkles clearly visible at an acute angle to the axis of a cotton fiber usually disappeared after hydrolysis and grinding, and were almost indetectable in the MCC.

It was observed with the scanning electron microscope that the structure of the surface of oxidized samples with 1-2 mass-% of CHO scarcely differed from the surface of the initial MCC described above. However, at a higher percentage of

Institute of the Chemistry and Physics of Polymers, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 44 26 61. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 632-638, July-August, 1997. Original article submitted April 4, 1996.

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TABLE 1. Position of the Signals of the ^{13}C Nuclei in the NMR Spectrum of the Amylose Isolated

^{13}C carbon atom nuclei (δ, ppm)					
C_1	C_2	C_3	C_4	C_5	C_6
101.1	73.9	72.1	79.6	72.5	61.3

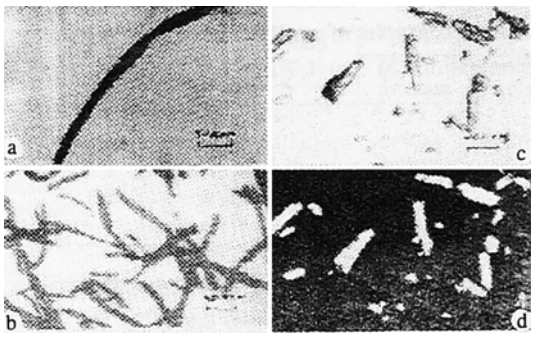

Fig. 1. Optical photographs in polarized light of samples before and after oxidation: *a*) MCC from CC; *b*) MCC oxidized by HIO_4 (11 mass-% CHO).

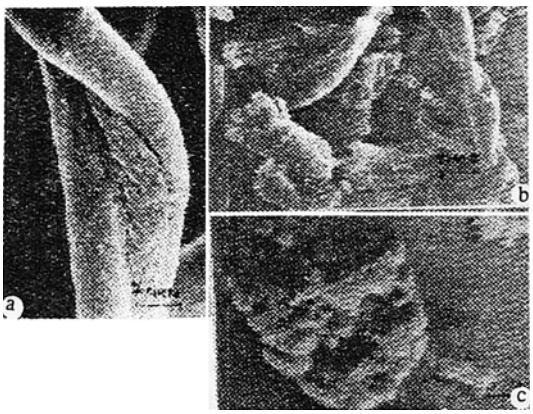

Fig. 2. SEM photographs of samples before and after oxidation: *a*) initial cotton cellulose; *b*) MCC from cotton cellulose; *c*) MCC after oxidation (23 mass-% CHO).

aldehyde groups we saw defects in the surface, fairly deep fissures, scaling, and the breakdown of some particles into very small fragments. In addition, there was an obvious tendency to the aggregation of the particles.

The electron microscope investigations showed that the surface of such natural cellulose fibers as cotton contain a system of fine wrinkles at an acute angle to the axis of the fibre. Such a structure was retained after hydrolytic degradation, but the surface had become inhomogeneous and the number of wrinkles had increased, some them being converted into fissures (Fig. 3). In addition to flattened sections, sections with a different type of defects in the form of depressions, dislocations, and excrescences were observed.

The investigations showed only slight changes in the structure of the surface of samples of MCC subjected to oxidation. Results obtained for dispersed preparations of oxidized MCCs are of considerable interest. Like the initial MCC, samples including a low level of CHO groups (less than 2 mass-%) broke down into crystallites and fragments. An increase in the level of aldehyde groups led to a decrease in the size of the fragments and to the appearance of defects in the crystallites and broken pieces of them.

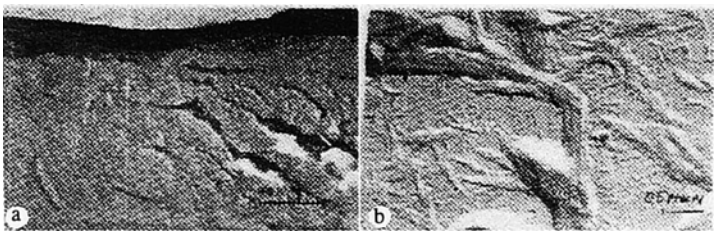

Fig. 3. Electron photomicrographs of replicas from the surface (*a*) and from dispersed samples after oxidation (*b*) : *a*) 4.7 mass-% CHO; *b*) 11.0 mass-% CHO.

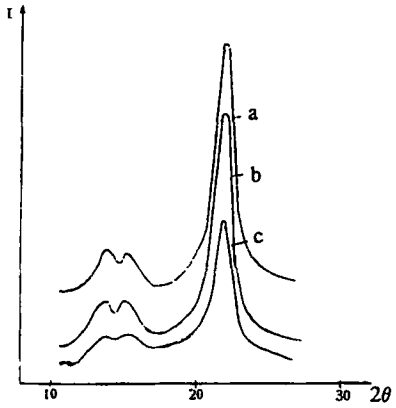

Fig. 4. Diffractograms of MCC before (*a*) and after (*b*, *c*) oxidation: *b*) 4.7 mass-% CHO; *c*) 11 mass-% CHO.

X-ray diffractograms of the initial samples of cotton cellulose and MCC corresponded to the crystalline modification cellulose-1, with maxima at the angles 2θ = 14.7, 16.8, 22.6, and 34.4°. The degree of crystallinity (DC) was 78% for the cotton cellulose and 80-82% for the MCC (Fig. 4).

As already mentioned, the results of the x-radiographic investigations correlated well with those of microscopy. In the initial stage, oxidation did not change the type of crystal lattice of the samples.

At a low degree of oxidation, the diffractograms of the preparations were, on the whole, identical with that of the initial MCC, although the intensities of the main reflections were somewhat lower (DC of the order of 75%). When the number of CHO groups was increased, the DC fell considerably, amounting to 66 and 61% for samples with DCs of 5 and 12 mass-% CHO, respectively. A broadening of the reflections, especially 002, was observed, witnessing a decrease in the dimensions of the crystallites, while the 101 and $10\bar{1}$ reflections fused into one broad maximum. MCC preparations with higher levels of aldehyde groups gave the diffractogram of a completely amorphous substance with one diffuse maximum in the region of 2θ = 20-24°.

Thus, intensive oxidation leads to very far-reaching changes in the supermolecular and fine structure of MCC and amorphous cellulose.

IR-spectroscopic investigations of the oxidized samples of MCC showed only slight changes at a low degree of modification. A rise in the level of CHO groups led to a fall in the intensity of the absorption band at 3200-3600 cm^{-1} (OH groups involved in hydrogen bonds) and to a worsening of the resolution of the structurally sensitive bands in the long-wave region. A weak diffuse band was detected in the region of C=O groups (1740 cm^{-1}). This was probably due to the formation of hemiacetal bonds between the CHO and OH groups of the cellulose. In favor of this hypothesis is the appearance of a band at 890 cm^{-1}, apparently due to hemiacetal bonds.

From the equilibrium sorption of water, we determined sorption isotherms of MCC before and after oxidation in the range of relative humidities from 0 to 100%. The isotherms of all the samples investigated had an S-shaped form, with a rapid rise in the region of high humidities, which is characteristic for materials with a heterogeneous structure.

TABLE 2. Sorption Properties of the Modified Samples

Sample	Sorption at the given relative humidity				
	10%	65%	100%	S, $sp \cdot m^2/g$	W_o, cm^3/g
MCC ox. by HIO_4	1.03	3.80	8.60	73.00	0.0860
MCC ox. by $NaIO_4$	1.00	2.50	5.50	55.00	0.0550
MCC, init.	0.70	2.50	6.20	48.14	0.0620

TABLE 3. Properties of the Modified Samples

Type of raw material	Modification conditions		CHO group content, mass-%	Activity, PU/g		
	oxidant	ratio		alk. protease	α-chymo-trypsin	trypsin
1. MCC	2% HIO_4	1:40	1.7	2.4	–	–
2. MCC	3% $NaIO_4$	1:16	4.7	6.0	–	–
3. MCC	6% HIO_4	1:16	7.55	16.0	–	–
4. MCC	13% HIO_4	1:19	23.5	41.0	–	–
5. Amylose	1% HIO_4	1:30	2.0	–	1.23	–
6. Amylose	1.5 HIO_4	1:30	5.0	–	–	0.96

All the results on the sorption of water together with the other structural characteristics calculated from them are given in Table 2. At a relative humidity of 65%, for MCC the sorption amounted to 2.5%.

An evaluation of the sorption capacities of the oxidized samples of cellulose permitted the following conclusions to be drawn. The oxidation of cellulose causes specific changes in its sorption capacity that depend on the content of CHO groups. The fact is that CHO groups introduced into cellulose are less hydrophilic than hydroxy groups, which must lead to a fall in the sorption capacities of the preparations. At the same time, depending on the conditions of the process and its degree, modification may cause definite disturbances of the structure, a fall in the DC, a decrease in the dimensions of the crystallites, and a change in the mutual orientation of structural elements.

Thus, two opposite directions of the process operate and the final results depend on which process prevails. By varying the conditions of oxidation it is possible to obtain cellulose matrices with different levels of CHO groups and, correspondingly, different capacities for immobilizing an enzyme (alkaline protease) with differences in its activity, on the basis of which medicinal compounds (profezim) are obtained in insoluble form for the treatment of pyonecrotic processes.

Matrices can be divided arbitrarily into those with low, medium, and high activities (Table 3).

The first group contains 0.8-2.0 mass-% of CHO groups. It has been found that on them the activity of the immobilized enzyme amounts to 1.5-2.5 PU/g. Matrices with medium activity contain 2.5-5.0 mass-% of CHO groups. The activity of protease immobilized on such matrices easily reaches 5-8 PU/g, but under more favorable conditions of immobilization it exceeds 10-15 PU/g. Finally, at more than 5 (up to 20) mass-% of CHO groups it is possible to obtain enzymes with an exceptionally high activity (up to 40 PU/g) immobilized on the matrices, many times exceeding the activity achieved on aminated matrices. This opens up completely new possibilities for using such materials in medicine, biotechnology, the food industry, biochemical investigations, and analytical chemistry.

Thus, by varying the conditions of modification it is possible to obtain preparations with different degrees of oxidation causing a change in the molecular and supermolecular structures of the modified polysaccharides, and this can be made use of for immobilizing proteolytic enzymes.

EXPERIMENTAL

The x-radiographic investigations were conducted on a DRON-3M diffractometer with monochromatized CuK_α radiation at a voltage of 20 kV and a current of 15 mA, which were selected in dependence on the preparation. The samples were prepared by molding the ground preparations in the form of tablets. Recording was carried out in the interval of 2θ = 10-36°.

IR spectra were obtained on a Specord-75IR spectrometer. Tablets were prepared by molding ground samples with KBr.

The microscope studies were made with a MBI-6 optical microscope, which permitted the determination of the external form, shape, and dimensions, the presence of pores and defects, and the degrees of homogeneity and swelling of the samples. To investigate the supermolecular structure of the surface of the preparations we used the method of two-stage polystyrene-

carbon replicas shadowed with Pt [4]. We first obtained imprints by pressing the samples onto a glass plate coated with polystyrene in a special molding press at 80°C for 2 h. Then, after the removal of the sample, carbon and Pt were sputtered on the primary imprint at an angle of 30-40°, after which the polystyrene was dissolved in xylene. Sputtering was carried out in a VUP-4K vacuum unit. The replicas were viewed in a PÉM-100 electron microscope. For the electron microscope investigations in the scanning regime the specimens were sputtered with silver in the VUK-4 vacuum unit and were then viewed in an RÉM-200.

The sorption measurements were carried out on a McBain quartz spiral vacuum balance in the interval of relative humidities of 0-100% at 25°C. Specific surfaces (S_{sp}), pore volumes (W_0), and mean effective radii of the capillaries (r_c) were evaluated by means of the BET equation from the results obtained.

High-resolution ^{13}C NMR spectra were taken on a Varian XI-100 instrument in the proton regime using solutions in DMSO-d_6. MCC was obtained by the acid hydrolysis of cotton cellulose in 3% HNO_3 at 100°C for 3 h.

The polysaccharides were modified by specific oxidation in the presence of 1-13% $NaIO_4$ at various weight ratios of polysaccharide to oxidant. The reaction was conducted at 30°C with constant stirring for 3 h, followed by fixation for 24 h, washing with water, filtration, and drying at room temperature.

The proteolytic enzymes trypsin and α-chymotrypsin were deposited on modified amylose as described in [5], while alkaline protease was immobilized on oxidized MCC by the method of [6].

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