ELSEVIER

0144-8617(95)00154-9

Carbohydrate Polymers, Vol. 29, No. 1, pp. 17–24, 1996

0144-8617/96 $15.00+.00

Compositional heterogeneity of heterogeneously deacetylated chitosans

Mette H. Ottøy, Kjell M. Vårum & Olav Smidsrød

Norwegian Biopolymer Laboratory (NOBIPOL), Department of Biotechnology, The Norwegian Institute of Technology (NTH), University of Trondheim, N-7034 Trondheim, Norway

(Received 13 March 1995; revised version received 10 September 1995; accepted 29 September 1995)

Commercial chitosans prepared by heterogeneous alkaline deacetylation with different F_A values were fractionated into acid-soluble and acid-insoluble fractions. The amount of acid-soluble fractions increased with increasing time of deacetylation. The acid-soluble fractions were characterized with respect to fraction of acetylated units (F_A), diad frequencies, intrinsic viscosities, molecular weights and molecular weight distributions. F_A values for the acid-insoluble fractions were obtained using CPMAS ^{13}C NMR spectroscopy. As a control, the F_A values for the acid-soluble fractions determined from CPMAS ^{13}C NMR spectroscopy were compared and found to be consistent with the F_A values from ^1H NMR spectroscopy. The diad frequencies indicated that the acetyl groups in the acid-soluble fractions of the commercially heterogeneously deacetylated chitosans were randomly distributed. Determination of $[\eta]$, M_w and M_w/M_n indicated negligible depolymerization of the acid-soluble fractions when deacetylation was performed under a nitrogen purge at 75°C. Chemical characterization revealed compositional heterogeneity in the chitosans, with chitin-like acid-insoluble fractions with F_A values between 0.88 and 0.95, and acid-soluble fractions with F_A values from 0.20 to 0.52. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chitin is the second most abundant polysaccharide in the world after cellulose. It is present as one of the main components in the exoskeleton of animals with an outer backbone. The main resources for commercial utilization of chitin are the crustaceans (crab and shrimp shell waste), because of their apparent abundance. Chitin is a linear polymer of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and it is insoluble in aqueous solvents.

Chitosan, the copolymer of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN), is, in contrast to chitin, soluble in aqueous solvents. Chitosan is rare in nature, almost exclusively found in Zygomycetes cell walls (Davis & Eveleigh, 1984). Commercial chitosan is obtained from chitin by heterogeneous alkaline deacetylation, and is positively charged at acidic pH values, which makes it interesting for several applications, e.g. agriculture, wastewater management, biotechnology, nutrition and cosmetics (Hirano, 1988; Sandford, 1988).

Chitin can be converted to chitosan by homogeneous or heterogeneous alkaline deacetylation (Kurita *et al.*, 1977; Vårum *et al.*, 1991*a*) or by enzymatic deacetylation (Araki & Ito, 1975). The effect of the fraction of acetylated units (F_A) and chain length on the solubilities of chitosans upon neutralization of the amino groups have been investigated (Sannan *et al.*, 1976; Vårum *et al.*, 1994) It has also been noticed that homogeneously deacetylated chitosans with F_A lower than 0.6 are fully soluble, whereas heterogeneously (e.g. commercially) deacetylated chitosans are only partially soluble in weak acidic solutions (Pelletier *et al.*, 1990; Terbojevich *et al.*, 1992).

^1H NMR (Vårum *et al.*, 1991*a*) has been used to determine F_A and distribution of acetyl groups (e.g. diad frequencies; F_{AA}, F_{DD}, F_{AD} and $F_{DA}V$) for acid-soluble chitosans, and from ^1H NMR and ^{13}C NMR results, it was concluded that the acetyl groups in both heterogeneously and homogeneously deacetylated chitosans were distributed randomly (Vårum *et al.*, 1991*a*, *b*). Sashiwa and co-workers used nitrous acid deamination (degradation of the glycosidic linkage at the reducing end of GlcN) and GPC of the GlcNAc oligomers to show the presence of randomly distributed acetyl groups in both heterogeneously and homogeneously deacetylated chitosans (Sashiwa *et al.*, 1991, 1993). The studies of Aiba (1991) and Kurita *et al.* (1977) suggested that chitosan obtained by heterogeneous alkaline deacetylation of chitin had a

block-type distribution of acetyl groups along the polymer chain. However, their conclusions were made on the basis of qualitative experiments (crystallinity, precipitation and swelling studies), with no quantitative measurements.

Determination of reliable F_A values for the acid-insoluble fractions by use of a solid-state technique is more complicated than determination of F_A values for the acid-soluble fractions. In a preliminary study we used elemental analysis (EA) to determine the chemical composition of the acid-insoluble fractions (Vårum *et al.*, 1992). However, this method turned out to have serious disadvantages (Pelletier *et al.*, 1990). Cross-polarization/magic angle spinning (CPMAS) ^{13}C NMR has been used to determine F_A values (Niola *et al.*, 1993; Pelletier *et al.*, 1990; Raymond *et al.*, 1993; Saito *et al.*, 1982).

Hasegawa *et al.* (1994) asserted in their recent paper that chitin was depolymerized when deacetylated to chitosan. In the work presented by Nud'ga *et al.* (1971), a depolymerization was noted under specific process conditions.

Very little is known about the differences in chemical composition between the acid-soluble and acid-insoluble fractions of heterogeneously (e.g. commercially) deacetylated chitosans (Pelletier *et al.*, 1990; Terbojevich *et al.*, 1992). To further investigate whether these chitosans have compositional homogeneity (as defined in the work of Painter *et al.*, 1968) or compositional heterogeneity, we fractionated five commercial chitosans with different F_A values into acid-soluble and acid-insoluble fractions.

The acid-soluble fractions of the commercial chitosans used in the present study were characterized by use of viscometry (i.e. determination of intrinsic viscosity) and HPGPC–LALLS–DRI (i.e. determination of molecular weight/polydispersity indices) in order to observe eventual degradation under the present experimental conditions. In the studies of Vårum *et al.* (1991*a*, *b*) and Sashiwa *et al.* (1991, 1993), the chitosans were heterogeneously deacetylated in the laboratory. In the present study we wanted to see whether the acetyl groups in heterogeneously (e.g. commercially) deacetylated chitosans were randomly distributed or had a block-type distribution. We used CPMAS ^{13}C NMR to determine F_A values in the acid-insoluble fractions, and we compared the F_A values for the acid-soluble fractions obtained by CPMAS ^{13}C NMR with the results obtained by ^1H NMR.

METHODS

Chitosans

Five commercial chitosans (Pronova Inc., Raymond, Washington, USA) made from Dungeness crab chitin (5–40 mesh size) were used in the experiments. The chitosans were made from chitin by heterogeneous alkaline deacetylation at 75°C and under a nitrogen purge. The ratio between 50% (w/v) caustic and chitin was 3.2:1. The chitosans were removed from the reactor at 2 h intervals (2 h, 4 h, 6 h, 8 h, 10 h).

Fractionation of heterogeneously deacetylated chitosans into acid-soluble and acid-insoluble fractions

Chitosan (1.0 g) was mixed with 1% (v/v) acetic acid (100 ml). The suspension was left on a shaker overnight, centrifuged and decanted. The precipitate was mixed with 1%(v/v) acetic acid once more, left on a shaker overnight, centrifuged and decanted again. The precipitate was washed twice with distilled water (100 ml), dried and weighed (acid-insoluble fraction). The combined supernatants were dialysed against 0.2 M NaCl and distilled water, freeze-dried and weighed (acid-soluble fraction).

Characterization of the acid-soluble fractions

Viscometry

The intrinsic viscosities $[\eta]$ were determined in a Schott-Gerate Ubbelohde viscosimeter as described previously by Draget *et al.* (1992). The solutions were filtered through Millipore AA-filters (0.8 μm) before determining $[\eta]$.

Determination of weight average molecular weights (M_w) *and polydispersity indices* (M_w/M_n)

The M_w values and (M_w/M_n) values were obtained using a HPGPC–LALLS–DRI system. This is a low angle laser light scattering photometer (Chromatix, KMX-6) in conjunction with a high-pressure gel permeation chromatography column and a differential refractive index detector (Shodex RI SE-61). The LALLS instrument had a light source of 633.5 nm and was operating at a scattering angle of 4.8°. The dn/dc-value used in all experiments was 0.162. The column was packed with macroporous, monodisperse particles based on poly(styrene-divinylbenzene) with a separation range of ca. $10^5 - 10^7$ (pullulan standards) (Christensen *et al.*, 1996). The solvent used in all experiments was 0.2 M ammonium acetate, pH 4.5, the sample concentration was 0.1 or 0.2 mg/ml and the amount of injected sample was 0.5 or 1 ml. Before injection, the solutions were filtered through Millipore HA filters (0.45 μm).

^1H NMR spectroscopy

The F_A values and the diad frequences of the acid-soluble fractions were obtained by ^1H NMR spectroscopy as described previously by Vårum *et al.* (1991a).

Characterization of the acid-insoluble fractions

Elemental analysis
The carbon and nitrogen content of the acid-insoluble fractions were determined in a Carlo Erba Elemental Analyser. The amount of sample was 500–700 μg, and 8 parallels were analysed for all the fractions.

Determination of protein content
Chitosan (50 mg) was mixed with 5 M urea (1 ml). The sample was left to react for 30 min at 95°C with periodic vortexing. Afterwards the sample was cooled to room temperature and distilled water (0.5 ml) was added. The sample was the centrifuged at high speed for 2 min and decanted. Distilled water (400 μl) was added to the supernatant (400 μl). The protein content of the sample was determined from the microassay procedure of the BIO-RAD protein assay.

Solid-state ^{13}C NMR spectroscopy
The spectra were recorded at 50.33 MHz on a BRUKER MSL 200 spectrometer. The cross-polarization pulse sequence was utilized for all the samples, which were spun at the magic angle at 4 kHz. A contact time of 1 ms and a pulse repetition time of 3 s were used, and 2048 scans were accumulated. Approximately 300 mg of sample was inserted into a 7 mm ceramic rotor. *Preparation of acid-soluble fractions*: The freeze-dried sample (0.5 g) was soaked in a 10:1 acetone/water mixture (55 ml) in a petri dish. The sample was left to dry at elevated temperature for several hours. The sample was ground in a mill (Mikro-Feinmühle-Culatti, Janke and Kunkel, IKA-werk). *Calculation of F_A values*: The F_A values were determined from the relative intensities of the resonances (Vårum *et al.*, 1991a) of the ring carbons (I_{C1}, I_{C2}, I_{C3}, I_{C4}, I_{C5}, I_{C6}) and the methyl-carbon (I_{CH_3}) from the following equation:

$$F_A = \frac{I_{CH_3}}{(I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6})/6} \quad (1)$$

RESULTS AND DISCUSSION

Fractionation of heterogeneously deacetylated chitosans into acid-soluble and acid-insoluble fractions

Five different commercially *N*-deacetylated chitosans (2 h, 4 h, 6 h, 8 h and 10 h) were fractionated into acid-soluble and acid-insoluble fractions using the fractionation scheme shown in Fig. 1. The percentage of acid-soluble chitosan as a function of time of deacetylation is shown in Fig. 2, and it is observed that the amount of acid-soluble material increases with increasing time of deacetylation. The present data were obtained from a full-scale commercial deacetylation process, and it should be noted that process parameters like chitin particle size, chitin source, temperature and NaOH concentration etc., will influence the time scale on the *x*-axis.

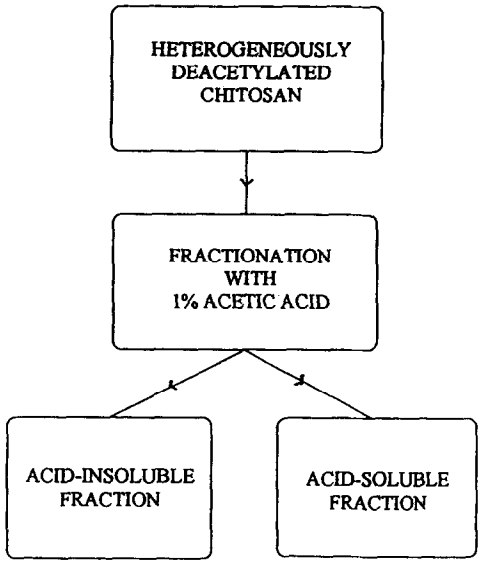

Fig. 1. Fractionation of heterogeneously deacetylated chitosans.

Characterization of the acid-soluble fractions

The results of the physical-chemical characterization of the acid-soluble fractions are summarized in Table 1. Reliable weight-average molecular weights of polysaccharides are sometimes difficult to obtain because of aggregate formation in solution. It has been demonstrated (Anthonsen *et al.*, 1994) that chitosan solutions may contain a small fraction (approximately 5%) of concentration-dependent aggregates in solution. The presence of a small fraction of high molecular weight aggregates will dramatically increase the value of the weight-average molecular weight (M_w), and thereby

Table 1. Intrinsic viscosities, weight-average molecular weights, polydispersity indices, fraction of acetyl groups and diades for the acid-soluble fractions

Sample	2 h	4 h	6 h	8 h	10 h
$[\eta]$ (ml/g)	1000	1220	1180	1220	1120
$M_w \times 10^{-5}$	5.5	5.9	5.7	5.3	5.2
M_w/M_n	2.0	2.1	2.1	1.9	2.2
F_A	0.52	0.35	0.24	0.23	0.20
F_{AA}*	0.28	0.15	0.08	0.08	0.06
	(0.27)	(0.12)	(0.06)	(0.05)	(0.04)
$F_{AD} = F_{DA}$*	0.24	0.21	0.15	0.15	0.13
	(0.25)	(0.23)	(0.18)	(0.18)	(0.16)
F_{DD}*	0.24	0.45	0.62	0.62	0.67
	(0.23)	(0.42)	(0.58)	(0.59)	(0.64)

*The captured figures are calculated with the assumption of a Bernoullian distribution.

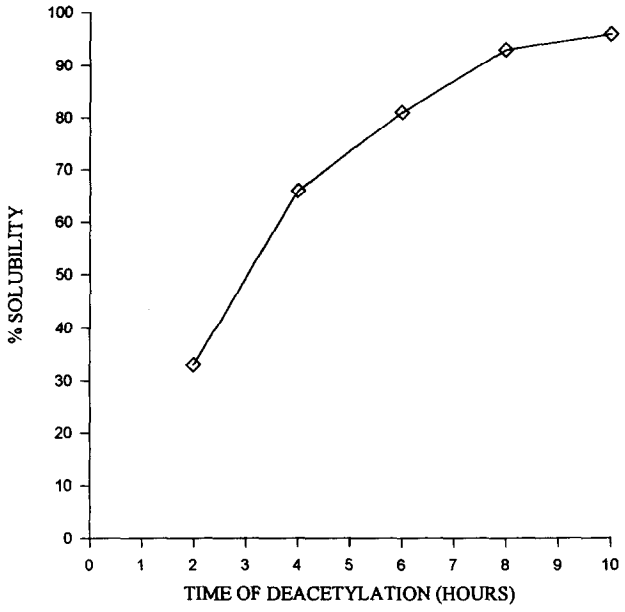

Fig. 2. Percent acid-soluble chitosan as a function of time of deacetylation.

make light scattering unsuitable for molecular weight measurements (Anthonsen *et al.*, 1994). In the present study we used HPGPC–LALLS–DRI as a method for determining M_w. The relatively high molecular weights of the samples made it possible to measure detectable scattered light on low-concentrated solutions in which the concentration of aggregates was negligible (Ottøy *et al.*, 1996). We have used HPGPC–LALLS–DRI because we also can achieve information on the molecular weight distribution, and the polydispersity indices (M_w/M_n) for the samples can be calculated.

The M_w and [η] values of all the samples are roughly the same (within the limits of experimental error), suggesting no degradation during the deacetylation process. However, it may be noted that the [η] value for the 2 h sample is somewhat lower than for the other samples. This is the most acetylated sample ($F_A = 0.52$), and hence, when the M_n value is higher than approximately 10^5, the [η] value corresponding to a certain molecular weight should be higher for this sample than for the other samples (Anthonsen *et al.*, 1993). The chitosans were fractionated into acid-soluble and acid-insoluble fractions with 1% (v/v) acetic acid (pH 2.7). The [η] and M_w values were determined in 0.02 M HAC/NaAC, pH 4.5 with 0.1 M NaCl, in which the acid-soluble fraction of the 2 h sample contained a small insoluble fraction, which was removed upon filtration before determining [η] and M_w. No correction was made for the [η] value, and this resulted in a too low [η] value. The M_w value was corrected taking into account the somewhat lower area under the elution curve from the DRI detector of this sample, compared to the other samples. The conclusion that no degradation took part during the deacetylation process does not agree with the work of Hasegawa *et al.* (1994) where it was claimed that chitin was degraded when heterogeneously deacetylated to chitosan. This may be explained from the lower temperature (75°C) used in our experiments compared to the work of Hasegawa *et al.* (1994) (95°C). In addition, the samples characterized herein were flushed with nitrogen during deacetylation and this probably prevented oxygen from degrading the polymer chain by oxidative–reductive depolymerization (ORD) (Korycka-Dahl & Richardson, 1978). However, it may be noted that Nud'ga *et al.* (1971) observed degradation of chitin when deacetylated to chitosan in a N_2 atmosphere at a higher temperature (140°C). Thus, the combination of low temperature and oxygen-free atmosphere seems beneficial for preparing high-viscosity chitosans.

The polydispersity indices (M_w/M_n) are approximately 2 for all the samples (Table 1). These M_w/M_n values are consistent with the so-called Kuhn distribution or random distribution, which results from random degradation of infinite long molecules or from a condensation polymerization process (Tanford, 1961). It is not possible from the present results to judge the molecular weight distribution of the native chitin, but the nearly constant M_w/M_n values observed here suggest that the isolated chitin is not monodisperse, in accordance with the results found by Hasegawa *et al.* (1994).

The F_A values decreased with time of deacetylation and the initial rate of deacetylation is high, levelling off with time, in agreement with previous results (Sannan *et al.*, 1976). The diad frequencies (F_{AA}, F_{AD}, F_{DA}, F_{DD}) were in accordance with the frequencies calculated with the assumption of Bernoullian (random) distribution of acetyl groups (Table 1). We therefore suggest that the acetyl groups in heterogeneously deacetylated chitosans are randomly or almost randomly distributed, independent of the scale of the deacetylation process (e.g. laboratory or industrial scale). However, the commercially deacetylated chitosans seem to contain more acid-insoluble material than the chitosans heterogeneously deacetylated in the laboratory (data not shown).

Characterization of the acid-insoluble fractions

Determination of F_A values (by elemental analysis (EA)) for the acid-insoluble fractions (Table 2)

Table 2. Fraction of acetyl groups for the acid-insoluble fractions determined by elemental analysis

Sample	2 h	4 h	6 h	8 h	10 h
F_A (±0.15)	0.74	0.67	0.75	0.59	0.63

revealed major differences between these F_A values and the F_A values from ^1H NMR for the acid-soluble fractions (Table 1) (Vårum *et al.*, 1992). The results show that the acid-insoluble fractions are much less deacetylated than the acid-soluble fractions. However, the error in the determination of F_A values obtained by EA is large (Table 2). This may be due to small differences in the N/C ratio as fully acetylated chitin contains 6.9% nitrogen, corresponding to an N/C ratio of 0.146, while fully deacetylated chitosan contains 8.7% nitrogen, corresponding to an N/C ratio of 0.194 (Roberts, 1992).

The large difference between the chemical composition for the acid-soluble and the acid-insoluble fractions shows large chemical heterogeneity in the unfractionated samples. If we assume, due to partial swelling during deacetylation, that this is true also within the acid-insoluble fractions of the samples, this may explain the large experimental errors. We used several duplicates and very small amounts of sample (in the μg scale), and assuming compositional heterogeneity in the acid-insoluble material, it is not unlikely that the composition of the different duplicates varies. In addition, it is crucial that no residual protein remains in the samples since the composition of proteins differ considerably from the composition of chitosan (Roberts, 1992). We therefore examined the protein content of some of the samples. It was found that all the samples analysed contained less than 1% protein. Thus, it is evident that variation in protein content cannot explain the large non-systematic variations in the F_A values for the acid-insoluble fractions. In spite of the relatively large error in the determination of F_A values by EA, it was clear that commercial chitosan prepared by heterogeneous deacetylation of chitin can be separated into an acid-soluble fraction with $F_A < 0.6$, and an acid-insoluble fraction of higher F_A.

In order to determine more accurate F_A values for the acid-insoluble fractions, we also determined F_A values from CPMAS ^{13}C NMR. This technique has the advantage over other solid-state techniques (i.e. IR spectroscopy) of better resolution, less difficulties in drawing baselines, no requirement for several replicates and no need for analysis of the water content of the sample (Domard, 1987; Domszy & Roberts, 1985; Raymond *et al.*, 1993). CPMAS ^{13}C NMR has been shown to give reliable results when the acetyl CH_3 signal is used to estimate the acetyl content in solid samples (Pelletier *et al.*, 1990; Raymond *et al.*, 1993; Saito *et al.*, 1982), while the C=O signal is less suitable for quantitative measurements because of longer relaxation time for the carbonyl C, resulting in underestimated F_A values.

Typical CPMAS ^{13}C NMR spectra of chitosan are shown in Fig. 3. The additional signal for the acid-soluble fraction (marked with an asterisk in Fig. 3b) is probably due to residual acetone from the sample

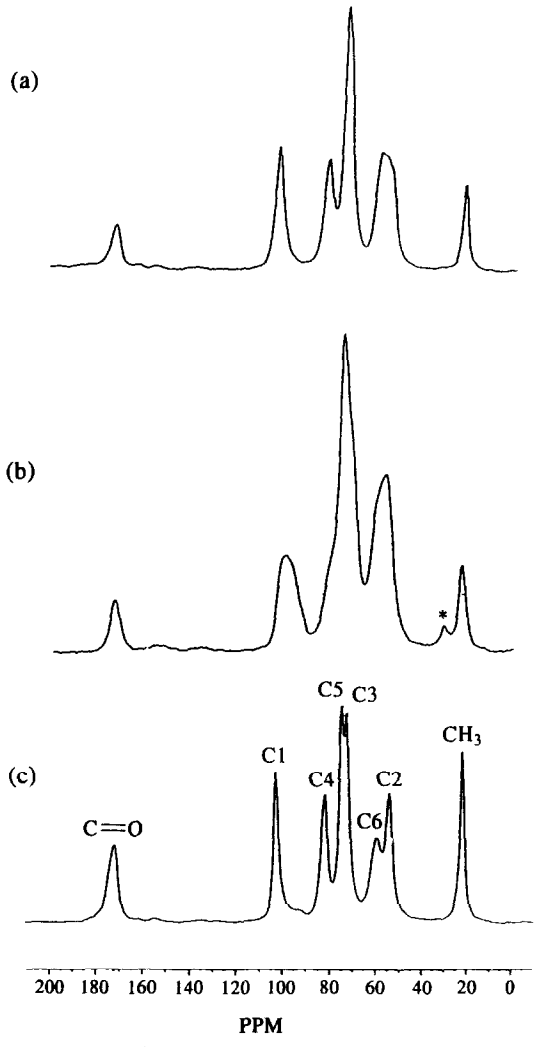

Fig. 3. CPMAS ^{13}C NMR spectra of the 4 h chitosan. (a) unfractionated chitosan; (b) acid-soluble fraction; (c) acid-insoluble fraction. The signal marked with an asterisk in (b) is caused by acetone impurities in the sample.

preparation even though the acid-soluble fractions were dried at an elevated temperature for several hours. The acid-soluble fractions were treated with acetone (see experimental part) because it was difficult to use the lyophilized material in the probe of the NMR machine. However, this did not cause problems when calculating F_A because the acetone CH_3 signal is sufficiently resolved from the acetyl CH_3 signal.

We have compared F_A values for the acid-soluble fractions obtained by ^1H NMR in solution and CPMAS ^{13}C NMR in the solid state. Figure 4 illustrates that the F_A values obtained from CPMAS ^{13}C NMR are consistent with the F_A values obtained from ^1H NMR.

The F_A values for the acid-insoluble fractions are shown together with the F_A values for the acid-soluble fractions in Fig. 5. In both cases the F_A values are obtained from CPMAS ^{13}C NMR The results show that the chemical composition of the acid-insoluble fractions are totally

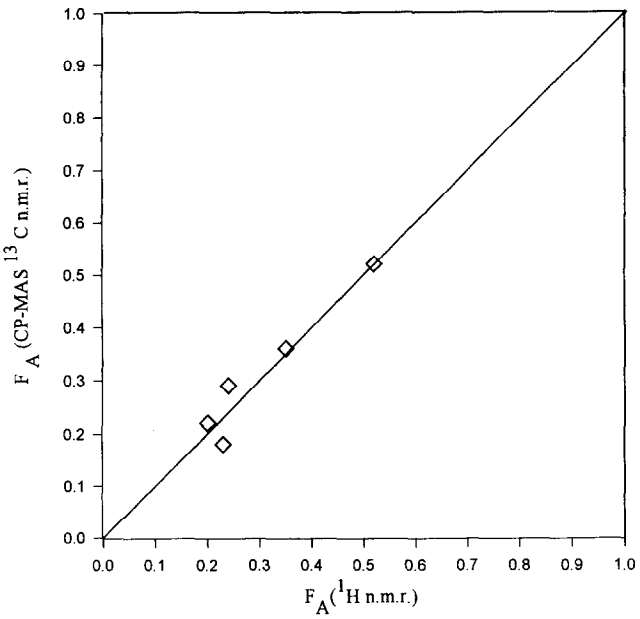

Fig. 4. F_A values (for the acid-soluble fractions) obtained from CPMAS ^{13}C NMR plotted against the F_A values obtained from ^1H NMR in solution.

different from the acid-soluble fractions, and we conclude that virtually no deacetylation has taken place in the chitin-like acid-insoluble fractions. One may note that the F_A values for the acid-insoluble fractions obtained by EA (Table 2) are lower than the F_A values obtained by CPMAS ^{13}C NMR (Fig. 5). This may be explained by a possible partial swelling of the acid-insoluble material during deacetylation. If this is the case, it will influence more the results obtained by EA (μg scale) than the results obtained by CPMAS ^{13}C NMR (mg scale).

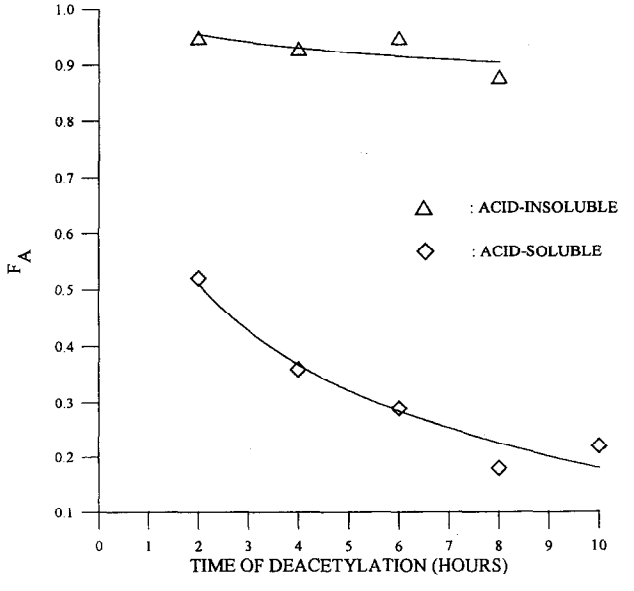

Fig. 5. F_A values obtained from CPMAS ^{13}C NMR as a function of time of deacetylation.

Characterization of unfractionated samples

We measured the F_A values for the unfractionated chitosans ($F_{A,exp}$) and compared this to the 'overall' F_A values ($F_{A,calc}$) from the equation:

$$F_{A_{calc}} = X \cdot (F_{A_{acid-soluble}}) + (1 - X) \cdot (F_{A_{acid-insoluble}}) \quad (2)$$

where X is the acid-soluble fraction of the chitosans (Figs 1 and 2), and $F_{A_{acid\text{-}soluble}}$ / $F_{A_{acid\text{-}insoluble}}$) are the F_A values obtained from CPMAS ^{13}C NMR. The results show that $F_{A,exp}$ is in good agreement with $F_{A,calc}$ for all the samples (Fig. 6). This confirms that the measured F_A is an average, but does not give any information about the distribution of F_A within the sample. We have recently investigated the solubility of fully acid-soluble heterogeneously deacetylated chitosans as a function of pH and depolymerization (Vårum *et al.*, 1994). We found that chitosan with $F_A = 0.37$ had a small fraction (~5%) of soluble material at pH 7.5. The F_A value of this small soluble fraction was determined to 0.49, significantly different from the F_A value of 0.37 of the unfractionated sample, indicating a distribution of F_A in partially deacetylated chitosans. Further work on the characterization of the distribution of F_A values in the acid-soluble fraction is in progress.

Proposed mechanism for the heterogeneous deacetylation process

A qualitative evaluation of the linewidth of the signals in the CPMAS ^{13}C NMR spectra revealed that the crystallinity of the acid-insoluble fractions does not change dramatically during deacetylation (data not shown), and that the linewidth resembles samples of α-chitin (e.g. crab chitin) (Focher *et al.*, 1992*a*, *b*; Tanner *et al.*, 1990), suggesting that these fractions are indeed

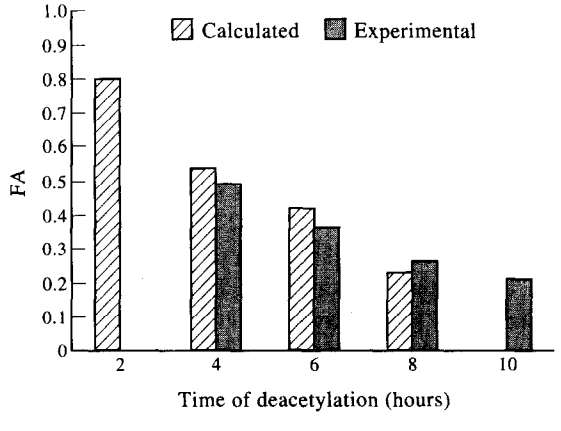

Fig. 6. F_A values for the unfractionated chitosans (called experimental) compared to F_A values for the unfractionated chitosans obtained when calculating from the F_A values obtained for the acid-soluble and acid-insoluble fractions (called calculated). All the F_A values were obtained from CPMAS ^{13}C NMR.

unreacted chitin. From this it seems unlikely that the chitin particles contain distinct amorphous and crystalline regions as described earlier (Kurita *et al.*, 1977; Sashiwa *et al.*, 1993). The acid-soluble fractions and the unfractionated samples give broader lines in their NMR spectra, implying amorphous regions in these chitosans, probably due to swelling and deacetylation—our data reveal a marked difference in chemical composition between the acid-soluble and acid-insoluble fractions of commercially heterogeneously deacetylated chitosans, and the differences in F_A increase with increasing time of deacetylation. In addition, the acid-soluble fractions of the samples increased with increased time of deacetylation; the 10 h sample was almost fully acid-soluble. As the chitin particles are suspended in an alkaline solution during the heterogeneous deacetylation process, it is likely that the chitin particles swell from the outside towards the interior (e.g. the particles are deacetylated from the outside towards the centre). Thus, the molecules at the surface of the particles are more deacetylated than those at the centre of the particles. A small fraction (3%) of the molecules at the centre of the particles were not deacetylated even after 10 h. This mechanism assume a broadening in distribution of F_A for the acid-soluble fractions with decreasing F_A.

We conclude here that the acetyl groups of heterogeneously deacetylated chitosans are randomly or almost randomly distributed, and that these chitosans contain a fraction of highly acetylated acid-insoluble material. We may say that the molecules have to be completely swollen (e.g. available for alkaline deacetylation) to become acid-soluble. If the molecules are only partially swollen (e.g. available for alkaline deacetylation), there may be parts of the molecules containing blocks of acetylated units, but these molecules are probably acid-insoluble. This may explain why previous investigations (Kurita *et al.*, 1977) concluded from qualitative solid-state experiments (X-ray crystallography) that heterogeneously deacetylated chitosans with F_A higher than 0.2 contained crystalline regions that gradually decreased with decreasing F_A.

Our proposed mechanism for the deacetylation process suggests that it is important to use small chitin particles or sufficient time of deacetylation in order to obtain chitosans with compositional homogeneity (e.g. with a unimodal distribution of F_A values) (Painter *et al.*, 1968) that are fully soluble.

ACKNOWLEDGEMENTS

We thank Dr Bjørn Erik Christensen for his assistance with the processing of the HPGPC–LALLS–DRI data. We also thank Ms Mildrid Myhr, Ms Lillian Nergaard and Ms Siv Moen for their skillful technical assistance. The chitosans were kindly provided by Pronova Biopolymers (Raymond, USA). Financial support has been provided by the Research Council of Norway (formerly NFFR).

REFERENCES

Aiba, S. (1991). Studies on chitosan: 3. Evidence for the presence of random and block copolymer structures in partially *N*-acetylated chitosans. *Int. J Biol. Macromol.*, **13,** 40–44.

Anthonsen, M.W., Vårum, K.M. & Smidsrød, O. (1993). Solution properties of chitosans: conformation and chain stiffness of chitosans with different degrees of N-acetylation. *Carbohydr. Poly.*, **22,** 193–201.

Anthonsen, M.W., Vårum, K.M., Hermansson, A.M., Smidsrød, O. & Brant, D.A. (1994). Aggregates in acidic solutions of chitosans detected by static light scattering. *Carbohydr. Polym.*, **25,** 13–23.

Araki, Y. & Ito, E. (1975). A pathway of chitosan formation in mucor rouxii (enzymatic deacetylation of chitin). *Eur J Biochem.*, **55,** 71–78.

Christensen, B.E., Mhyr, M., Aune, O., Hagen, S., Berge, A. & Ugelstad, J. (1996). Macroporous, monodisperse particles and their application in aqueous size exclusion chromatography of high molecular weight polysaccharides. *Carbohydr. Polym.*, accepted manuscript.

Davis, B., Eveleigh, D.E. (1984). In *Chitin, Chitosan and Related Enzymes*, ed. J.P. Zikakis. Academic Press, New York, pp. 161–179.

Domard, A. (1987). Determination of *N*-acetyl content in chitosan samples by c.d. measurements. *Int. J. Biol. Macromol.*, **9,** 333–336.

Domszy, J.G. & Roberts, G.A.F. (1985). Evaluation of infrared spectroscope techniques for analysing chitosan. *Macromol. Chem.*, **186,** 1671–1677.

Draget, K.I., Vårum, K.M., Moen, E., Gynnild, H. & Smidsrød, O. (1992). Chitosan cross-linked with Mo(VI) polyoxyanions: a new gelling system. *Biomaterials,* **13,** 635–638.

Focher, B., Naggi, A., Torri, G., Cosani, A. & Terbojevich, M. (1992a). Structural differences between chitin polymorphs and their precipitates from solution — evidence from CPMAS ^{13}C-NMR become FT-IR and FT-Raman spectroscopy. *Carbohydr. Polym.*, **17,** 97–102.

Focher, B., Naggi, Torri, G., Cosani, A.A. & Terbojevich, M. (1992b). Chitosans from Euphausia superba. 2: Characterization of solid state structure. *Carbohydr. Polym.*, **18,** 43–49.

Hasegawa, M., Isogai, A. & Onabe, F. (1994). Molecular mass distribution of chitin and chitosan. *Carbohydr. Res.*, **262,** 161–166.

Hirano, S. (1988). Production and application of chitin and chitosan in Japan. In *Chitin and Chitosan*, eds G. Skjåk-Braek, T. Anthonsen & P. Sandford. pp. 37–43.

Korycka-Dahl, M.B. & Richardson, T. (1978). Activated oxygen species and oxidation of food constituents. *CRC Crit. Rev. Food Sci. Nutr.*, **10,** 209–241.

Kurita, K., Sannan, T. & Iwakura, Y. (1977). Studies on chitin, 4. *Makromol. Chem.*, **178,** 3197–3202.

Niola, F., Basora, N., Chornet, E. & Vidal, P.F. (1993). A rapid method for the determination of the degree of *N*-acetylation of chitin-chitosan samples by acid hydrolysis and HPLC. *Carbohydr. Res.*, **238,** 1–9.

Nud'ga, L.A., Plisko, E.A. & Danilov, S.N. (1971). Preparation of chitosan and study of its fractional composition. *Zhurnal Obshcei Khimii;* **41**(11), 2555–2558.

Ottoy, M.H., Vårum, K.M., Christensen, B.E., Anthonsen, H.W. & Smidsrød, O. (1996). Preparative and analytical size-exclusion chromatography of chitosans. *Carbohydr. Polym.*, accepted manuscript.

Painter, T., Smidsrød, O., Larsen, B. & Haug, A. (1968). A computer study of the changes in composition-distribution occurring during random depolymerisation of a binary linear heteropolysaccharide. *Acta Chem. Scand.*, **22,** 1637–1648.

Pelletier, A., Lemire, I., Sygusch, J., Chornet,, E. & Overend, R.P. (1990). Chitin/chitosan transformation by thermo-mechano-chemical treatment including characterization by enzymatic depolymerization. *Biotechnol. Bioeng.*, **36,** 310–315.

Raymond, L., Morin, F.G. & Marchessault, R.H. (1992). Degree of deacetylation of chitosan using conductometric titration and solid-state NMR. *Carbohydr. Res.*, **246,** 331–336.

Roberts, G.A.F. (1992). *Chitin Chemistry*, The Macmillan Press Ltd., Hong Kong.

Saito, H., Tabeta, R. & Hirano, S. (1982). A high resolution ^{13}C nmr study of chitin, chitosan and *N*-acyl chitosans by cross polarization/magic angle spinning (CP/MAS) nmr spectroscopy. Conformational behaviour and gelation mechanism. In *Chitin and Chitosan* (Proceedings of the Second International Conference on Chitin and Chitosan), eds S. Hirano & S. Tokura, Sapporo, Japan, pp. 71–76.

Sandford, P.A. (1988). Chitosan: commercial uses and potential applications, In *Chitin and Chitosan*, eds G. Skjåk-Braek, T. Anthonsen & P. Sandford, pp. 51–69.

Sannan, T., Kurita, K. & Iwakura, Y. (1976). Studies on chitin, 2. Effect of deacetylation on solubility. *Makromol. Chem.*, **177,** 3589–3600.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R. & Tokura, S. (1991). Distribution of the acetamide group in partially dacetylated chitins. *Carbohydr. Polym.*, **16,** 291–296.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R. & Tokura, S. (1993). *N*-acetyl group distribution in partially deacetylated chitins prepared under homogeneous conditions. *Carbohydr. Res.*, **242,** 167–172.

Tanford, C. (1961). *Physical Chemistry of Macromolecules*, John Wiley, New York.

Tanner, S.F., Chanzy, H., Vincendon, M., Roux, J.C. & Gaill, F. (1990). High-resolution solid-state carbon-13 nuclear magnetic resonance study of chitin. *Macromolecules*, **23,** 3576–3583.

Terbojevich, M., Cosani, A., Focher, B., Naggi & Ajorri, G. (1992). Chitosans from Euphausia superba. 1: Solution properties. *Carbohydr. Polym.*, **18,** 35–42.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. & Smidsrød, O. (1991). Determination of the degree of *N*-acetyl groups in partially *N*-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr. Res.*, **211,** 17–23.

Vårum, K.M., Anthonsen, M.W., Grasdalen, H. & Smidsrød, O. (1991). ^{13}C-N.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydr. Res.*, **217,** 19–27.

Vårum, K.M., Anthonsen, M.W., Ottoy, M.W., Grasdalen H. & Smidsrod, O. (1992). Chemical composition and sequences in chitosans determined by high-field proton and carbon N.M.R. spectroscopy — relation to solubility. In *Advances in Chitin and Chitosan. (Proceedings from the 5th International Conference on Chitin and Chitosan)*, eds, C.J. Brine, P.A. Sandford & J.P.Zikakis. Princeton, New Jersey. pp. 127–136.

Vårum, K.M., Ottøy, M.H. & Smidsrød, O. (1994). Water-solubility of partially *N*-acetylated chitosans as a function of pH: Effect of chemical composition and depolymerization. *Carbohydr. Polym.*, **25,** 65–70.