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On the preparation and characterization of chitosan hydrochloride

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Summary

The procedures for the purification of commercial chitosan samples as hydrochlorides and in the neutralized form are described. Thermal analysis reveals that, independently of the purification method, all samples are highly pure. The agreement between values of degrees of acetylation determined by conductimetric titrations and by ^{1}H nmr spectroscopy is very good. The aqueous solutions of both forms of purified chitosan were free of aggregation as evaluated by their Huggins constants. The potential use of solutions of chitosan hydrochloride in acid-free aqueous NaCl for the studies aiming to characterize the solution behavior of chitosan is demonstrated.

Introduction

Chitin is a biodegradable and nontoxic polysaccharide widely spread among marine and terrestrial invertebrates and fungi (1). It is usually obtained from waste materials of the sea food-processing industry, mainly shells of crab, shrimp, prawn and krill. Native chitin occurs in such natural composite materials usually combined with inorganics, proteins, lipids and pigments. Its isolation calls for chemical treatments to eliminate these contaminants (2,3), some of which may be commercially explored (4,5). By treating crude chitin with aqueous 40-50% sodium hydroxide at 110-115°C chitosan is obtained (6). However, the fully deacetylated product is rarely obtained due to the risks of side reactions and chain depolymerization (7). Chitosan and chitin are closely related since both are linear polysaccharides containing 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units joined by ß(1→4) glycosidic bonds. They can be distinguished by their contents of the above-mentioned units and by their solubilities in aqueous media. The acetylated units predominate in chitin while chitosan chains contain mostly deacetylated units. Chitin is soluble in a very limited number of solvents while chitosan is soluble in aqueous dilute solutions of a number of mineral and organic acids, being the most common ones, the hydrochloric and acetic acids (8). In aqueous dilute acid media chitosan forms salts, producing polyelectrolyte chains bearing positive charges on the nitrogen atoms of their amine groups. In fact the salt of chitosan may be formed in a separate step or as a consequence of the presence of acid in the water suspension of the neutralized form of chitosan. The latter is the most usual practice but it implies that acid media must be used for the dissolution of the sample and for the study of chitosan in solution. Polyelectrolytes are usually purified as water soluble salts (9), which are then studied in aqueous media containing low molar mass salt for maintaining the ionic strength but, in general, without any added acid. Some authors claim that solutions formed by the dissolution of chitosan in aqueous NaCl 0,2 mol/L / acetic acid 0,1 mol/L are not good for such studies since the aggregation of the macromolecules occurs as a consequence of the poor thermodynamic quality of the solvent (10). This work describes the methodologies employed for the purification of chitosan samples as

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hydrochlorides and in the neutralized form. The degrees of acetylation and intrinsic viscosities are determined by employing different techniques and in different solvents, according to the characteristics of the purified samples. The intrinsic viscosity of chitosan purified as hydrochloride, a water soluble form of the polysaccharide, is determined in aqueous NaCl 0.1 mol/L and 0.2 mol/L without adding acid to them. The occurrence of aggregation is evaluated by determining the values of the Huggins constants in both solutions.

Experimental

Preparation of pure chitosan in the neutral and hydrochloride forms

The samples of chitosan were commercial products of high (sample A) and medium molecular weight (sample B), as informed by the manufacturer (Fluka-Biochimika / Swtizerland). Chitosan hydrochlorides were obtained by the dissolution of the crude commercial products (approximately 1 g) in 100 mL of dilute acetic acid (1% by weight) followed by filtration, dialysis and freeze-drying. The dissolution of the sample was assured by stirring the initial suspension in dilute acid during 18 hours. After that, the final solution was filtered through Millipore membrane (5.0 μm) and exhaustively dialyzed against aqueous solution of NaCl 0.2 mol/L and deionized water. White flakes of water soluble chitosan hydrochlorides were obtained by freeze-drying the solution. The purification of chitosan in the neutralized form was attained by carefully adding concentrated alkali to the filtered solution of the sample in dilute acetic acid. After the occurrence of the precipitation of the polysaccharide, it was filtered and exhaustively washed with water until neutrality and with methanol. The final product was a white powder soluble in dilute acetic and hydrochloric acids but insoluble in pure water.

Characterization of the purified forms of chitosan

The humidity degrees and the ash contents were determined by thermogravimetry, heating the samples (5-10 mg) under ambient air at a rate of 10°C/min between 25°C and 700°C. A thermogravimetric modulus DSC 2010 (TA Instruments) was used in those experiments. The average degrees of acetylation were determined by ^{1}H nmr spectroscopy of both purified forms of chitosans dissolved in D_2O/HCl (100:1 v/v) at 80°C, by using a 200 MHz spectrometer from Bruker. . The chitosan hydrochlorides average degrees of acetylation were also determined by the conductimetric titrations of their solutions in pure water at 25°C ± 0.1°C, by using a CD-21 conductivity meter from Digimed. The intrinsic viscosities were determined by capillary viscometry at 25°C ± 0.01°C of aqueous solutions previously filtered through 0.22 μm membranes. The chitosan hydrochlorides and the neutralized form of chitosans were studied in 0.1 mol/L and 0.2 mol/L NaCl and in 0.3 mol/L acetic acid / 0.2 mol/L sodium acetate aqueous solutions, respectively. The AVS-350 viscometer coupled to the AVS-20 automatic burette, both from Schott-Geräte, was used in those determinations. A glass capillary (ϕ=0.53 mm) was used and the solutions of the purified chitosans were sequentially diluted directly into it, by adding previously programmed volumes of the appropriate solvent.

Results and Discussion

Chitosan purification

Both methods of purification applied to the commercial chitosans resulted in losses of mass as evaluated by the amount of purified products which were recovered (70% - 85%). The higher recovery rates were observed in the preparation of chitosans purified in the neutralized form and for the medium molecular weight chitosan (sample B). The lower amounts of purified products were recovered when the purification procedures

were applied to the higher molecular weight chitosan (sample A). That may be attributed to the higher viscosities of its solutions, resulting in higher losses of mass during the purification procedures. The need of additional steps may be responsible for the lower recovery rates observed in the preparation of chitosan hydrochlorides. The losses of mass observed in the purification of chitosans in the neutralized form are comparable to the acid insoluble fractions recovered from the fractionation of commercial chitosans in terms of their solubilities in dilute acetic acid solutions (11). Therefore, those losses may be attributed, essentially, to the exclusion of acid insoluble materials and of aggregates, observable as gel-like substances retained in the filtration step. Their presence in commercial chitosans is a consequence of the fact that those products are prepared by heterogeneous deacetylation of chitin. Hence, they may correspond to poor deacetylated, chitin-like chains, which impart chemical heterogeneity to the commercial products (11).

Characterization of purified chitosans

The humidity degrees and the ash contents of the purified chitosan samples were both determined by thermogravimetric analyses. The former ones were determined as the mass losses up to 100°C and the latter, as the residual masses after burning at 700°C. The ash contents of the purified chitosan samples are very low in the case of hydrochlorides and virtually zero for the neutralized form (Table 1). That fact suggests that both methods of purification resulted in highly pure products. The chitosan hydrochlorides are, as expected from the presence of ionic charged groups along their chains, more hydrophilic than the samples purified in the neutralized form. Therefore, both purified forms of chitosan have polar sites, mainly hydroxyl groups, but only the chitosan hydrochlorides have protonated amine groups, which are responsible for their increased affinities for water. As a consequence, the humidity degrees of chitosan hydrochlorides are three to four times higher than those of chitosan samples prepared in the neutralized form (Table 1). The same behavior has been reported concerning the water adsorption capacities of ionic and neutral polysaccharides (12) and lignocellulosic materials (13). Also, cellulose derivatives containing carboxyl groups were more hydrophilic when in the sodium salt form (13).

Table 1: Humidity degrees (HD) and ash contents of purified chitosan samples determined by thermogravimetry [(a)].

SAMPLE	HD (%)	ASH (%)
A_N	5.1	0.0
A_{Cl}	19.0	3.4
B_N	5.0	0.0
B_{Cl}	16.6	0.7

a) the subscripts "N" and "Cl" stand for chitosans purified in the neutralized form and as hydrochlorides, respectively.

The most important region of the ^{1}H nmr spectrum of chitosan for the determination of its degree of acetylation is shown in Figure 1, with the attribution of signals as done in the literature (14,15). The degrees of acetylation were determined from the integral intensities of methyl protons from the acetamide group, I_{Met}, by relating them to the integral intensities due to the glycosidic ring protons.

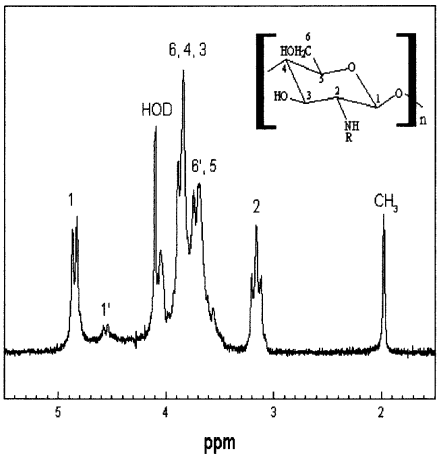

Figure 1: ^{1}H nmr spectrum of 1% solution of chitosan hydrochloride (C_{Cl}) in D_2O/HCl (100:1 v/v) obtained at 80^{0}C.

The calculations were done by using the expressions presented below and the degrees of acetylation were expressed as the values averaged from the use of both expressions.

$$\%DA = [(I_{Met} / 3) / (I_{H2})] \times 100 \quad (1)$$
$$\%DA = [(I_{Met} / 3) / (I_{H2}/6 / 6)] \times 100 \quad (2)$$

where:

- I_{Met} = integral intensity of the signal from the methyl protons of acetamide groups;
- I_{H2} integral intensity of the signal from the H atom bonded to the carbon 2 of the glycosidic ring.
- $I_{H2/6}$ = sum of integral intensities of the signals from the H atoms bonded to carbons 2, 3, 4, 5 and 6 of the glycosidic ring.

Table 2: Values of degrees of acetylation (DA) determined by ^{1}H nmr spectroscopy and by conductrimetric titrations of purified chitosans [(a)].

SAMPLE	DA_{NMR}	DA_{TIT}
A_N	24.2	-
A_{Cl}	21.4	23.0
B_N	21.4	-
B_{Cl}	19.8	21.4

a)the subscripts "NMR" and "TIT" stand for the determinations of degrees of acetylation by nuclear magnetic resonance spectroscopy and by conductimetric titrations, respectively.

The agreements between the values of the average degrees of acetylation determined by ^{1}H nmr spectroscopy and by titrations and of the samples purified in different forms are

very good (Table 2), indicating that both methods of purification are adequate to commercial chitosan samples.

The relationships between intrinsic viscosity and average molecular weight are commonly used to evaluate the degree of polymerization of macromolecules, as polymers and polysaccharides. If a whole set of data are properly determined, the resulting **K** and **a** parameters of the correspondent Mark-Houwink equation can be used to calculate average molecular weights. It may be done from practical and precise viscosity measurements performed at the very same experimental conditions as employed for the determination of **K** and **a**. The use of purified, monodisperse polymer samples in the determinations of intrinsic viscosity and molecular weight is called for obtaining a reliable relationship. However, various practical limitations make this ideal condition almost unattainable in most cases. The use of high performance gel permeation chromatography (HPGPC) systems, able to simultaneous determinations of concentration, viscosity and molecular weight of each fraction of the polymeric sample, is an expensive but very efficient alternative.

The curves relating reduced viscosities and polymer concentrations for the purified chitosans (Figure 2 and Figure 3) show that all experimental points are very well aligned along straight lines ($r > 0.999$) of similar slopes. It strongly suggests that an environment of constant ionic strength has been maintained around the dissolved polyelectrolyte chains during the dilution procedure, so that precise estimatives of their hydrodynamic volumes have been determined. The viscosity measurements performed with the purified chitosans allowed the determinations of their intrinsic viscosities, Huggins constants and viscosity average molecular weights (Table 3).

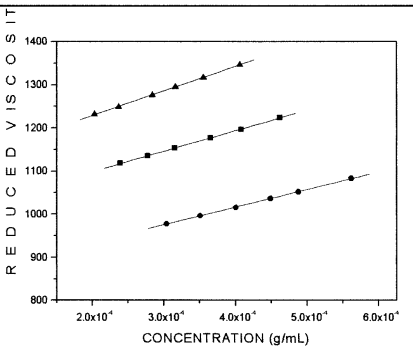

Figure 2: Curves of reduced viscosity against concentration for purified chitosans: (▲) sample A_N in aqueous acetic acid 0.3mol/L/sodium acetate 0.2mol/L; (■) sample A_{Cl} in aqueous 0.1mol/L NaCl; (●) sample A_{Cl} in aqueous 0.2mol/L NaCl.

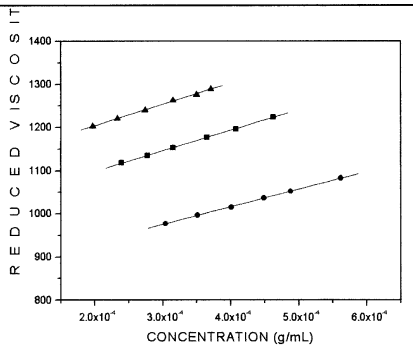

Figure 3: Curves of reduced viscosity against concentration for purified chitosans: (▲) sample B_N in aqueous acetic acid 0.3mol/L/sodium acetate 0.2mol/L; (■) sample B_{Cl} in aqueous 0.1mol/L NaCl; (●) sample B_{Cl} in aqueous 0.2mol/L NaCl

Table 3: Intrinsic viscosities of purified chitosans in aqueous 0.1 mol/L NaCl ($(|\eta|_{NaCl})^{0.1}$), 0.2 mol/L NaCl ($(|\eta|_{NaCl})^{0.2}$) and 0.3 mol/L acetic acid / 0.2 mol/L sodium acetate ($|\eta|_{AcOH}$), and viscosity average molecular weights ($\mathbf{M_V}$)[a].

SAMPLE	$(\|\eta\|_{NaCl})^{0.1}$	$(\|\eta\|_{NaCl})^{0.2}$	$\|\eta\|_{AcOH}$	$\mathbf{M_V}$
A_{Cl}	1003 (0.47)	853 (0.56)	-	1,260,000[b]
B_{Cl}	840 (0.43)	765 (0.50)	-	1,120,000[b]
A_N	-	-	1115 (0.46)	315,000[c]
B_N	-	-	1106 (0,40)	310.000[c]

a)values of Huggins constants are shown in parenthesis.
b)values calculated by using **K** and **a** from reference (16).
c)values calculated by using **K** and **a** from reference (10).

The intrinsic viscosities of the chitosan samples purified in the neutralized form, dissolved in aqueous acetic acid 0.3 mol/L / acetate of sodium 0.2 mol/L allowed the determination of their viscosity average molecular weights by using the expression proposed by Rinaudo et al. (10). The resulting values permit to qualify both samples as medium molecular weight chitosans but they are so close to each other, that one cannot say that the purified chitosans are different, when their viscosity average molecular weights are compared. If the parameters **K** and **a** adopted by Roberts et al. (16) for chitosans dissolved in aqueous 0.2 mol/L NaCl / acetic acid 0.1 mol/L are used, the differences between the calculated values of Mv for the two samples purified as hydrochlorides are slightly greater. However, from those values, one must classify both samples as high molecular weight chitosans.

The **K** and **a** parameters given by Rinaudo et al. (10) seem to be more coherent since they were determined by gel permeation chromatography (GPC) with multidetection. It means that intrinsic viscosity, concentration and molecular weight of fractionated chitosan samples were simultaneously determined for the construction of the resulting Mark-Houwink equation. Moreover, these **K** and **a** parameters seem to be adequate to our data, since in both studies commercial chitosans were purified by the same methodology and the purified samples were dissolved in the same solvent for the use in viscosity measurements performed at the same temperature. On the other hand, the second set of **K** and **a** parameters are not adequate, since it was determined in a different solvent, the main difference being the presence of acetic acid in its composition when compared with the solvent used in the present work. Moreover, some authors (10) claim that the overestimated values of the viscosity average molecular weights obtained by using these parameters may be attributed to the presence of large aggregates of chitosan chains due to the thermodynamic quality of that solvent. The values of the Huggins constant, *k'*, are usually employed to evaluate the thermodynamic quality of polymeric solutions and the presence of aggregates. Although a very wide range of *k'* values is attributed to them (17) and despite its use as a valid estimative of the solution quality has long been questioned, it is generally accepted that to polymers dissolved in good solvents correspond small *k'* values. On the other hand, the polymeric solutions of poor quality have the higher *k'* values. For a polyelectrolyte, *k'* increases as the ionic strength of its solution increases. Reflecting the progressive screening of electrostatic repulsions as the ionic strength increases, the macromolecular chains aggregate and the intrinsic viscosity

decreases. Considering the range of *k*' values described in the literature (10,18) for aggregate-free chitosan solutions, most of the solutions of chitosan studied in the present work are free of large aggregates. In fact, most of the Huggins constants shown in Table 3 are below 0.5 and only one attains more than 0.55. The values of the Huggins constants determined in aqueous 0.2 mol/L NaCl for both purified chitosans are not so higher than those determined in aqueous 0.1 mol/L NaCl (≅ 20%). If the absence of aggregation can be assumed, solutions of chitosan hydrochlorides in aqueous NaCl could be adequate for the determination of rigidity / flexibility of the chitosan chain and for its comparison with other polyelectrolytes from experimental measurements performed in this medium. Indeed, a study of the dependence of the intrinsic viscosity of the acid-free solutions of chitosan hydrochlorides in terms of the ionic strength, showed that the Huggins constants ranged from 0.35 to 0.63, while the ionic strength was varied from 0.06 mol/L NaCl to 0.3 mol/L NaCl (18). These values of the Huggins constants also indicate that these solutions are free of aggregates and adequate to the estimation of the chitosan chain rigidity from viscosity measurements. Supporting this assertive, the Smidsrod rigidity parameter (19) of the chitosan hydrochloride was determined and its value (B ≅ 0.060) (20) agrees with previously reported data (10).

Conclusions

The results show that the methodologies employed for purification of chitosan in the present work resulted in highly pure samples. The losses of 15% - 30% of the initial mass of the crude commercial products as insoluble materials and aggregates promoted the fractionation of the chitosan samples. As a consequence of this process, most of the high molecular weight chitosan chains were also excluded, making the two purified samples indistinguishable, concerning their viscosities in the studied solvents. The Huggins constants of the aqueous solutions of chitosan hydrochlorides in 0.1 mol/L and 0.2 mol/L NaCl and of chitosan samples purified in the neutralized form in 0.3 mol/L acetic acid / 0.2 mol/L sodium acetate are small and indicatives of the good solubilities of the purified samples in these solvents. The data suggest that both media, with and without low molar mass acid, are adequate for studies of chitosan in aqueous solution, indicating the potential use of the acid-free solutions of chitosan hydrochloride in studies aiming to compare chitosan and other polyelectrolytes. Also, they could be useful in the determinations of **K** and **a** parameters to relate intrinsic viscosity and average molecular weight of chitosan, as well as in other studies aiming to understanding the solution behavior of chitosan.

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