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Chitosans from *Euphausia superba*. 2: Characterization of solid state structure

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Chitosans from *Euphausia superba* of different degrees of acetylation (42%, 28% and <5%) were characterized by X-ray diffractometry, CP-MAS ^{13}C-NMR and FTIR spectroscopy to study the influence of both the regeneration process and the degree of polymerization on chitosan structure.

The X-ray powder patterns indicated a decrease of order with decreasing the degree of acetylation as well as after regeneration and acid treatments. Similarly, the CP-MAS ^{13}C-NMR and FTIR spectra exhibited a general broadening of signals suggesting the occurrence of new conformations for chitosans after regeneration. Furthermore, samples treated with hydrochloric acid showed some structural modifications which accounted for their behaviour in solution as reported in the first part of this study.

INTRODUCTION

Solubility and aggregation properties of chitosans from *Euphausia superba*, prepared under heterogeneous conditions of deacetylation, mainly depend on their degree of acetylation as well as on depolymerization conditions as a result of a structural heterogeneity due to the presence of blocks of N-acetyl-D-glucosamine and D-glucosamine (Aiba, 1991; Terbojevich *et al.*, 1992). In the solid state, structural heterogeneity of chitosans should be revealed as a consequence of formation in the polymer lattice of ordered regions separated by disordered ones.

The aim of this second part of the present study is to investigate the properties of chitosans from *Euphausia superba* in the solid state and to find some relationships with those observed in solution.

EXPERIMENTAL SECTION

Materials

The same samples of chitosan described in the preceding paper (Terbojevich *et al.*, 1992) were used. Their characteristics, evaluated as previously referred, are collected in Table 1.

α-Chitin from *Euphausia superba* (Antarctic krill) was kindly supplied by the Sea Fishering Institute, Gdynia, Poland. Its viscosity in N,N dimetylacetamide (DMAc) containing 5% LiCl was 24 dl g^{-1} (Terbojevich *et al.*, 1988).

METHODS

X-ray powder patterns

X-ray powder patterns were recorded using Ni-filtered CuKα radiation from a Siemens 500 D (Munchen) diffractor equipped with a scintillator counter and a linear amplifier.

The crystallinity index (CrI) and the apparent crystal size $(D_{app})_{110}$ in the direction of (110) crystal plane was calculated as previously reported (Focher *et al.*, 1990).

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Table 1. Samples characteristics

Sample	Degree of acetylation (%)	$[\eta]$ dl/g[e]
α-Chitin	—	—
Chitosan A[a]	42	23·5
B[a]	28	18·1
C[b]	0–5	—
Chitosan A_R[b]	42	16·6
B_R[b]	28	14·8
C_R[b]	0–5	8·1
Chitosan A_B[b]	42	17·0
Chitosan A_{HCl}[c]	35	0·9
A_{HAc}[d]	42	2·0

[a]Supplied by Sea Fishering Institute, Gdynia, Poland.
[b]Prepared as described in Part 1 of this paper.
[c]Prepared hydrolysing sample A with 0·6N HCl at 50°C for 71 h.
[d]Prepared hydrolysing sample A with 1% HAc refluxing for 37 h.
[e]0·1M HAc–0·2M NaCl at 25°C.

CP-MAS ^{13}C-NMR spectra

CP-MAS ^{13}C-NMR spectra were obtained with a Bruker (Karlsruhe) CXP-300 spectrometer at 75·46 MHz. The cross polarization was 1 ms while the repetition time and ^{1}H 90° pulse were 4 s and 4·75 μs, respectively.

The chemical shifts were measured with respect to TMS with benzene as a secondary substitution reference (128 ppm). Usually, 1000–3000 scans were taken, and the spinner was spun at about 3·4 kHz.

FTIR spectra

IR spectra were obtained with a Bruker (Karlsruhe) IFS 66 FTIR interferometer using KBr pellets prepared according to the literature (Abbott *et al.*, 1988). One hundred scans were taken with a resolution of 2 and 4 cm^{-1}. In order to visualize details in band shapes and unresolved band components, the Fourier self-deconvolution procedure was applied (Kauppinen *et al.*, 1981; Faix & Beinhoff, 1988). Values of 15 cm^{-1} for the half width Lorentzian line and 1·3 for the resolution enhancement factor (Kauppinen *et al.*, 1981) avoided side lobes and preserved the constancy of band areas of all examined samples. The signal-to-noise ratio was better than 500.

Both absorbance and width of undeconvoluted spectra bands were calculated by a base line method using a Bruker program. The well defined band at 2922 cm^{-1} due to the CH vibration mode represented a good internal reference for the comparison of band absorbance.

RESULTS AND DISCUSSION

The process for production of chitosans with decreasing acetyl group content requires gradually stronger reaction conditions (Kurita *et al.*, 1977; Muzzarelli, 1977; Muzzarelli *et al.*, 1986; Focher *et al.*, 1990) which affect not only the primary structure but also the physical structure and the supramolecular morphology of chitosan, as a result of changes in chain conformation and crystalline packing. Hence, diffraction and spectroscopic data reflect chemical and structural modifications of the polymer and the regeneration process. Normalization of the preparation of samples, thus allows their homogeneous evaluation.

X-ray diffraction

Figure 1 compares X-ray powder patterns of chitosans A, B and C with that of the parent α-chitin. A decrease in order on going from chitin to variously deacetylated chitosans is clearly observed. Some of the reflections of α-chitin disappear, while a variation of the intensity of (020) equatorial reflection at 9·2° (2θ) is shown. Similarly, CrI and $(D_{app})_{110}$ (Table 2) decrease on going from chitosan A to chitosan C. The absence of the 9·2° reflection in chitosan C cannot be attributed to a very low extent of acetyl groups, because the mechanical treatment of beating of chitosan A (Fig. 2) causes the disappearance of the same reflection without modifying the acetyl groups' content. The preparation of chitosan C, performed on its acetate salt, could account for its structural modification. In fact, the regeneration treatment of chitosan A and B has the same effect (Fig.

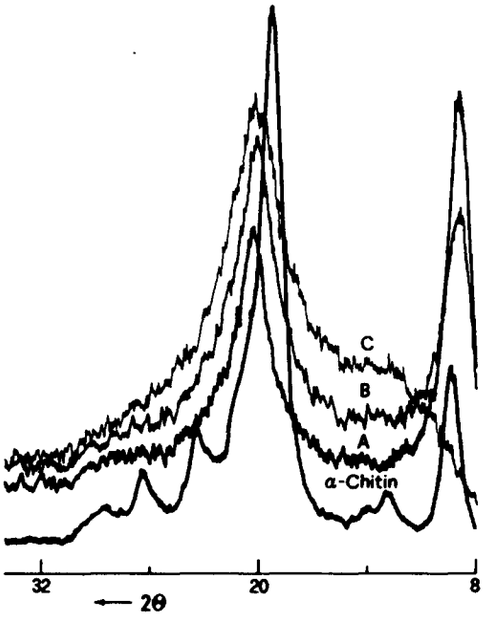

Fig. 1. X-ray powder patterns of α-chitin and chitosans A, B and C.

Table 2. Crystallinity index (CrI) and apparent size $D_{app(110)}$ of chitosan crystals

Samples	CrI (%)	$D_{app(110)}$ Å
Chitosan A	68·4	24·4
B	64·0	21·1
C	57·7	13·7
Chitosan A_R	49·1	10·6
B_R	38·0	8·5
C_R	56·0	12·9
Chitosan A_B	59·3	15·0

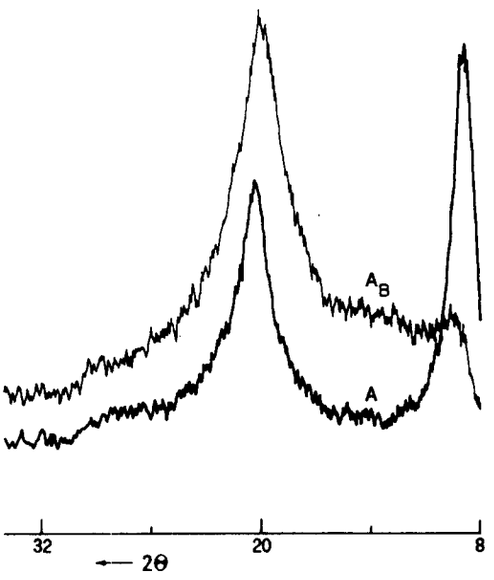

Fig. 2. X-ray powder patterns of chitosans A and A_B.

2; Table 2). As this structural modification is already present in the parent polymer C, no further structural changes are observed in chitosan C_R (Table 2).

The higher values of CrI and $D_{app(110)}$ of sample C_R in comparison with A_R and B_R may be attributed to its chemical homogeneity which prevents the occurrence of a marked disorder during the regeneration treatment. On the contrary this induces a modification of the overall architecture of chitosans A and B with a reduction of lateral order, in disagreement with literature data obtained under different experimental conditions (Struszczyk, 1987). The well-known dimorphism that occurs when cellulose I is regenerated from its solution to give cellulose II has no counterpart in the case of chitosan. This is likely to be due to the inability of chitosan chains to rearrange in an ordered form with new intra- and intermolecular hydrogen bonds as in cellulose II.

Beating of chitosan induces (Table 2) a slight decrease of structural parameters CrI and D_{app}, and the almost complete destruction of the (020) crystallographic plan (Fig. 2), suggesting an anisotropic resistance of material to shear constraints as previously observed for cellulose (Centola & Borruso, 1962).

Chemical treatment of the chitosan A with 0·6N HCl at 50°C reduces the intensity of both (110) and (020) reflections, while the same sample treated with HAc does not exhibit any difference with respect to the A_R chitosan.

CP-MAS ^{13}C-NMR spectroscopy

On going from α-chitin to chitosan A, B and C, a progressive decrease in the intensity of signals of both methyl and carbonyl groups as well as the downfield shift of C-2 signal are observed (Fig. 3(a)). In the case of chitosan A and B, the broadening of all signals is likely to be due to the presence in their macromolecules of both acetylated and deacetylated arrangements, which prevent suitable packing of the macromolecules.

CP-MAS ^{13}C-NMR spectra of chitosan A_R and B_R (Fig. 3(b)) show, in comparison with those of the parent polymers, a multiplicity of C-2 and C-6 signals as well as of the CH_3 and carbonyl signals of acetamide groups. In particular, as noticed also for chitosan A_B, chitosan B_R shows a new component of the C-4 signal. The origin of the signal multiplicity is still a matter of controversy also in the case of cellulose, for which either a unit cell containing four inequivalent glucose units with two different types of glycosidic linkages or a different symmetry and packing of chains in the unit cell were suggested (VanderHart & Atalla, 1984; Cael *et al.*, 1985; Horii *et al.*, 1987). In the case of chitosan one would consider mainly the chemical inhomogeneity of a chain which may induce different crystallographic sites due to independent chains in the unit cell.

Chitosan C_R shows a NMR spectrum very similar to that of the parent polymer C suggesting, in agreement with the X-ray analysis, that the initial supramolecular disorder is not further increased by the precipitation treatment. The C-1 signal is sharper than those of chitosan A_R and B_R, while in the C-4 signal the inversion of the intensity of components with respect to the B_R sample is observed.

In a previous work (Focher *et al.*, 1990), it was found that this new component of the C-4 signal was independent of the extent of acetylation, being rather associated with the deacetylation temperature. In fact, both in the amorphous and in the semicrystalline polymers the overcoming glass transition (T_g) (Goring, 1963) induced the occurrence of new conformations. In the present study, the deacetylation treatment to obtain sample C was performed at a lower temperature (90°C) than T_g, but the sample was completely submerged in mixed medium (DMSO:H_2O, 4:1) and, as is known, the presence of water markedly decreases the T_g value (Ogura *et al.*, 1982).

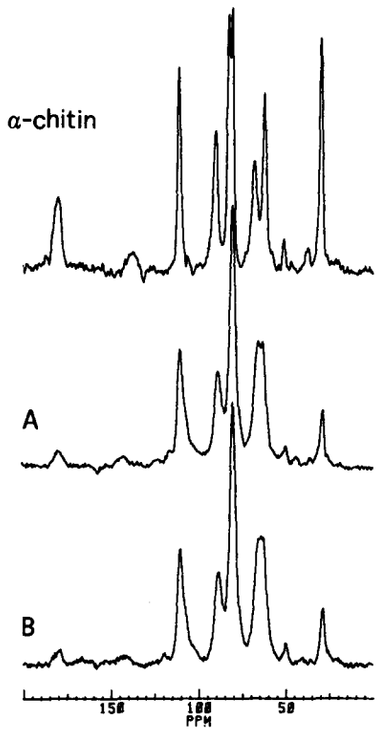

Fig. 3. CP-MAS ^{13}C-NMR spectra of (a) α-chitin, and chitosans A and B, and (b) chitosans A_R, B_R and C_R.

In the spectra of chitosans A_{HCl} and A_{HAc}, the CH_3 signal is more composite than in the parent polymer exhibiting a different molecular environment due to inter- or intramolecular inequivalence within the solid

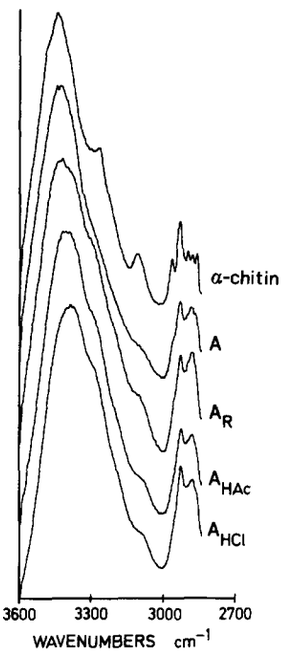

Fig. 4. FTIR spectra in the OH and CH stretching region of α-chitin, and chitosans A, A_R, A_{HAc} and A_{HCl}.

matrix. Furthermore both the A_{HCl} and A_{HAc} samples show a splitting of the C-4 signal whereas sample A_{HAC} shows a splitting also of the C-1 signal, with the concomitant reduction of the multiplicity of CH_3 signals with respect to the A_{HCl} sample. In the A_{HCl} sample a marked depolymerization of the polysaccharide chain occurs at the level of the acetylated residues. The lower acetyl extent as well as the reduced molecular weight can decrease the non-homogeneity of solid matrix.

FTIR spectroscopy

In the stretching vibration region of OH and NH groups (3600–3000 cm^{-1}), the deconvoluted spectrum of α-chitin exhibits a more detailed structure than indicated by the spectra of chitosans A, B and C. The difference is due not only to the different primary structure, but also to the occurrence of a more ordered packing of macromolecules as suggested by the band line-widths of the non-deconvoluted spectra (Fig. 4). In particular, the shoulder at 3479 cm^{-1} in the spectrum of α-chitin attributed (Pearson *et al.*, 1960; Iwamoto *et al.*, 1982; Shrivastava *et al.*, 1982) to an intramolecular hydrogen bonding involving the OH(6)...C=O disappears in the spectra of chitosan samples. Furthermore, the deacetylation and the regeneration processes, disrupting the initial crystal lattice of the parent chitin, induce in chitosans A and A_P a rearrangement of hydrogen bonding as indicated both from the band broadening at 3444 cm^1 [(OH(3)...O′(5))] and from the lower frequency shift of the OH stretching. The shift, which is a measure of the strength of intermolecular hydrogen bonding, is more evident in the

spectrum of the sample A_{HCl} for which the depolymerization process involved the disruption of the architecture of the hydrogen bonds also in the ordered regions.

The bands at 3264 cm^{-1} and 3106 cm^{-1} attributed (Pearson *et al.*, 1960; Iwamoto *et al.*, 1982; Shrivastava *et al.*, 1982) for α-chitin to vibrational modes of NH amide (intermolecular hydrogen bonding C=O ... NH and H bonded NH groups) disappear in chitosans spectra, while unexpectedly they occur as a shoulder in the spectra of both the regenerated and the treated-with-acid chitosans.

In the 750–500 cm^{-1} region, thought to be more sensitive to change of crystallinity (Tul'Chinsky *et al.*, 1976), the deconvoluted spectra of α-chitin and chitosan show a weak shoulder at 706 cm^{-1} and a well resolved band at 690 cm^{-1} assigned to out-of-plane bending of NH (amide V) and of OH groups participating in hydrogen bonding formation, respectively. The intensity of the 690 cm^{-1} band markedly decreases on going from α-chitin to commercial chitosans and it is further reduced by acid treatment with HCl more than with HAc. Some other differences between the samples are observed in the 550–500 cm^{-1} region which may be explained in terms of formation of various sets of hydrogen bonds.

In agreement with the X-ray analysis, the overall rearrangement of intra- and intermolecular hydrogen bonding, due to deacetylation, regeneration and depolymerization processes, induces a progressive decrease of infrared crystallinity index ($A_{1423\ cm^{-1}}/A_{823\ cm^{-1}}$) (Nelson & O'Connor, 1964) on going from chitosan A to chitosans A_R, A_{HAc} and A_{HCl}.

The amide I mode of deconvoluted spectrum (Fig. 5) of α-chitin is split in two well resolved peaks at 1660 cm^{-1} and 1623 cm^{-1}, while a shoulder is observed at 1636 cm^{-1}. The splitting of the polymer infrared bands is usually associated with interchain interaction in the crystals (Painter *et al.*, 1982) and, in the case of α-chitin, the component at 1660 cm^{-1} should be assigned to C=O groups hydrogen bonded only to NH groups, whilst the component at 1623 cm^{-1} should be ascribed to C=O groups bonded with a bifurcated hydrogen both to NH and OH-6 groups (Iwamoto *et al.*, 1982). The splitting itself is in accordance with a crystal structure (Minke & Blackwell, 1978) with two non-equivalent C=O and OH-6 groups belonging to two different conformations. The curve fitting of the amide I mode shows few well defined components with different measurable areas which reflect relative populations of various conformational states assigned to individual bands (Focher *et al.*, 1992). The splitting occurs with very weak intensity at 1651 cm^{-1} and 1620 cm^{-1} for chitosan A and B and completely disappears in chitosan C, where a single broad band between 1660 and 1580 cm^{-1} is observed. The splitting of the amide I is not present after regeneration of chitosans

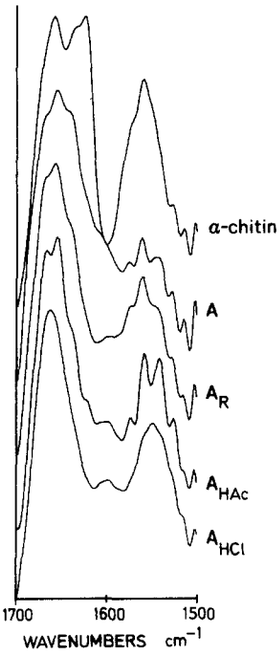

Fig. 5. FTIR spectra of α-chitin, and chitosans A, A_R, A_{HAc} and A_{HCl} in the 1700–1500 cm^{-1} region.

(Fig. 5) suggesting a significant conformation change during the treatment similar to that observed for cellulose. After acid treatment with HCl, the splitting is no longer observable, while a more detailed structure is exhibited by amide I mode of sample A_{HAc} (Fig. 5).

The intensity of amide II at 1553 cm^{-1} in the undeconvoluted spectrum of α-chitin, progressively decreases on going from chitin to commercial regenerated and depolymerized chitosans (Fig. 5). Unexpectedly, this vibration mode is split in the case of sample A_{HAc} suggesting a peculiar rearrangement of N-acetylglucosamine residues after acetic acid treatment.

Similarly to amide II, the intensity of amide III vibration mode at 1313 cm^{-1} progressively decreases on going from α-chitin to chitosans but it is not influenced by further treatments.

In the CH-stretching region (3000–2800 cm^{-1}), the deconvoluted spectrum of α-chitin shows (Fig. 4) five well resolved bands. C-CH_3 deformation and wagging modes appeared at 1381 cm^{-1} and 974 cm^{-1}, respectively. The latter mode as well as that at 2960 cm^{-1} (ν_s CH_3 stretching) appear in the spectra of commercial chitosans as shoulders which significantly weaken in the spectra of regenerated as well as of depolymerized samples.

A marked modification of shape and intensity of CH_2 stretching bands of chitosans with respect to that of the corresponding α-chitin is observed, suggesting a different arrangement of primary hydroxyl groups in the two cases. This fact is confirmed by the analysis of the bending mode of CH_2 groups in the 1420–1435 cm^{-1}

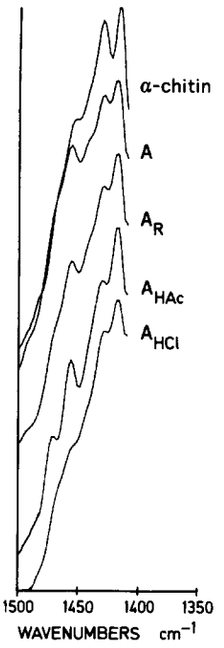

Fig. 6. FTIR spectra of α-chitin, and chitosans A, A_R, A_{HAc} and A_{HCl} in the 1450–1350 cm^{-1} region.

frequencies range, this spectral region being considered for polysaccharides as conformation sensitive (Cael *et al.*, 1974). Both shift and intensity changes of the bands have been related to modifications in the environment of CH_2OH group, although the high degree of band coupling in this region makes it difficult to assess the kind of chain packing or hydrogen bond network. In the deconvoluted spectrum of α-chitin (Fig. 6), a band is observed at 1433 cm^{-1} which gradually weakens in the spectra of chitosans A, B and C as well as in those of regenerated and depolymerized chitosans suggesting a change of environment of primary hydroxyl group, hence of the hydrogen bonding network.

CONCLUSIONS

Solid state study

On going from α-chitin to highly acetylated commercial chitosans A and B, the X-ray diffraction patterns show a regression in the number of reflexions as well as a lowering of the crystallinity index. Similarly, the CP-MAS ^{13}C-NMR spectra evidenced a general broadening of signals, and the FTIR spectra suggest a progressive rearrangement of hydrogen bonds. Furthermore, some modifications of intensity of infrared bands sensitive to the conformation of primary hydroxyl groups and of the polymer chain account for the weaker packing of chitosan chains with respect to chitin.

The regeneration treatment induces further significant modifications in the chain conformation and, hence, in the lateral order of chitosans A and B. In fact, the dissolution process, disrupting completely the hydrogen bond lattice of chitosans A and B, removes the memory of the parent α-chitin structure. The regeneration process is unable to exactly reproduce it, or any other polymorphs, thermodynamically favoured, as in the case of cellulose II.

The highly deacetylated chitosan C, which undergoes a concomitant precipitation process during the deacetylation reaction, shows a recovery of order and the appearance of new conformations evidenced by CP-MAS ^{13}C-NMR and FTIR spectroscopy. Depolymerization treatments of chitosan A with acids induce new structural modifications, especially due to the rearrangement of the hydrogen bond network. Changes are more evident for the sample treated with hydrochloric acid rather than with acetic acid.

Correlations between solid state and solution properties of chitosan

Useful insights into solution properties of chitosans reported in the accompanying paper (Terbojevich *et al.*, 1991) can be given through the solid state study:

- The heterogeneous deacetylation of α-chitin causes a progressive modification of pre-existing ordered regions. Hence the different solubility of commercial chitosans A and B is attributable to a lower structural order of sample B, as suggested in the CP-MAS ^{13}C-NMR spectra by the broadening of signals and particularly by a higher complexity of the signal of the primary hydroxyl group; these results are consistent with those obtained by X-ray and FTIR analysis.
- Regenerated chitosans A_R and B_R, evidencing in the solid state a less ordered architecture, exhibit solubility properties comparable with those of commercial chitosans of higher degree of deacetylation. The mechanical treatment of beating, which leads to modifications in the solid state similar to those of regeneration process, allows a substantial dissolution of sample A.
- The solid state study shows a remarkable difference between the depolymerized chitosans A_{HCl} and A_{HAc} which accounts for the aggregation state indicated by the light scattering analysis. Thus, the CP-MAS ^{13}C-NMR spectra indicate a loss of 'cluster' crystalline regions in the samples treated in 0·6 N HCl at 50°C for which a molecular dissolution is observed. These results agree both with the higher structural order found for the sample A_{HAc} by X-ray analysis and with a more pronounced disruption of the hydrogen bond lattice found for the sample A_{HCl} by FTIR analysis.

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