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Preparation of low-molecular-weight chitosan using phosphoric acid

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Two types of low degree of polymerisation (DP) chitosan were prepared by homogeneous hydrolysis of chitosan in 85% phosphoric acid at room temperature for 1–6 weeks. The hydrolysates were collected by addition of excess ethanol, and were fractionated by solubility in water. The changes in yields of water-insoluble (higher DP) and water-soluble (lower DP) fractions were determined as a function of hydrolysis time. The hydrolysis proceeded with further deacetylation of chitosan, resulting in degree of deacetylation of more than 90%. The water-insoluble fraction prepared after the hydrolysis for 4 weeks (43% yield) had a weight-average DP ($\overline{DP_w}$) of 16·8, and showed the 'tendon' type X-ray diffraction pattern. The water-soluble fraction (12·5% yield) had a $\overline{DP_w}$ of 7·3, and showed the 'annealed' type pattern.

INTRODUCTION

Chito-oligosaccharides, oligomers of β-1,4-linked D-glucosamine, are useful compounds for model experiments of chemical modificaiton, solubilisation and structural analyses of chitosan (Domard & Cartier, 1989; Cartier *et al.*, 1990; Domard *et al.*, 1991). These chito-oligosaccharides have lately attracted the authors' attention, because they were found to have biological activities such as medicinal actions to living bodies and anti-fungal activities (Kendra & Hadwiger, 1984; Tokoro *et al.*, 1988). Chitohexaose and *N*-acetyl chitohexaose are known to have anti-tumour activities (Tokoro *et al.*, 1988). Chitoheptaose seemed to have an optimal molecular weight required for anti-fungal activities (Kendra & Hadwiger, 1984).

The methods for preparing chito-oligosaccharides are classified into enzymic and chemical hydrolyses. While the enzymic method gives chito-oligosaccharides with relatively high yields, it is not effective in preparing products with molecular weights greater than chitoheptamer (Izume & Ohtakara, 1987).

Various acids have been used for hydrolysis of chitosan to obtain chito-oligosaccharides. However, the reaction conditions were so strong that it seemed to be difficult to obtain chito-oligosaccharides with degrees of polymerisation (DP) greater than six in high yields without large amounts of by-products. Hydrolysis with hydrochloric acid gave mostly monomer with a small amount of low-DP oligosaccharides (DP = 2 to 5) (Horowitz *et al.*, 1957). A modified hydrolysis method using concentrated hydrochloric acid produced glucosamine oligomers with a wide distribution of DP, from monomer to approximately 40 DP (Domard & Cartier, 1989). Hydrolysis with nitrous acid provided chito-oligosaccharides with a DP of 9–18; however, the products contained 2,5-anhydromannose residue, which was formed by deamination with nitrous acid (Yaku *et al.*, 1977).

In the study of acid hydrolysis of cellulose, concentrated phosphoric acid was found to give a cello-oligosaccharides mixture (Atalla *et al.*, 1980) by homogeneous hydrolysis. The products were separated to two fractions: a higher DP fraction of number average DP ($\overline{DP_n}$ = 15, $\overline{DP_w/DP_n}$ = 1·15) and a lower DP fraction of $\overline{DP_n}$ = 7 ($\overline{DP_w/DP_n}$ = 1·07). These low-DP celluloses seem to be useful compounds for studying crystal structures and chemical modifications of cellulose (Atalla *et al.*, 1984; Isogai *et al.*, 1989; Isogai & Usuda, 1990). In view of the structural similarity between cellulose and chitosan, hydrolytic action of phosphoric acid on chitosan attracts special attention. Recently, the method for preparing chito-oligomers with hot phosphoric acid has been reported as a patent (Omura *et al.*, 1991). Although the yields and DP values of hydrolysates were reported as 10–20% and 6–8, respectively,

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the hydrolysates have not been characterised, in terms of the structure of amine groups, DP distributions, or solid-state structures. In this study, the authors applied the homogeneous hydrolysis of chitosan with 85% phosphoric acid, in anticipation of obtaining chitosan oligomers with narrow DP distributions.

MATERIALS AND METHODS

Chitosan samples

'Chitosan PSH' derived from crab shell (Yaizu Suisan Kagaku Co. Ltd, Shizuoka, Japan) was used as an initial material without further purification. The degree of deacetylation was determined to be 75% by ^{1}H-NMR analysis, as described below.

Hydrolysis with phosphoric acid

The procedure and conditions of hydrolysis were basically the same as those for cellulose (Atalla *et al.*, 1980; Isogai & Usuda, 1991). Figure 1 shows the scheme of preparing the low-molecular-weight chitosans. A 5 g sample of chitosan was placed in 300 ml of an Erlenmeyer flask with a stopper, and 100 g of 85% phosphoric acid was added. It usually took 1–2 days with intermittent stirring at room temperature for complete dissolution of chitosan, resulting in a brown viscous liquid. This solution was allowed to stand at room temperature for 1–6 weeks. After standing, chitosan was regenerated as a white precipitate by pouring the solution into excess ethanol. After stirring the mixture for 1 day, free phosphoric acid was removed with ethanol by decantation, and phosphoric acid forming salt at amine groups of hydrolysed chitosan was removed by repeated treatments with 1% triethylamine in ethanol. The precipitate was thoroughly washed with ethanol. The water-soluble fraction was separated from the precipitate by extraction with 1000 ml of water at room temperature for 2 days. The water-insoluble fraction was treated with 1% aqueous sodium hydroxide for the complete removal of phosphate ions, and then was washed thoroughly with water. This water-insoluble low-DP chitosan was collected by filtration followed by freeze-drying. On the other hand, the water-soluble fraction was concentrated by evaporation, and was obtained as a reprecipitate by addition of ethanol to the mixture. The precipitate was treated with 1% triethylamine in ethanol, and then was washed thoroughly with ethanol by centrifugation. This water-soluble low-DP chitosan was collected by drying *in vacuo*.

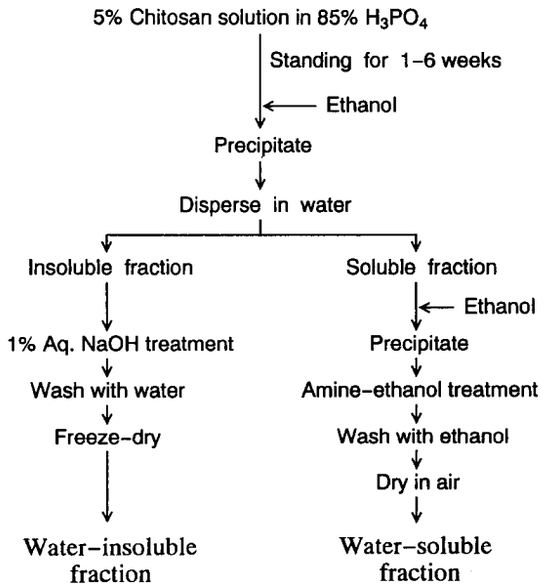

Fig. 1. Scheme for preparing low-molecular-weight chitosans by homogeneous hydrolysis in 85% phosphoric acid.

Analyses

The X-ray diffraction patterns of the hydrolysed chitosan fractions were measured by a JEOL-5B diffractometer in the reflection mode by using nickel-filtered CuK_α radiation.

The chitosan samples were analysed by high-performance size-exclusion chromatography (HPSEC) after conversion to phenyl carbamate derivatives. The derivatisation was performed as follows: dry chitosan (20 mg) was treated with phenyl isocyanate (0·16 ml) in a 15 ml screw-capped reaction tube with dry pyridine (1 ml) at 75°C. The mixture was periodically swirled. After complete dissolution of the solid (usually within 8 h), 0·2 ml of dry methanol was added to the mixture in order to consume excess phenyl isocyanate. Excess methanol and pyridine were removed by evaporation using azeotropic distillation with toluene, and the reaction mixture was dried *in vacuo*. The products, containing phenyl-carbamated chitosan and methyl phenyl carbamate, were dissolved in tetrahydrofuran to prepare ~ 0·01 wt% of a solution, and were analysed by HPSEC.

HPSEC was carried out on a system of a Shimadzu LC-6A pump, a SOMA S-310A model-II variable wavelength UV detector operated at 254 nm, and a column set of TOSOH GMHXL and G2500H (exclusion limits of 4×10^8 and 2×10^4 for polystyrene , respectively). Injected volume was 0·1 ml and flow rate was 0·5 ml/min. The DP_w and DP_n values were calculated from the chromatograms by a calibration curve determined by using polystyrene standards (Isogai & Usuda, 1991). The numerical analysis was carried out with programs developed in the authors' laboratory.

^{1}H- and ^{13}C-NMR spectra of chitosan samples were recorded on a Bruker AC-300 at 323 K (magnetic field = 7·05 T, frequencies = 300·1 MHz for ^{1}H and 75·5 MHz for ^{13}C). A 2% solution of chitosan samples

in 5% D_2SO_4–D_2O was used for the measurements. Chemical shifts were determined on the δ scale (ppm) relative to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as a reference. Degrees of deacetylation were determined by the relative signal intensities between the H1 signal of glucosamine residue and the CH_3 signal of the amide group in ^{1}H-NMR chart.

Fourier transform infrared (FT-IR) spectra were obtained with a Shimadzu FT-8000 spectrometer equipped with process controller, using the KBr-disc-pellet method.

RESULTS AND DISCUSSION

When the original chitosan sample was added to 85% phosphoric acid, a small part of the sample remained insoluble at the initial stage. This part, however, became completely soluble in the solution within 2 days, and the subsequent hydrolysis seemed to proceed under homogeneous conditions. This process was accompanied by a decrease in viscosity of the solution, indicating a decrease in DP of chitosan. Two chitosan fractions were obtained from this solution after various hydrolysis time. The low-DP chitosans thus prepared were found to be free from phosphate salts at the amine groups by FT-IR analysis.

Figure 2 shows changes in yields of the water-insoluble and water-soluble fractions as a function of hydrolysis time. The yields of the water-insoluble fraction continuously decreased from 70 to 30% with increasing hydrolysis time in 85% phosphoric acid. On the other hand, the yields of the water-soluble fraction gradually increased up to approximately 15% during the hydrolysis for 6 weeks. The total yields of the water-insoluble and water-soluble fractions decreased from 70 to 43% during the hydrolysis from 1 to 6 weeks. DP values of the rest may be too low, probably lower than 3, to have been obtained as a precipitate under the conditions used in the present study.

Figure 3 shows changes in degrees of deacetylation of the water-insoluble fraction as a function of hydrolysis time. While the original chitosan had a degree of deacetylation of $\sim$ 75%, the values for the water-insoluble fraction increased rapidly during the hydrolysis for 1 week and then became more than 90%.

As described above, therefore, hydrolysed chitosan samples were obtained in relatively high yields, by the homogeneous hydrolysis with 85% phosphoric acid. Then, in this study, DP values and crystal structures were analysed for the water-insoluble and water-soluble fractions obtained by the hydrolysis for 4 weeks.

As to the determination of DP values of chitosan, several methods have been reported so far, primarily using chitosan solutions in aqueous acid media. Viscosity measurements, light-scattering measurements, and HPSEC analyses of chitosan in acid buffer solutions were used for this purpose (Roberts & Domszy, 1982; Kobayashi *et al.*, 1988; Terbojevich *et al.*, 1981; Wang *et al.*, 1991). However, it seems to be difficult to evaluate correct DP values of such ionic polysaccharides, and the DP values of chitosans reported so far have varied, depending on measuring conditions (Wang *et al.*, 1991). The carbanilation/HPSEC technique was adopted for determining DP values and distributions of DP of chitosan, as this technique has been evaluated as a preferable method for determining DP values of cellulose in terms of little degradation of cellulose molecules (Evans *et al.*, 1989).

The results of HPSEC analysis of the water-insoluble and water-soluble fractions are shown in Fig. 4 and Table 1, and they had $\overline{DP_w}$ values of about 17 and 7, respectively. Furthermore, both the water-insoluble and water-soluble fractions had relatively narrow DP distributions, with $\overline{DP_w}/\overline{DP_n}$ of 2·1 and 1·35, respectively. Thus, low-DP chitosans were prepared by this method in

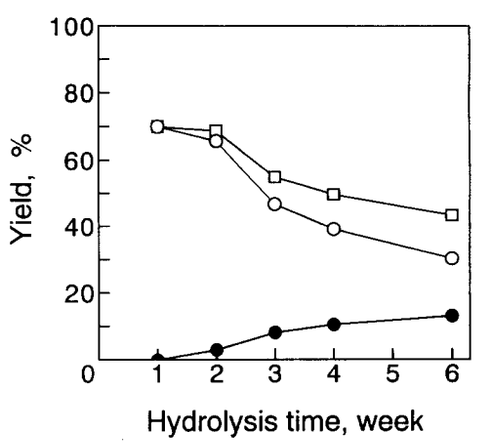

Fig. 2. Yields of water-insoluble and water-soluble chitosan fractions as a function of hydrolysis time using 85% phosphoric acid at room temperature. (○) Water-insoluble chitosan fraction, (●) water-soluble chitosan fraction; and (□) gross.

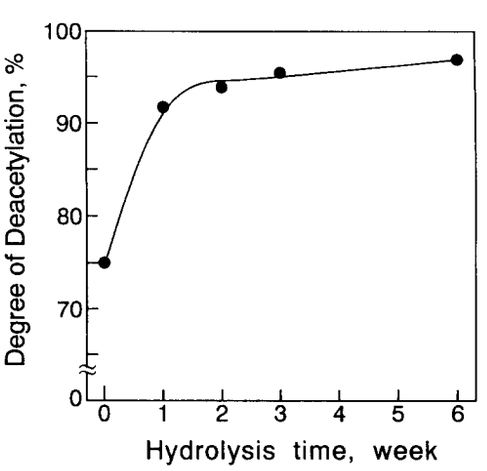

Fig. 3. Changes in degree of deacetylation of water-insoluble chitosan fraction during hydrolysis in 85% phosphoric acid at room temperature.

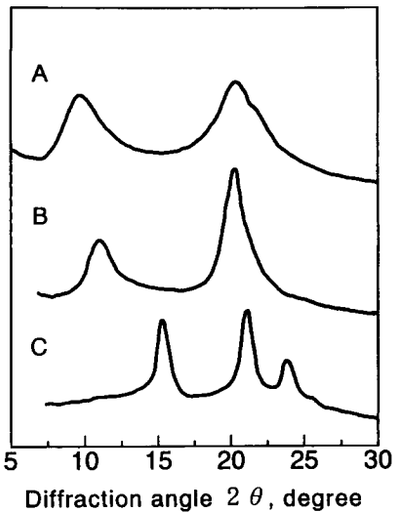

Fig. 4. X-ray diffraction patterns of water-insoluble and water-soluble chitosan fractions, and of original chitosan. These chitosan fractions were obtained after 4 weeks standing of the chitosan/85% phosphoric acid solution. (A) Original chitosan; (B) water-insoluble chitosan fraction; and (C) water-soluble chitosan fraction.

high yields. Although various hydrolysis procedures and conditions using acids and enzymes have been proposed for preparing chitosan oligomers, it seems to be difficult to obtain the oligomers with the DP ranges of 10–15. On the other hand, since the chitosan fractions of these DP ranges were found to have some physiological activities, the method of hydrolysis using 85% phosphoric acid seems to be useful for preparation of chitosan oligomers with such range of DP in high yields.

Figure 5 shows the X-ray diffraction patterns of the water-insoluble and water-soluble fractions. The pattern of the water-insoluble fraction was identical to that of the 'tendon' type crystal structure, as observed for the original chitosan (Saito *et al.*, 1987). On the other hand, the water-soluble fraction showed the 'annealed' type pattern, which was identified by the three characteristic diffraction peaks at $2\theta = 15{\cdot}1°$, $20{\cdot}9°$ and $23{\cdot}7°$ (Ogawa *et al.*, 1984; Saito *et al.*, 1987). The 'annealed' type chitosan was formed from the 'tendon' type chitosan by heating at more than 200°C, probably as a result of removal of water molecules from the crystal lattice (Ogawa *et al.*, 1984). Therefore, the formation of the 'annealed' type chitosan at room temperature, observed for the water-soluble fraction in this study, seems to be

Table 1. Degrees of polymerisation of hydrolysed chitosans[a]

Sample	$\overline{DP_w}$	$\overline{DP_n}$	$\overline{DP_w}/\overline{DP_n}$
Water-insoluble fraction	16·8	8·0	2·10
Water-soluble fraction	7·3	5·4	1·35
Original chitosan	476	104	4·57

[a]Obtained after 4 weeks standing of the chitosan/85% phosphoric acid solution.

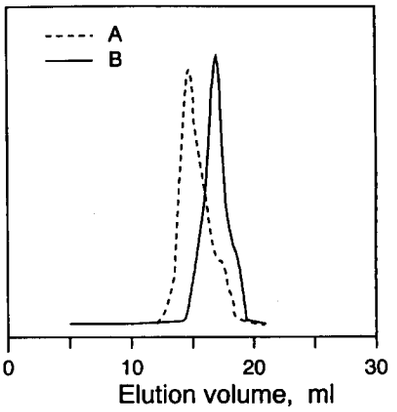

Fig. 5. Size exclusion chromatograms of water-insoluble and water-soluble chitosan fractions. These chitosan fractions were obtained after 4 weeks standing of the chitosan/85% phosphoric acid solution. (A) Water-insoluble fraction; and (B) water-soluble fraction.

characteristic of the present hydrolysis system using 85% phosphoric acid.

Single-crystalline chitosan samples with lamella structures, which showed a diffraction pattern similar to that of 'annealed' type, were reported to be prepared by recrystallisation of low-DP chitosan at 125°C with ammonia (Cartier *et al.*, 1990). Since the water-insoluble chitosan fraction, with $\overline{DP_w}$ of 16·8, formed the 'tendon' type crystal structure, the DP values and/or the precipitation procedure seem to have influence on the crystal structures of low-DP chitosans prepared.

Thus, two types of low-DP chitosans, which had $\overline{DP_w}$ values of approximately 17 and 7 with relatively narrow DP distributions, were prepared in high yields by the homogeneous hydrolysis of chitosan with 85% phosphoric acid. These low-DP chitosans may be useful not only as model compounds of chitosans but also as bio-active compounds. Incidentally, the homogeneous hydrolysis with 85% phosphoric acid was conducted also to chitin in anticipation of obtaining low-DP chitin. It was found, however, that not only depolymerisation but also deacetylation took place on chitin, and thus low-DP chitin could not be obtained by this method.

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