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| D:\Rinat\Rinat\доки\журнал\статьи\logo.jpg | **Oligochitosan hydrochloride: preparation and characterization** | | |
| Cite this: *INEOS OPEN*, **20XX**, X (X), XX–XX  *Received XX Month 20XX,*  *Accepted XX Month 20XX*  http://ineosopen.org | | V.E. Tikhonov, B.B. Berezin, I.V.Blagodatskikh, E. A. Bezrodnykh\* | |
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| Abstract  Most common chemical methods used for chitosan depolymerization and oligochitosan preparation are discussed, and an approach to preparation of oligochitosan hydrochloride with molecular weight below 16 kDa from the parent industrial high molecular weight chitosan by means of joint application of hydrochloric acid and hydrogen peroxide is described. A series of analytical protocols are used to determine physicochemical properties and the quality of oligochitosan hydrochloride as required by European Pharmacopoeia 4.0. Illustrative data include one table and four figures. | | | **C:\Users\Vladimir Tikhonov\Desktop\ineos open\20210312_110156.jpg** |
| **Key words:** chitosan, oligochitosan | | | |

**Introduction**

Since Fleming have found first penicillin, dozens of antibiotics were discovered and introduced to medical practice. At the same time, bacteria, molds and yeasts have become more resistant towards classic antibiotics and more tolerant to natural and synthetic preservatives used in pharmaceutical, food and cosmetic products. Nowadays, misuse and overuse of antibiotics has become a major global problem. On the other side, number of new antibiotics introduced to medical practice is constantly reducing. The World Health Organization has warned that the abuse of antibiotics risks taking the world back to a time when infections were incurable. In order to combat the growing threats from "superbacteria" and pathogenic fungi, all developed countries have planned the reduction of the use of antibiotics and high speed the elaboration of new antibiotics and synthetic chemicals possessing a wide range of antimicrobial activity [1]

Among the last ones, chitosan has been considered as a non-toxic fungicide and bactericide for warm-blooded animals and plants. The US Food and Drug Administration (FDA) in 1983 allowed the use of chitosan as a food additive, and the Environmental Protection Agency [2] allowed the use of chitosan in agriculture and the food industry. In the European Union, it is allowed to use chitosan hydrochloride in the medical, food and cosmetic industries [3].

Chitosan represents a polysaccharides consisting of glucosamine and N-acetylglucosamine, and it has been industrially manufactured by deacetylation of crab/shrimp shell chitin - [(poly(N-acetylglucosamine)] - in the presence of 30-50% sodium hydroxide solution at 70-1300C in 1-8 hr. The conditions of chitin deacetylation determine molecular weight (MW), degree of acetylation (DA) (or, vice versa, degree of deacetylation DD), polydispersity, solubility and antimicrobial activity. The increase in sodium concentration, temperature and process duration decreases MW, DA, solubility and crystallinity. The recognition criterion between chitin and chitosan is complete solubility of the product in aqueous acidic media. Usually, industrially produced chitosan has DA= 4-25% (DD=75-98%) and MW= 60-1500 kDa [4, 5].

The product is called "chitosan" if its MW is more than 16 kDa, and "oligochitosan" if its molecular weight is less or equal to 16 kDa [ 6, 7].

Many publications devoted to antimicrobial activity and safety of chitosan and its derivatives against bacteria and fungi have been published so far. As a result, chitosan and oligochitosan were recognized as biocompatible non-toxic to higher organisms antimicrobial polysaccharides providing synergistic or additive effect in combination with antibiotics [8-13].

Among the chemical methods usually used for oligochitosan preparation, the main ones are acidic hydrolysis, oxidative decomposition and deamination methods. Although each method has its own peculiarities, advantages and drawbacks, acid hydrolysis [14,15] and application of hydrogen peroxide [16] seem preferable since they introduce lower chemical changes to final structure of depolymerized chitosan [17,14].

In this work we describe a modified approach for preparation of medical-grade oligochitosan hydrochloride suitable for application in pharmaceutical, food and cosmetic products and compositions.

Results and discussion

Acid hydrolysis of chitosan

As it has been shown, the rate of chitosan depolymerization by hydrochloric acid depends on DA and acetyl-groups distribution along chitosan polymer chains of parent chitosan, acid concentration, and temperature. Acid depolymerization of chitosan is very specific towards disruption of the chitosan chain bonds. The rate of depolymerization reduces in the order: between two N-acetylglucosamine (A-A) > N-acetylglucosamine-glucosamine (A-G or G-A) >> glucosamine-glucosamine (G-G) fragments. Partial deacetylation is also occurred and, therefore, DA of oligochitosan obtained by acid hydrolysis is lower than that of starting chitosan. The rate of depolymerization and deacetylation depends on the acid concentration so that the rate of depolymerization by 12M HCl acid is about one order faster than that of the rate of chitosan N-dеaсеtylation. Otherwise, the rate of depolymerization in 1-6M HCl is equal to that of the rate of deacetylation [18].

From the practical point of view, the method of chitosan depolymerization by 1M HCl seemed attractive since it produced no impact on the oligochitosan chemical structure although it represented a longer-lasting process than the concentrated acid is applied.



Figure 1. Impact of 1M HCl (a), 1.5% H2O2 in 1% acetic acid (b) and 1M HCl/1.5% H2O2 (c) on the weight average molecular weight of chitosan *versus* the reaction time (t)

As it is shown in (Fig.1a), the MW of CHI-p having the parent Mw 350 kDa and DA 12% falls very fast on the beginning of the process but afterwards the rate of hydrolysis is significantly reduced. This can be attributed to faster disruption of the glycoside bonds between two conjunct N-acetylglucosamine (A-A) groups and further slower disruption of the residual A-G and G-G fragments.

Chitosan depolymerization by hydrogen peroxide

Similar to chitosan hydrolysis in acidic media, the reaction rate and depolymerization depth depend on chitosan MW, DA and acetyl-groups distribution along chitosan polymer chains, hydrogen peroxide concentration, solution pH, and temperature. The mechanism of depolymerization involves the cleavage of glycoside bonds between non-protonated G-A and G-G fragments which concentration increases with increase of solution pH [19].

As it is shown in Fig. 1b, the depolymerization rate is very fast on first stage of the reaction but reduces as soon as most of G-A fragments exhaust. As a result, deeper depolymerization of chitosan by hydrogen peroxide represented a long-lasting process at low hydrogen peroxide concentration and requires higher hydrogen peroxide concentration and higher process temperature. Last parameters have been crucial for the product quality since the treatment of chitosan by hydrogen peroxide results both in reduction of MW and polymer chain backbone oxidation. These changes in the chemical structure and browning greatly increase with the decrease in MW of the product [20].

Oligochitosan preparation by the modified method

The joint application of acid and hydrogen peroxide (Fig.1c) leads to a much faster reduction of chitosan molecular weight so that oligochitosan with MW< 16 kDa and very low solution viscosity (Fig.2) can be obtained.



Figure 2. Kinematic viscosity of 1% oligochitosan solution *versus* the molecular weight values

The application of combined hydrochloric acid/hydrogen peroxide mixture allows both the producing of the product with a high yield (70-90%) and minimal chemical changes in the structure of oligochitosan and obtaining a narrow dispersed (Mw/Mn 1.2÷1.6) samples of oligochitosan with reduced content of acetylated units. The proposed method takes 40-60 min and allows obtaining oligochitosan hydrochloride with the quality corresponding to EU Pharmacopoeia requirements (Table 1).

Oligochitosan hydrochloride characterization

Oligochitosan hydrochloride with MW 10 kDa with reduced DA value as a consequences of a partial acid deacetylation of CHI-p was characterized in accordance with the prescriptions of EP 2004 (Table 1), and its chemical structure can be imaged as shown in Fig.4.



Figure 3. Chemical image of oligochitosan hydrochloride

Following the protocol, chitosan hydrochloride solution was characterized by the appearance of 1% solution, solubility in water, solution pH, degree of acetylation, loss on drying and the content of chlorides, sulphated ash and heavy metals.

Table 1. Quality characteristics of oligochitosan hydrochloride compared with the requirements of EP 4.0 for chitosan hydrochloride

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| Characteristics | EP 4.0 acceptable criteria for chitosan hydrochloride | Actual data for oligochitosan hydrochloride |
| Appearance (solid state) | white or almost white | From snow white to chalky white |
| Solubility in water | soluble | soluble |
| Matter insoluble in water,% | ≤0.5% | ≤0.02% |
| Color of 1% solution (brownish-yellow standards) | ≤ standard solution BY5 | ≤ standard solution BY6 |
| Appearance of solution | Opalescence ≤ the standard reference suspension II | Opalescence ≤ the standard reference suspension I |
| Viscosity of 1% solution in water | not specified (80-120% of the value stated on the label) | 1.03-1.20 cSt |
| Solution pH (1% w/v in water) | 4.0-6.0 | 2.6-2.8 |
| Degree of acetylation? % | unstandardized value | 1-2% |
| Chlorides, % | 10.0-20.0% | 16.0-16.5% |
| Heavy metals (ICP-MS), ppm | Total ≤40 ppm | Fe < 15 ppm; Cr <1 ppm; Ni < 1ppm |
| Loss on drying (100-105 0C), % | ≤10% | ≤10% |
| Sulfated ash, % | ≤1% | ≤0.01% |

As it follows from the Table 1, quality characteristics of oligochitosan hydrochloride are equal to the requirements or better than those required by EP 4.0 with the only exception: 1% oligochitosan solution has pH 2.6-2.8. This contradiction arises from the uncertainty of the required content of chlorides in chitosan hydrochloride. As it is shown in Fig. 5, pH value of 1% chitosan hydrochloride solution strongly depends on the molar glucosamine/hydrogen hydrochloride ration.

Figure 4. Dependence of 1% chitosan (DD = 98%) solution pH on hydrochloric acid/chitosan amino groups molar ratios

The content of chlorides (16.0-16.5%) found in oligochitosan hydrochloride is equal to 0.96-0.98 molar ratio of HCl/glucoacamine ratio and corresponds with the chitosan solution pH 2.6-2.8 as shown in Fig.4.

Experiment and Calculations

Materials

Chitosan (CHI-p) with MW 350 kDA and DA 12% was purchesed from BIOPROGRESS (Russia). Analytical grade hydrochloric acid (HCl) and 30% hydrogen peroxide (H2O2) were purchased from the MERCK.

Methods

Hydrolysis of 10 % chitosan (MW=350 kDa, DA 12%) solution in 1M hydrochloric acid

10 g of chitosan is loaded into a 150 ml flask equipped with a stirrer and a reverse refrigerator and placed in a water bath heated to 70 C. In the flask, pour 100 ml of 1% (weight/volume) acetic acid solution, or 100 ml of 1 M hydrochloric acid and 5 ml of 30 % hydrogen peroxide with stirring.

Degree of deacetylation (DD, mol. %) was determined by 1H-NMR method [14] using the facility of the Center for molecular composition studies of INEOS RAS.

Apparent weight average (Mw) and number average (Mn) molecular weights (MW) of oligochitosan hydrochlorides were determined by the high performance size exclusion chromatography as described in [21].

The kinematic viscosity of 1% aqueous solutions of oligochitosan was determined using a capillary viscometer (diameter 0.56 mm, viscometer constant 0.0093109 mm2/s2). Each solution was filtered through a Millipore filter with a pore diameter of 1 mm to remove impurities. The measurements were carried out at 25±0.5 0С.

Content of chlorides in oligochitosan hydrochloride was determined using the facility of the Laboratory for microanalysis (INEOS RAS).

Quality of oligochitosan hydrochloride was determined in accordance with the prescriptions of EP 2004 [3].

**Conclusions**

Preparation of medical-grade oligochitosan hydrochloride is a complicated process and requires a specific conditions combining application of hydrochloric acid and hydrogen peroxide application in order to avoid undesirable impacts of process condition on the quality of the final product. The quality of oligochitosan hydrochloride corresponding with the requirements of European Pharmacopoeia can be achieved at precise selection of parent chitosan and under precise control of the reaction parameters. It should be noted here that in order to prepare a medical-grade oligochitosan, precise conditions for depolymerization an initial chitosan should be selected dependently on its specific molecular weight and degree of acetylation.

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