CHARACTERISTICS OF N,O-CARBOXYMETHYLCHITOSAN SYNTHESIZED BASED ON CHITIN FROM *ARTEMIA PARTHENOGENETICA* CYSTS

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Abstract: The paper presents the results of a study of carboxymethyl chitosan synthesized based on chitin/chitosan from cysts of the Karakalpak population of *Artemia parthenogenetica* in the Aral Sea. The resulting N, O-carboxymethyl chitosan (N, O- CMCS) is hydrophilic and readily soluble in acidic, neutral, and alkaline media. ^{1}H and ^{13}C NMR spectroscopy and pH-metric titration confirmed the formation of N, O-CMCS with predominant nitrogen substitution. The degree of substitution (DS), according to pH-metric titration, is DS=1.289.

Keywords: *Artemia parthenogenetica*, cysts, chitin, chitosan, carboxymethylchitosan.

Introduction

Chitin and chitosan are copolymers of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) linked by β-D-(1→4) glycosidic bonds.When the degree of deacetylation (DD) is less than 50%, the biopolymer is called chitin. When the DD is more than 50% (usually more than 60%), the polysaccharide becomes soluble in dilute solutions of organic and inorganic acids. Such a polysaccharide was named chitosan [1,2].Due to the limited solubility of chitin/chitosan, very often their use is limited in areas where solubility is a major factor. The solubility of chitosan can be improved by depolymerization and chemical modifications [1].The introduction of a carboxymethyl group into the structure of chitosan can dramatically increase the solubility of chitosan at neutral and alkaline pH values without affecting its other characteristics, which significantly expands the possibilities of its practical application.

Carboxymethylchitosan (CMCS) is superior to the original biopolymer not only in solubility. It also has properties such as the ability to bio adhesion, absorption, and sustained release, and the biodegradability and non-toxicity of the final degradation products ensure its environmental friendliness [3].

Reactive centers for carboxymethylation of chitosan are amino groups and hydroxyl groups present in its chains. According to literature data, the selection of appropriate reaction conditions and reagents makes it possible to obtain N-, O-, N, O- or N, N-CMCS. O-CMCSis generally obtained when the reaction is carried out at room temperature, in an isopropanol/water suspension and the presence of monochloroacetic acid and sodium hydroxide. However, when this reaction is carried out at high temperatures, N- and N, O-carboxymethyl chitosan are formed. Also, N-CMCS can be formed by the reaction of chitosan with glyoxylic acid followed by reduction with sodium cyanoborohydride, the degree of substitution of the derivative being determined by the stoichiometry of the reaction and the properties of the starting chitosan [4].The properties and applications of carboxymethyl-chitosan depend on its structural features,

mainly on the degree of substitution and the place of carboxymethylation, amino group or hydroxyl groups.

The aim of this work is a more detailed study of some physicochemical and structural characteristics of N, O-carboxymethylchitosan synthesized based on chitin from cysts of the Karakalpak population of *Artemia parthenogenetica* in the Aral Sea.

Materials and methods. Methods for obtaining chitin, chitosan and carboxymethyl-chitosan

In early publications, we described in detail the methods for preparing cysts for the analysis and isolation of chitin, the synthesis of chitosan, and carboxymethyl-chitosan based on it. The results of the chemical composition of the feedstock are summarized, and the main qualitative and structural characteristics of chitin/chitosan and CMCS are determined by chemical, IR spectroscopic, and other instrumental methods [5]. When obtaining chitin from cysts of *Artemia parthenogenetica* of the Aral Sea, the classical method was taken as a basis [6], and for chitosan, the method of the "hard method for obtaining chitosan" [7–8]. The synthesis of N, O- CMCS was carried out by the interaction of chitosan with monochloroacetic acid in an alkaline medium at a temperature of 45°C for 3 hours according to the method described in the article by N. Celikci et al. [9].

Nuclear magnetic resonance (NMR)

The nuclear magnetic resonance spectra of chitosan and CMCS(^{1}H and ^{13}C NMR) were recorded at 30°C in D_2O and CF_3COOH using a JNM-ECZ600R 600 MHz NMR spectrometer (JEOL, Japan).

pH metric titration method

Based on the titration data obtained using a Bante 210 pH meter, the degrees of deacetylation (DD) of chitosan and substitution (DS) of CMCS, respectively, were calculated [10–11]. To do this, we took a sample of 200 mg of each sample (with an accuracy of 0.001 mg). Then 20 ml of a 0.1 N HCl solution was added and stirred on a magnetic stirrer. Each sample is titrated with a 0.1 M NaOH solution to pH 12–13. The titration curve is shown in Figure 5. The DD and DS values were calculated using the following formulas: (1), (2), and (3):

$$\mathrm{DD}\ (\%) = \left(\frac{203 * \mathrm{C}\ (\mathrm{V_2} - \mathrm{V_1})}{42\ * \mathrm{C}\ (\mathrm{V_2} - \mathrm{V_1}) + m_{Chitosan}}\right) * 100 \qquad (1)$$

where: C is the molarity of the basic titrant solution (NaOH); (V_1–V_2) is the volume in milliliters of stock solution consumed between the first and second titration equivalence points; $m_{Chitosan}$ - is the used sample of chitosan (mg); 203 and 42 are the molar masses of the chitin residue and the acetyl group, respectively.

$$DS = \frac{203 * \mathrm{C}\ (\mathrm{V_2} - \mathrm{V_1})}{m_{CMCS} - 80\ * \mathrm{C}\ (V_2 - V_1) + 22\ * \mathrm{C}\ (\mathrm{V_3} - \mathrm{V_0}) + 42\ * \mathrm{C}\ (V_3 - V_2)} \quad (2)$$

$$DS = \frac{V_2 - V_1}{V_3 - V_2} * DD \qquad (3)$$

where: C is the molarity of the basic titrant solution (NaOH); V_0 is the volume (ml) of sodium hydroxide consumption consumed for hydrochloric acid in a blank sample;V_1 is the

volume of sodium hydroxide consumed by excess hydrochloric acid (ml);V_2, V_3 - volumes in milliliters of sodium hydroxide corresponding to the amount consumed between the first, second and third equivalence points of titration of COOH and -NH_3^+, -NH_2^+ ,CH_2COO^-;m_{CMCS}- is the used sample of chitosan (mg); 203 and 42 and 80 are the molar masses of the chitin residue and the acetyl and -CH_2COONa groups, respectively; 22 represents the difference in the molar masses of CH_2COONa and -CH_2COOH.

Results and discussion

According to the results of a number of studies, during the interaction of chitosan with monochloroacetic acid, depending on the reaction conditions, the carboxymethyl group can be introduced into atoms at C-2 (NH_2 group) and C-3 (OH group), as well as C-6 (CH_2OH group) positions.In early publications, we noted the formation of N,O-CMCS according to the results of the analysis of IR spectra.In this work, ^1H and ^{13}C NMR spectroscopy and pH-metric titration were used to confirm the formation of N,O-CMCS.According to the results of these studies, the carboxymethyl group binds to C-3 atoms (OH group) and C-6 (CH_2OH group) of chitosan, as well as to C-2 atoms (NH_2 group).The resulting N,O-CMCS is hydrophilic and readily soluble in water.Comparative characteristics of the obtained chitosan and CMCS are presented below in table 1.

Table 1

Comparative characteristics of chitosan and CMCS

Characteristics	Chitosan	CMCS
Appearance	fine powder	fine powder
Colour	white	white
Odor	odorless	odorless
Deacetylation degree, %	84,5	-
Degree of substitution	-	1,289
Molecular weight, kDa	16	0,8-3
Solubility	in acidic medium	in water

The formation of chitosan and N,O-CMCS is also confirmed by pH-metric titrations. According to this the degree of deacetylation of the obtained chitosan corresponds to DD=84.5% and the degree of substitution of CMCS- DS=1,289. The molecular weight of the samples of chitosan and N, O-CMCS was determined using Size exclusion chromatography [5]. Given the low stereoselectivity of carboxymethylation at one specific reaction center of chitosan and the formation of N, O-CMCS, we below present an analysis of NMR spectroscopy and pH metric titration data to confirm its structural features and degree of substitution.

NMR spectra of chitosan and N,O-CMCS

The ^1H-NMR spectrum of chitosan is shown in fig.1. The signal at δ1.901 ppm corresponds to residual acetamido groups. The signal observed at 3.008 ppm corresponds to the proton at the C-3 atom associated with the amino group. The relative integral intensity of these two signals (~1:7) corresponds to the degree of deacetylation of 87%, which is in good agreement (within the integration error) with the data of potentiometric titration. The signals of the remaining protons of the glucosamine cycle appear at 3.550, 3.612 and 3.75 ppm.

The ^{13}C NMR spectrum of chitosan is shown in fig. 2. The signal at δ162.5 ppm refers to the carbonyl carbon of the -$COCH_3$ group. The signal of the C-3 atom associated with the amino

group has a characteristic chemical shift of 56.85 ppm. The signals at δ 70.17, 74.82, and 76.37 are characteristic of carbon atoms associated with oxygen atoms and refer to C4, C-5, and C-6 atoms, while the C-2 signal associated with two oxygen atoms (oxygen in the cycle and bridging between two glucosamine fragments), appears at 97.66 ppm [12].

The ^{1}H-NMR spectrum of N,O-CMCS is shown in fig.3. The signal at 1.932 ppm corresponds to the acetyl of the amide group. Its integral intensity indicates a low content of acetylamino groups in the product. The signal observed at 3.303 ppm corresponds to a proton at C-3 bonded to the nitrogen atom. Narrow band signal at 3.81ppm corresponds to the N-CH_2-COOH methylene group, and the signal at 4.85, partially overlapped by the solvent signal, corresponds to the O-CH_2COOH group. The signals of other protons appear as a series of broadened bands at 3.55, 3.65, and 3.88 ppm.

The results of ^{13}C NMR spectra also support the structure of N,O-CMCS (Fig.4). When examining the spectra, it can be seen that the carboxymethylated product's spectrum contains extra signals, including an acidic carbonyl group at 178.1 ppm and signals in the range of 50–80 ppm that are indicative of carbon atoms linked to nitrogen and oxygen atoms [4].

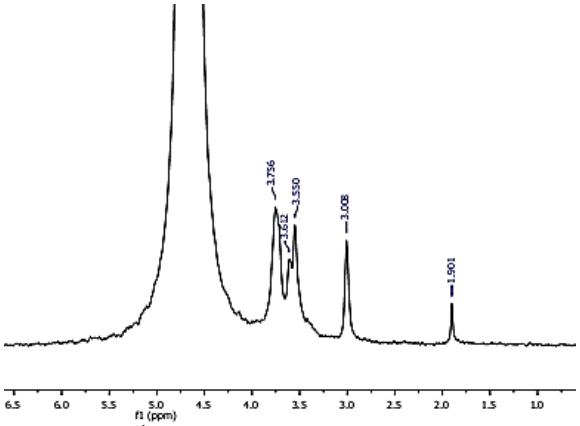

Fig. 1. ^{1}H-NMR spectrum of chitosan

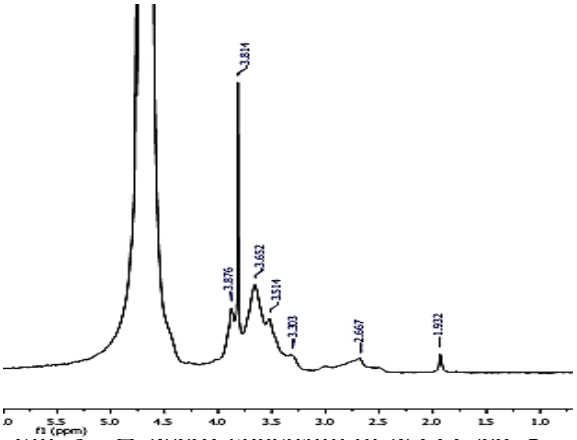

Fig. 3. H-NMR spectrum of N,O-CMCS

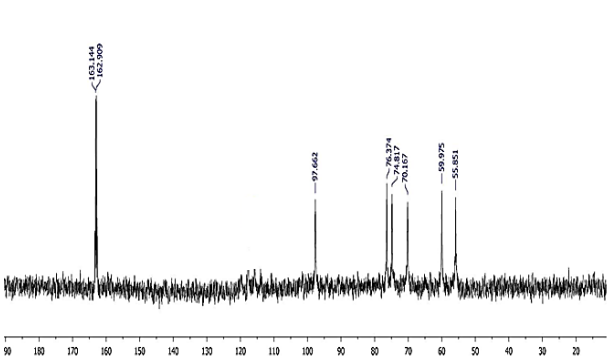

Fig.2. ^{13}C-NMR spectrum of chitosan

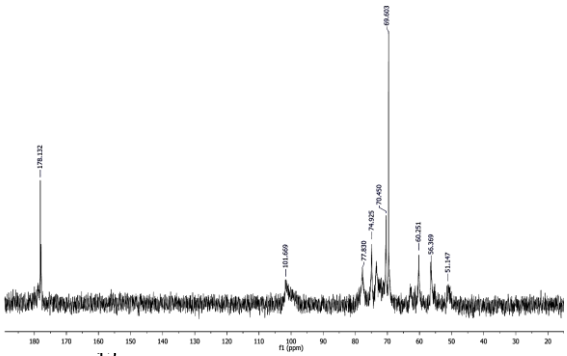

Fig. 4. ^{13}C-NMR spectrum of N,O-CMCS

pH-metric titration of chitosan and N,O-CMCS

Fig. 5a shows one inflection point in a blank titration. To determine the amount of free (not acetylated) amino groups of the resulting chitosan, its sample was dissolved in a known excess of dilute hydrochloric acid so that all NH_2 groups were converted into a salt form and titrated with an aqueous solution of NaOH [13]. The chitosan titration curve (Fig.5b) has two inflection points. The first inflection on the titration curve, a (Fig.5b) refers to the neutralization of free

HCl, the second - to its salt form. The amount of free amino groups calculated from the titration data (NaOH V_2 volume) was 84.5%.

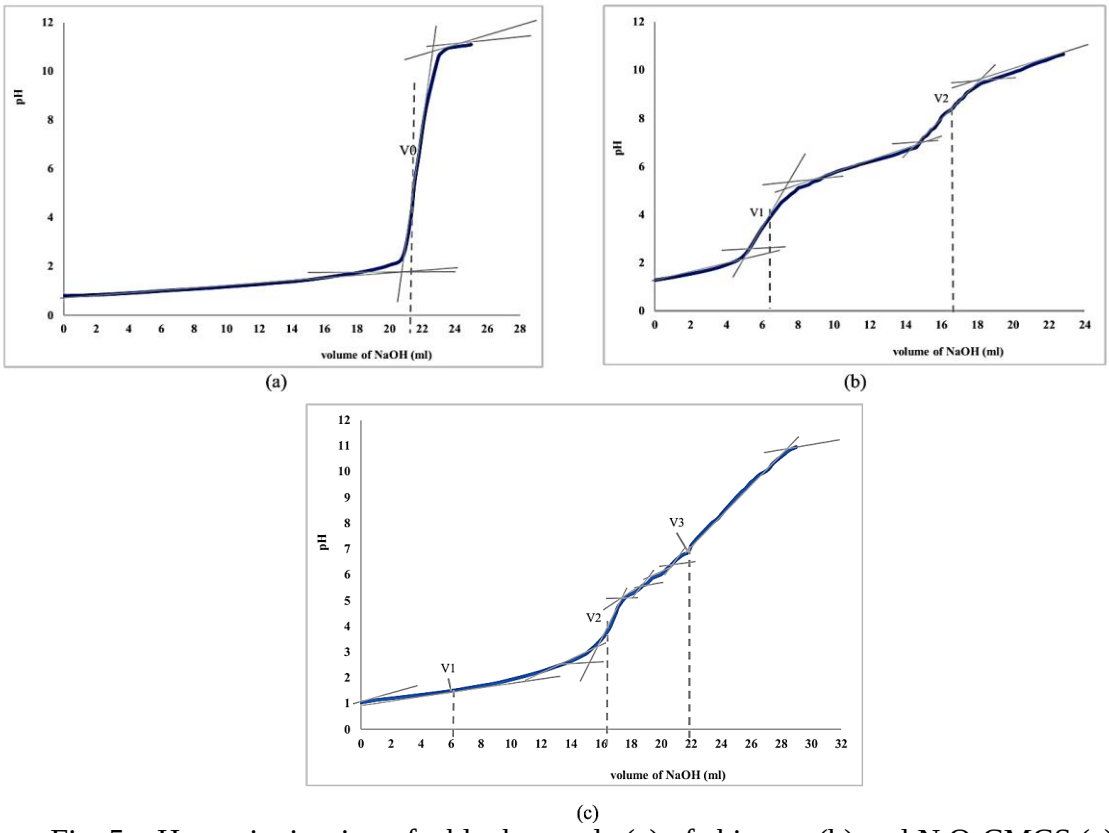

Fig. 5. pH-metric titration of a blank sample (a) of chitosan (b) and N,O-CMCS (c)

The N, O-CMCS titration curve (Fig.5c) has 3 inflections since there are 4 acids in the reaction mixture: HCl, formed as a result of the interaction of chloroacetic acid with chitosan and most likely associated with the amino group; free (unreacted) chloroacetic acid, and chemically bound to the chitosan link - N- and O-methyl carboxyl group. Probably HCl is titrated first, as this is a strong acid (pKa 7), the second is free chloroacetic acid (medium strength acid, pKa =1,38.10-3) and, most likely, O-methyl carboxylic acid, because its strength should be comparable to chloroacetic acid (for comparison: glycolic acid - $HOCH_2COOH$ has a pKa of 1,32.10-4). The last inflection should be attributed to the N-methyl carboxy group, as the weakest of the acids (for comparison, glycine, also known as amino acetic acid–pKa= 1,66.10-10). Thus, the degree of N-carboxymethylation of chitosan can be calculated from V3 and is 1,289. It is not possible to calculate the degree of O-substitution from the titration data, because it is impossible to differentiate the volumes of NaOH spent on the titration of free chloroacetic acid and that which reacted at the hydroxy group. Nevertheless, the comparability of the volumes of NaOH consumed for the titration of HCl (V1) and $N\text{-}CH_2COOH$ (V3) testifies in favor of the predominant N-substitution of chitosan.

Conclusions

The formation of N, O-CMCS synthesized by the interaction of chitosan obtained on the basis of chitin from substandard cysts of the Karakalpak population of the crustacean *Artemia parthenogenetica* of the Aral Sea, with monochloroacetic acid was confirmed by ^{1}H and ^{13}C NMR spectra and the result of pH-metric titration. The results of the analysis testify in favor of

the predominant N, O-carboxymethylation of chitosan, the degree of substitution of which is 1,289.

The synthesized CMCS can be used in many areas, such as the food industry, medicine, pharmaceuticals, agriculture, and others [14].

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