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0144-8617(94)00031-X

Carbohydrate Polymers **25** (1994) 65–70

0144-8617/94/$07.00

Water-solubility of partially *N*-acetylated chitosans as a function of pH: effect of chemical composition and depolymerisation

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(Received 25 March 1994; revised version received and accepted 9 May 1994)

The solubility of four partially *N*-acetylated chitosans with fraction of acetylated units (F_A) of 0.01, 0.17, 0.37 and 0.60 as a function of pH was investigated. The chitosan with $F_A = 0.60$ was soluble at all pH-values between 4 and 9. The solubility versus pH curve of the other chitosans, showed that all chitosans precipitated between pH 6 and 7.5, but with increasing solubility at higher pH-values with increasing F_A. Such solubility differences may have profound effects on enzyme accessability and biological effects of chitosans.

The three chitosans with the lowest F_A values were depolymerised by treatment with nitrous acid, and the fraction of water-soluble material at pH 7.5 was determined. The almost fully deacetylated chitosan was completely insoluble at pH 7.5 in the depolymerisation range investigated, while the most acetylated chitosan ($F_A = 0.60$) was fully soluble at all pH-values. However, the two chitosans with $F_A = 0.17$ and 0.37 could be fractionated into a neutral-soluble and a neutral-insoluble fraction. The amount of neutral-soluble material increased with decreasing depolymerisation. The neutral-soluble and the neutral-insoluble fraction differed in both chemical composition and degree of polymerisation. Generally, the neutral-soluble fraction had a higher fraction of acetylated units and a lower degree of depolymerisation than the neutral-insoluble fraction. This compositional heterogeneity of the degraded chitosans was shown to be consistent with what is expected from the theoretical random degradation of chitosans with a Bernoullian (random) distribution of acetylated and deacetylated units.

INTRODUCTION

Chitosan is commercially prepared by *N*-deacetylation of chitin (1→4-linked 2-acetamido-2-deoxy-β-D-glucopyranose—GlcNAc) and may be considered as a family of unbranched binary hetero-polysaccharides of β(1→4)-linked GlcNAc (**A**-unit) and 2-amino-2-deoxy-β-D-glucopyranose—GlcN (**D**-unit) of varying composition. The **A**- and **D**-units have been shown to be randomly distributed in water-soluble partially *N*-acetylated chitosans (Vårum *et al.*, 1991*a*, *b*).

It is well known that chitosan is insoluble in water at neutral and basic pH-values. Commercial chitosans usually contain a fraction of acetylated units (F_A) between 0 and 0.2, and are only soluble in aqueous solutions at acidic pH-values. This lack of water-solubility of commercial chitosans at neutral pH-values may be an advantage for certain applications (i.e. easy to remove from solutions by adjusting the pH) while it may complicate the use of chitosans in other applications (i.e. biological effects at physiological pH-values). Considerable efforts have been made in synthesising derivatives of chitin and chitosan (Roberts, 1992), and some of these derivatives, i.e. *N*-carboxymethylchitosan and chitosan phosphates, have been prepared in order to extend the water-solubility of chitosans to neutral pH.

It has, however, been shown that chitosans which are soluble at neutral pH-values can be made by controlling the fraction of acetylated units (F_A). Chitosans with full neutral-solubility were obtained with relatively high molecular weight chitosans with F_A between 0.4 and 0.6 (Sannan *et al.*, 1976). Both the physical-chemical and biological properties of chitosans are dependent on the chemistry of the polymer (F_A and the distribution of the monomers along the chain), the molecular weight, the pH, and the ionic strength of the solution. This study was aimed at comparing the solubilities of chitosans as

a function of pH, and at preparing well-defined chitosans with full neutral-solubility at pH 7.5. Some of the neutral-soluble fractions have been applied in a study where pH is restricted to neutrality (i.e. soluble chitosans' ability to induce TNF-α production from human monocytes (Otterlei *et al.*, 1994)).

MATERIALS AND METHODS

Chitosans

Four chitosans with different fractions of acetylated units were used in this study. All chitosans, except for Chit4, were prepared by heterogeneous deacetylation, and the acid-soluble fraction prepared as previously described (Vårum *et al.*, 1992). Chit1 ($F_A = 0.01$) was prepared by further heterogeneous deacetylation of a commercial chitosan, while Chit4 was prepared by homogeneous deacetylation. The chitosans were converted into the chloride salt (chitosan-HCl) by dissolution in acetic acid, dialysis against excess 0.2 M NaCl and further dialysis against distilled water. The chitosan-hydrochlorides were finally filtrated and lyophilised. No detectable amount of acetate could be determined in the 500 MHz NMR of the chitosans, where the acetate methylprotons are well separated from the other protons on chitosan (including the acetyl protons)

Fractionation of undegraded chitosans

Equal volumes of a 1% chitosan solution in 0.1 M NaCl and a solution containing a certain amount of NaOH were mixed. The pH-value obtained was determined after leaving the solution for 30 min, and thereafter centrifuged. The distribution of the chitosan between the centrifugate and the precipitate was determined by weighing the fractions (after dialysis and lyophilisation). The procedure is based on pH-solubility experiments with alginates described by Haug and Larsen (1963).

Nitrous acid degradation of chitosans

Solutions of chitosan hydrochloride (chitosan-HCl) were made by dissolving the chitosan in distilled water overnight, which was then diluted with an equal volume of 4% acetic acid, and the desired amount of $NaNO_2$ added. The next day the solution was reduced conventionally with sodiumborohydride. The chitosan was converted to the hydrochloride salt, as described above, filtrated and lyophilised.

Hydrochloric acid degradation of chitosans

To a solution of chitosan-HCl (Chit3) in distilled water was added HCl to pH 2.5. The solution was heated to 80°C for approximately 100 h, cooled and the reaction stopped by adjusting the pH to 4.5 with NaOH. The chitosan was converted to the chitosan-HCl salt, filtrated and lyophilised.

Fractionation of degraded chitosans

Solutions of the degraded chitosans were made by dissolving the chitosan in distilled water overnight. NaOH was added dropwise to the chitosan solution, while the pH was continually monitored until it became stable at pH 7.5 for 30 min. The precipitates were easily separated from the supernatant, with little or no gel-like precipitates, while the sodiumhydroxide was added. The solutions were centrifuged, and the supernatant and precipitate separated by decanting. The supernatant was converted to the chitosan-HCl salt, lyophilised and weighted (neutral-soluble fraction). The precipitate was solubilised by adding 2% acetic acid, converted to the chitosan-HCl salt, lyophilised and weighted (neutral-insoluble fraction).

Chemical composition

The fraction of acetylated units and the diad frequencies were determined by high-field proton NMR-spectroscopy as previously described (Vårum *et al.*, 1991*a*).

Physical properties

Intrinsic viscosities of chitosans were determined as previously described (Draget *et al.*, 1992). The number-average molecular weights were estimated from the Mark–Houwink–Sakurada equation as reported by Anthonsen *et al.* (1993). The degree of scission was calculated as $1/\overline{DP_n}$, where $\overline{DP_n}$ is the number-average degree of polymerisation.

Calculation of compositional distribution of oligomers

The compositional distributions of oligomers of different lengths when a binary copolymer is randomly degraded and the distribution of monomer units along the chain is random, are binomial (Frensdorff and Pariser, 1963). Thus, the equation for the calculation of the mole fractions (f) of an n-mer containing x **A**-units and $(n-x)$ **D**-units is:

$$f(\mathrm{A} = x) = \binom{n}{x} F_A^x (1 - F_A)^{n-x},$$

where F_A is the fraction of acetylated units in the undegraded chitosan molecule.

RESULTS AND DISCUSSION

The insolubility in water at neutral and basic pH-values is a well-known property of commercial chitosans.

Table 1. Chemical composition and physical properties of chitosans

Sample	F_A	F_{AA}	$F_{AD} = F_{DA}$	F_{DD}	$\overline{N_A}$	$\overline{N_D}$	$[\eta]$ (ml/g)	$\overline{M_n}$
Chit1	<0·01	—	—	1·00	—	—	852	310 000
Chit2	0·17	0·05	0·12	0·71	1·4	6·9	887	230 000
Chit3	0·37	0·17	0·18	0·47	2·1	3·5	956	250 000
Chit4	0·60	0·40	0·21	0·18	2·9	1·9	813	164 000

Except for the study of Sannan and coworkers (Sannan *et al.*, 1976), little is known about the factors other than chemical composition influencing the precipitation of chitosan by addition of alkali. In the present investigation, standardised conditions have been used for the precipitation, and the solubility versus pH curves of undegraded chitosans varying in chemical composition were studied. In addition, the effect of depolymerisation on the solubility of the chitosans at neutral pH-values was investigated.

Four chitosans with fractions of acetylated units (F_A) of 0.0, 0.17, 0.37 and 0.60 were chosen in this study. It should be stressed that all four chitosans were fully water-soluble at acidic pH-values. Their chemical composition and physical properties are summarised in Table 1.

Solubility of undegraded chitosans as a function of pH

The solubility of the chitosans as a function of pH was investigated by mixing a solution of the chitosan and dilute sodium hydroxide. The amount of precipitated chitosan was determined at increasing pH-values. Figure 1 shows the results for the four chitosans with F_A of 0.01 (Chit1), 0.17 (Chit2), 0.37 (Chit3) and 0.60 (Chit4), which were all of approximately equal intrinsic viscosities ($[\eta]$ of 852, 887, 956 and 813 ml/g, respectively). Chit4 showed the expected full water-solubility at all pH-values. By the technique used in this work, the precipitates for Chit1 and Chit2 were well-defined with little water entrapped, while the precipitates for Chit3 were more gel-like particles as obtained by precipitation of alginate with acid (Haug & Larsen, 1963). However, the volume fraction of the gel was in all cases less than 10% of the total volume. The result in Fig. 1 shows that most of the material is precipitated within a pH-range of about one pH-unit. With these three chitosans, all of the material is precipitated at pH 7.5, except for Chit3 where a small fraction (~5%) is soluble also above pH 7.5. Although the precipitation curves of the three chitosans presented in Fig. 1 are similiar, it can be seen that the solubility at higher pH-values increases with increasing F_A. This may be explained by a small increase in the appearent pK_a-value with increasing F_A, as observed by Domard (1987). However, Anthonsen and Smidsrød (1994) found that the pK_a-value of low molecular weight chitosans varied very little with their chemical composition. The differences in solubility may also be explained by a decreased possibility of aligning the polymer chains (precipitates) caused by the increasing amount of A-units.

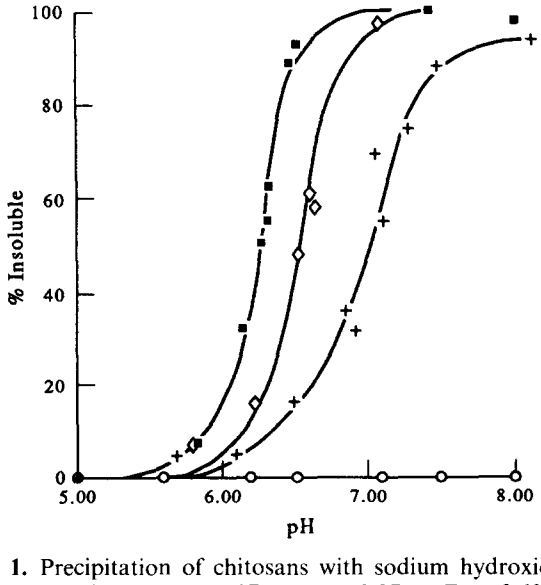

Fig. 1. Precipitation of chitosans with sodium hydroxide. ■ $F_A = 0.01$, ◇ $F_A = 0.17$, + $F_A = 0.37$, ○ $F_A = 0.60$.

It should be emphasised that the solubility differences between chitosans demonstrated in Fig. 1 may have profound influence on accessibility of chitosans to enzymes (many enzyme assays are performed at pH 6.5) and biological effects of chitosans (testing of chitosans *in vivo* and *in vitro* are often performed at neutral pH-values). Thus, solubilities of the chitosans may be as important as their chemical compositions to the effect in question, as demonstrated recently by Otterlei *et al.* (1994).

Solubility of degraded chitosans at neutral pH

Chit3 contained a small fraction of material soluble above pH 7.5 (see Fig. 1) with a chemical composition and degree of depolymerisation different from the original material. This encouraged us to study systematically the effect of depolymerisation on the neutral-solubility of the three chitosans. A convenient way of depolymerising chitosan is by nitrous acid, as first described by Ambrecht (1919). Nitrous acid attacks D-units but not A-units in the chitosan molecules, the following glycosidic linkage towards the reducing end is cleaved, and a 2,5-anhydro-D-mannose unit is generated at the new reducing end, which is then reduced by $NaBH_4$. This reduction was performed in order to

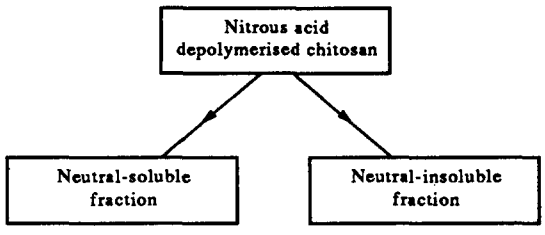

Fig. 2. Fractionation of depolymerised chitosan.

eliminate the reactive aldehyde groups (both on the original reducing ends and on the new reducing ends after nitrous acid degradation) to less reactive hydroxyl groups. Each chitosan was depolymerised using nitrous acid to different chain lengths, and the amount of neutral-soluble material was determined. The fractionation scheme is shown in Fig. 2.

The almost fully deacetylated chitosan was completely neutral-insoluble in the depolymerisation range investigated (22–50 mg $NaNO_2$/g chitosan, giving intrinsic viscosities from 20–50 ml/g). It seems therefore that a fully deacetylated chitosan must be degraded to oligomers in order to be soluble at neutral pH (Domard *et al.*, 1991).

The partially acetylated chitosans ($F_A = 0.17$ and $F_A = 0.37$) could, however, be separated into a neutral-soluble and a neutral-insoluble fraction. For both chitosans, the fraction of neutral-soluble material increased with increasing depolymerisation, as shown in Fig. 3. The more acetylated chitosan ($F_A = 0.37$) gives higher amounts of neutral-soluble material at lower degrees of depolymerisation. Thus, the neutral-solubility of partially *N*-acetylated chitosans is determined by both the chemical composition *and* the degree of depolymerisation (molecular weight) of the polymer. Relatively high molecular weight chitosans are only neutral-soluble when $0.4 < F_A < 0.6$, as shown by Sannan *et al.* (1976). However, we demonstrate here that low-viscosity neutral-soluble chitosans can also be made by depolymerisation of commercially available chitosan products with $F_A \approx 0.2$.

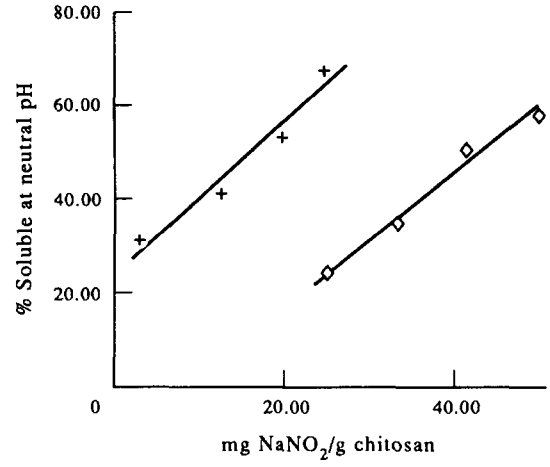

Fig. 3. Solubility of chitosans at neutral pH as a function of depolymerisation.

The chemical compositions and the intrinsic viscosities (which can be converted into a molecular weight) of the neutral-soluble and the neutral-insoluble fraction were determined. The results are shown in Table 2 for the two more acetylated chitosans (Chit2 and Chit3). Firstly, it is noted that the neutral-soluble and the neutral-insoluble fraction have different chemical compositions (F_A values). For Chit2 with F_A equal to 0.17, the F_A values of the neutral-soluble fractions

Table 2. Fractionation of nitrous acid degraded chitosan samples by precipitation at pH 7·5

Sample	Amount $NaNO_2$ (mg/g chitosan)	% soluble/ insoluble	F_A	$[\eta]$ (ml/g)	$\overline{M_n}$	Degree of scission (α)	$\overline{DP_n}$
Chit2 (F_A = 0·17)							
SOLUBLE	25	24·0	0·30	30	3800	0·046	22
INSOLUBLE		76·0	0·14	60	6000	0·028	36
SOLUBLE	33·5	34·4	0·27	24	2900	0·060	17
INSOLUBLE		65·6	0·13	54	5100	0·033	30
SOLUBLE	41·5	49·4	0·25	21	2500	0·080	13
INSOLUBLE		50·6	0·12	38	3200	0·053	19
SOLUBLE	50	57·3	0·25	20	2300	0·081	12
INSOLUBLE		42·7	0·12	32	2500	0·067	15
Chit3 (F_A = 0·37)							
SOLUBLE	3	31·0	0·45	195	42 000	0·0043	230
INSOLUBLE		69·0	0·34	235	48 000	0·0037	270
SOLUBLE	12·5	41·1	0·44	79	16 000	0·011	91
INSOLUBLE		58·9	0·34	103	19 000	0·0094	110
SOLUBLE	20	52·8	0·45	57	12 000	0·015	67
INSOLUBLE		47·2	0·37	76	14 000	0·013	77
SOLUBLE	25	66·9	0·46	50	10 000	0·018	56
INSOLUBLE		33·1	0·37	52	9000	0·020	50

vary from 0.25 to 0.30, while the F_A vlaues of the neutral-insoluble fractions vary from 0.12 to 0.14. For Chit3, with F_A equal to 0.37, the F_A values of the neutral-soluble fractions vary from 0.44 to 0.46, while the F_A values of the neutral-insoluble fractions vary from 0.34 to 0.37. The neutral-soluble fractions contain chitosan with higher F_A values than the neutral-insoluble fractions at all degrees of depolymerisation. It should be stressed that the undegraded chitosans (see Table 1) were fully water-soluble at acidic pH, and with a close to random distribution of acetyl groups, as judged from the diad frequencies (F_{AA}, F_{AD}, F_{DA} and F_{DD}; see Table 1). The fractionated chitosans also had a close to random distribution of acetyl groups (data not shown). Secondly, it is noted that the neutral-soluble and the neutral-insoluble fractions have different intrinsic viscosities and therefore also different molecular weights. The neutral-soluble fractions contain chitosan with lower molecular weights than the neutral-insoluble fractions. Thus, the fractionation of partially acetylated chitosan at pH 7.5 results in a soluble chitosan fraction with high F_A and low molecular weight, and an insoluble fraction with low F_A and high molecular weight.

In order to exclude the possibility that the non-random degradation method (nitrous acid) caused the compositional heterogeneity, a control experiment was performed by acid-depolymerisation of the more acetylated chitosan (Chit3). The chitosan was degraded at pH 2.5 (using hydrochloric acid) at 80°C and fractionated as described above. The result of the fractionation was a soluble fraction with $F_A = 0.44$ and $[\eta] = 126$ ml/g and an insoluble fraction with $F_A = 0.33$ and $[\eta] = 171$ ml/g. The result was as expected for the compositions of the fractions after nitrous acid degradation, and we conclude that the nitrous acid degradation does not cause the present results.

It is clear that any binary heteropolysaccharide, even when originally unimodally distributed, upon degradation will become heterogeneous with respect to composition, since a simple mixture of the monomers will finally be obtained. The degree of scission at which compositional heterogeneity first appears, and its relation to the distribution of the monomers along the chain in the undegraded copolymer, have been analysed theoretically (Painter *et al.*, 1968). The degree of scission (α) in our degradation experiments varies from 0.0037 to 0.081 ($\overline{DP}_n$ from 12 to 270). So can the fractionation in Table 2 be explained by the random degradation of a binary heteropolysaccharide, assuming that the **A**- and **D**-units are randomly distributed along the chain? In order to answer this question, the compositional distribution was calculated of oligomers of length 12, 15 and 20, when a chitosan with $F_A = 0.17$ is degraded. The result is shown in Fig. 4. From the compositional distribution of 12-mers (Fig. 4a), it is seen that ~10% of the 12-mers do not contain any **A**-units, while ~10% contain four **A**-units. The effect of increasing chain length is also shown in Fig. 4(b) and (c). We conclude that the degradation of a chitosan with a random distribution of **A**- and **D**-units, and the fractionation shown in Table 2, is clearly possible. The fractionation of the degraded chitosans into neutral-soluble and neutral-insoluble fractions is governed by their chemical composition, degree of polymerisation and, possibly charge, and we have so far not attempted to model the experimental data in Table 2 in more detail.

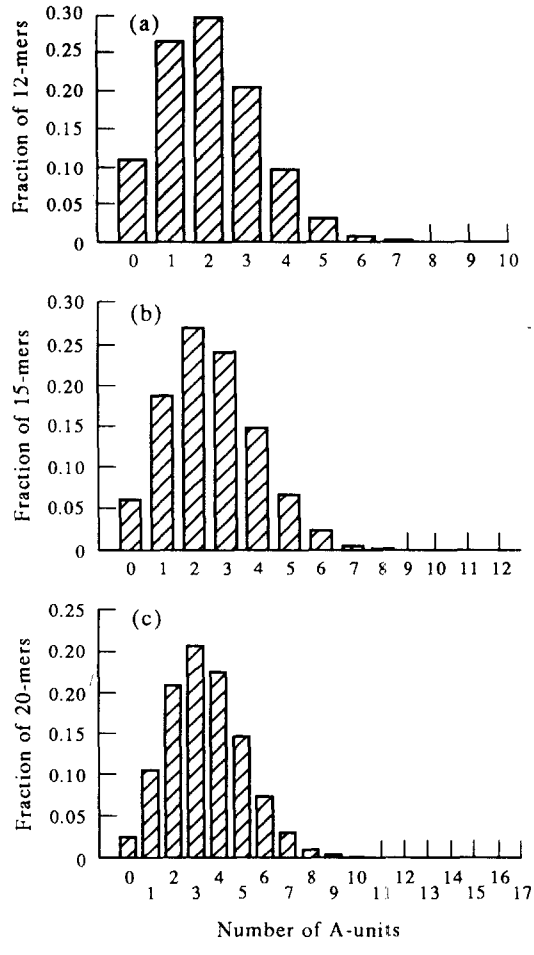

Fig. 4. Compositional distribution of oligomers of different lengths (model data) assuming a random degradation of a chitosan with $F_A = 0.17$, and a random distribution of A and **D**-units. a, b and c show the compositional distribution of oligomers of length 12, 15 and 20, respectively.

ACKNOWLEDGEMENTS

We thank Lillian Nergaard and Siv Moen for skilful technical assistance. Financial support has been provided by the Research Council of Norway (formerly NFFR and NTNF) and Pronova Biopolymer (Norway).

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