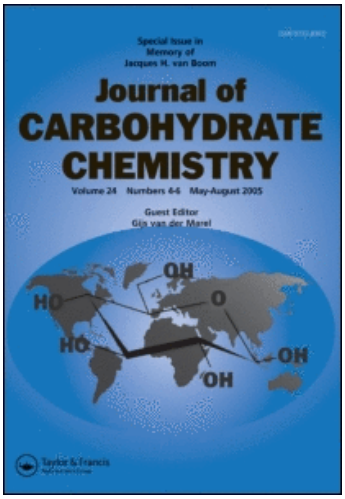


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# CRYSTALLINE TRANSFORMATION OF CHITOSAN FROM HYDRATED TO ANHYDROUS POLYMORPH VIA CHITOSAN MONOCARBOXYLIC ACID SALTS[1]

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## ABSTRACT

Spontaneous removal of monocarboxylic (formic, acetic, propionic or butyric) acids accompanying dehydration of the corresponding chitosan salts was observed from X-ray fiber diffraction diagrams obtained during the storage of these salts for a given period of time. The first three salts were prepared by immersing a tendon chitosan (a hydrated crystal) in an aqueous solution of respective monocarboxylic acid and 2-propanol. The salts showed similar fiber patterns not only to one another but also to the "Eight-fold" polymorph of the original chitosan, indicating that they are Type II salts, hydrated crystals, where the backbone chitosan molecule takes up an eight-fold helical conformation. The temperature required for

the salt formation depended on the hydrophobicity of the acid, e.g., the chitosan formic acid salt could be prepared at room temperature, whereas, formation of the propionic acid salt was carried out at 4 °C. All the acids spontaneously evaporated accompanied by dehydration during storage of the salts, resulting in formation of anhydrous crystalline chitosan. Removal of the monocarboxylic acids was accelerated by increasing the hydrophobicity of the acid: at 100% relative humidity approximately 3 months for the formic, 1 month for the acetic and 3 weeks for the propionic acid salts. In the case of butyric acid the anhydrous polymorph of chitosan was obtained immediately after the reaction, suggesting that the water removing action of this acid was too fast to detect a Type II salt by the present X-ray method. The anhydrous crystals of chitosan were irreversibly prepared by annealing a hydrated crystal in water at a high temperature, such as 240 °C, leading to a little loss of orientation and to thermal decomposition of the sample specimen to some extent. But it was found that, through Type II salts of monocarboxylic acids, the hydrated crystals of chitosan can be dehydrated even at room temperature without any loss of orientation and decomposition of the chitosan specimen.



## INTRODUCTION

Chitosan, a linear polymer of β-(1→4)-linked 2-amino-2-deoxy-D-glucose and prepared from chitin by *N*-deacetylation, is an important biopolymer and has been utilized for various industrial, agricultural, medicinal and pharmaceutical applications because of its biodegradable, biocompatible,[2] polyelectrolyte,[3] chelating[4] and flocculating[4] properties. These properties undoubtedly depend on the molecular conformation of chitosan. So far, five crystalline polymorphs of chitosan have been found by X-ray diffraction measurements; four hydrated forms and one anhydrous form. Hydrated polymorphs are called "Tendon" (the most abundant),[5,6] "Form II",[7] "L-2",[8] and "Eight-fold"[9] although the last polymorph is unstable. In the former three polymorphs, chitosan molecules take up an extended two-fold helix and in the last, an 8/5 helical structure.[9] The anhydrous polymorph is prepared by heating a hydrated crystal of chitosan in water at a high temperature, such as 240 °C, depending on the molecular weight of chitosan.[10,11] This polymorph is called an "Annealed" form where chitosan

has an extended two-fold helix.[10] Recently, we have analyzed molecular and crystal structures of "Tendon"[6] and "Annealed"[12,13] polymorphs, and found that the transformation from the former to the latter occurred irreversibly[11] and involved a drastic change in the chain arrangement of chitosan although no change in the molecular conformation of each chitosan chain was observed.[6] Anhydrous crystalline chitosan does not dissolve in aqueous acid solution, nor form a complex with any transition metal ion.[12,14] Consequently, the anhydrous chitosan may be considered to be inert material. However, the high temperature treatment required to generate this crystalline transformation results in not only a little loss of the orientation but also in thermal decomposition of chitosan to some extent, particularly, on the surface of the specimen. We found that a chitosan specimen of the hydrated "Tendon" polymorph changed to the anhydrous "Annealed" polymorph without any decomposition when it was stored in water at room temperature for 18 months,[15] although the period is too long for practical application.



Our X-ray diffraction studies on the crystals of various inorganic[16] and organic[17-19] acid salts of chitosan have revealed that these crystals are classified into two types depending on the structure of the acid and sometimes temperature of salt preparation. One called Type I salts are anhydrous, and in these crystals the backbone chitosan chains retain the extended two-fold helix of the unreacted chitosan molecule.[6] The other, Type II salt, is a hydrated crystal and has an eight-fold helical conformation in the crystal. Despite different anion sizes, all the Type II salts give not only similar fiber patterns to one another[16,18] but also the "Eight-fold" polymorph of chitosan molecule;[9] that is, they have identical unit cell dimensions. These facts suggested that anions were not present regularly in the respective crystals of the salts and consequently that only the backbone chitosan chains contributed to the fiber pattern.[16]

Recently, Demarger-Andre and Domard reported that anhydrous chitosan crystals can be obtained at room temperature from chitosan salts of several monocarboxylic acids (acetic, butyric or valeric acids) by spontaneous removal of the acids accompanied by dehydration.[20] We call it "water removing action of acid".

These findings stimulated us to develop procedures to prepare the anhydrous "Annealed" crystal, the inert chitosan, without any decomposition. We studied the spontaneous water removing action of acid by preparing chitosan salts of many different (monocarboxylic, inorganic and organic) acids, and examined their structures by using X-ray diffraction measurements.



## RESULTS

The water removing action was observed with the monocarboxylic acid salts of chitosan.[20] All other acid salts[16-18] (including dicarboxylic acids[21]) studied so far have not shown any spontaneous removal of the acid accompanying dehydration even after their storage at room temperature (23 °C) and 100 % r.h. for one year or more. Recently, we have found from X-ray diffraction measurements on fiber diagrams that the action occurs in the crystals of chitosan acetic acid salt.[19] The salt prepared at 110 °C showed a diagram of typical Type II salt (Figure 1, bottom, left), a hydrated crystal, where the chitosan molecule has an eight-fold helix.[16] A completely different fiber pattern (Figure 1, bottom, right), which was regarded as the anhydrous "Annealed" polymorph of chitosan, was observed when the salt specimen was stored at the room temperature and around 80% r.h. for three months. Measurements of density and the FT-IR spectrum of the specimen also supported the transformation from the acetic acid salt to an anhydrous crystal of chitosan.[19] This change was accelerated when the salt was stored at higher humidity, e.g., at 100% r.h. for approximately one month.

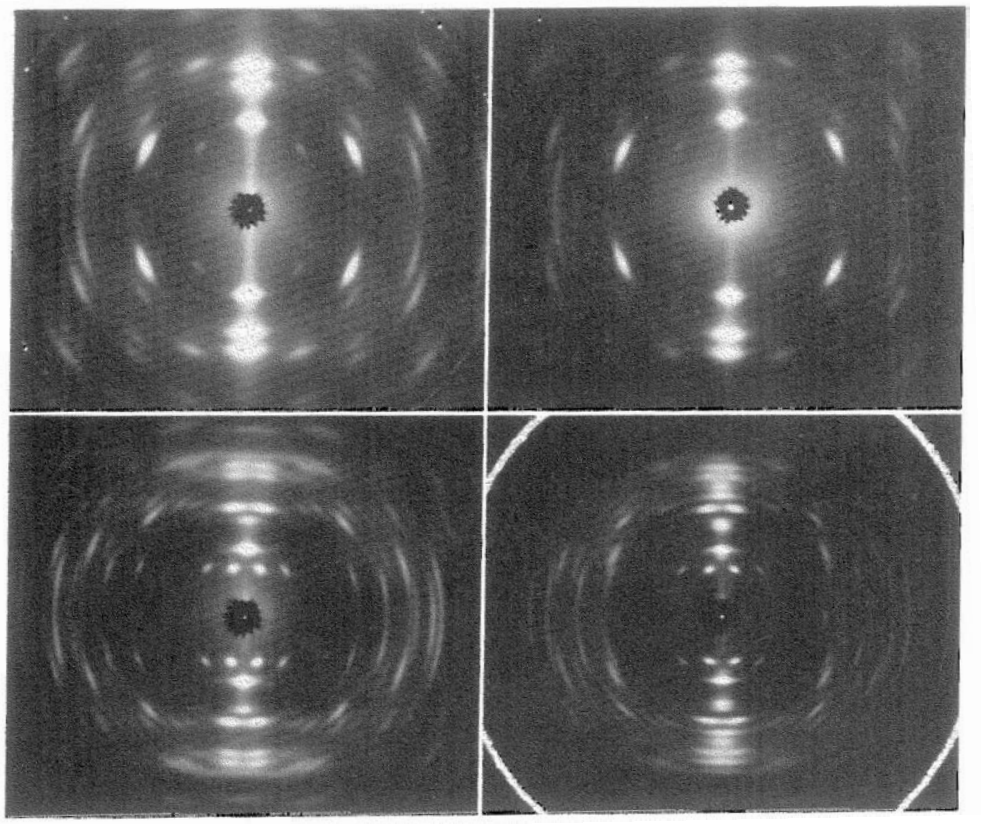

**Figure 1.** Fiber patterns of the chitosan formic (top) and acetic (bottom) acid salts. Left: freshly prepared, right: aged at 100% r.h. Fiber axes are vertical. The formic acid salt was prepared at 25 °C for 3 h, and the acetic acid salt, at 110 °C for 10 min.

A fiber pattern of Type II chitosan formic acid salt (Figure 1, top, left) was obtained with specimens prepared at lower temperatures between 4 and 25 °C. In addition, this salt differs from the chitosan acetic acid salt, in that no change in the fiber pattern of the formic acid salt was observed even after one year storage at room temperature and around 80% r.h. This may be the cause of the speculation by Demarger-Andre and Domard that the spontaneous water removing action did not occur in chitosan formic acid salt.[20] However, when the salt was kept at 100% r.h. for approximately

three months, the fiber pattern of the resultant specimen was completely changed to that shown in Figure 1 (top, right) which is obviously regarded as the "Annealed" chitosan polymorph.

In the case of chitosan propionic acid salt, when prepared at 25 °C for 3 hours, the resultant specimen showed a fiber diagram indicating a mixture of three components: a large amount of unreacted ("Tendon") chitosan, a little "Annealed" (anhydrous) chitosan, and a trace of Type II salt. At 70 or 110 °C no change in the fiber diagram was observed, suggesting no reaction. Whereas, using the 4 °C preparation, the sample showed a pattern which indicated the clear appearance of Type II salt although unreacted chitosan still remained. When the 4 °C preparation was prolonged to 3 days, diffraction spots of unreacted chitosan disappeared. However, this pattern indicates that the specimen consists of not only Type II salt but also the "Annealed" chitosan judged by the presence of (020) and (200) reflections of the crystal[10] (Figure 2, top, left). These facts indicate that a fiber diagram showing solitary Type II salt for chitosan propionic acid salt cannot be obtained because the water removing action of propionic acid is faster than that of formic and acetic acids. After this specimen was kept at 100% r.h. for approximately 3 weeks, the fiber diagram changed to the complete pattern of the "Annealed" polymorph (Figure 2, top, right).

When tendon chitosan was immersed in a mixture of aqueous butyric acid and 2-propanol at 25 °C for 3 h, the resultant sample showed a fiber diagram of the original "Tendon" chitosan, indicating no reaction. Prolongation of the reaction time to one week was not effective. No reaction was observed at 70 °C. These results suggest that chitosan does not react with butyric acid at 25 °C or higher, whereas, when prepared at 4 °C the resultant sample gave a fiber pattern (Figure 2, bottom). This pattern was judged to be the "Annealed" chitosan for the following reason. The relative intensities of the three strong reflections on the equator which were observed with the "Annealed" polymorphs made from the other three acid salts (Figure 1, rights and Figure 2, top right) are different from those

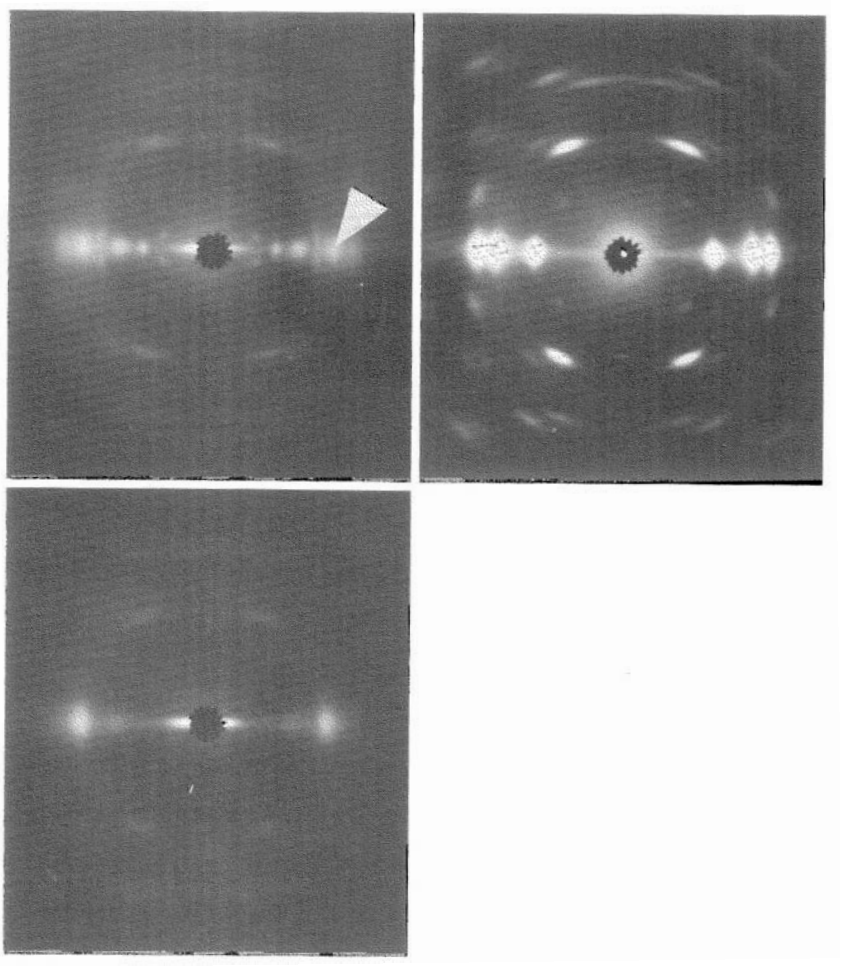

**Figure 2.** Fiber patterns of propionic acid salt (top) and the chitosan butyric acid salt (bottom). Left: freshly prepared, top right: aged at 100% r.h. Fiber axes are vertical. A white triangle indicates the presence of (020) and (200) reflections of "Annealed" chitosan polymorph. The propionic acid salt was prepared at 4 °C for 3 days, and the chitosan / butyric acid reaction was carried out at 4 °C for 3 h.

prepared with the butyric acid salt (Figure 2, bottom). The differences in reflection intensities may be attributed to a disorder in the latter crystal because of the lower crystallinity. The present result may indicate that the water removing action of butyric acid is too fast to detect Type II salt by the present X-ray measurement.

## DISCUSSION

Salt formations. All the monocarboxylic acids of chitosan studied were Type II salts, the hydrated crystal, where the backbone chitosan chains had eight-fold helical conformations. The chitosan formic acid salt could be prepared at a lower temperature than the acetic acid salt, which may be due to stronger acidity of formic acid. Whereas, the temperature trend for salt formation was acetic > propionic > butyric acid regardless of the acidity, the Type II salt of chitosan butyric acid could not be detected in the present study presumably because of fast water removing action of the acid. The temperature trend for salt formation may be due to the solubility of the monocarboxylic acid to water because the relative hydrophobicity of these acids is acetic < propionic < butyric, and because monodispersity (i.e., no molecular aggregation) of more hydrophobic material in water solution requires lower temperature.[22]



Water removing action of monocarboxylic acid. Present results suggested that all the monocarboxylic acids studied were spontaneously removed accompanied with dehydration from the salts during storage for a given period of time. The period depended not only on the acid but also on relative humidity of the storage. When stored at 100% r. h., three months, one month and three weeks were required to produce the complete fiber pattern of the "Annealed" chitosan polymorph from formic, acetic and propionic acid salts of chitosan, respectively. In contrast, chitosan butyric acid salt gave the "Annealed" pattern of chitosan (Figure 2, bottom) immediately after the preparation. These facts suggest that the water removing action of monocarboxylic acid is accelerated by the hydrophobicity in addition to p*Ka*, solubility and boiling point of acid as speculated by Demarger-Andre and Domard.[20]

The mechanism of the spontaneous water-removing action of monocarboxylic acid has not been well defined yet, but may be attributed to the conformational stability of the chitosan molecule. As shown in our

previous reports[6,12] the extended two-fold helix of chitosan is stabilized by O(3)----O(5') intramolecular hydrogen bonds, whereas, energy calculations done by us[16] and Cairns *et al.*[9] have revealed that no intrachain hydrogen bond is observed in the 8/5 helix. In addition, the latter authors have indicated that the "Eight-fold" polymorph is unstable and is easily converted to the two-fold helix. Therefore, the 8/5 conformation is less stable than the former. The *h* value, which is one of helix parameters showing an axial rise per glucosamine residue and is calculated from the fiber repeat divided by number of residues composing the repeat, is 0.52 nm[6,12] for the two-fold helix and 0.51 nm[16] for the eight-fold helix, indicating that the latter is an extended helix close to the former. It is reasonable to consider that the chitosan molecule having a two-fold helix in the hydrated crystal is twisted slightly by the formation of type II salt. The resulting 8/5 helix structure may be stabilized by an anion of the salt. However, if the interaction between the anion and chitosan is weak, the chitosan molecule tends to go back to the more stable two-fold conformation by throwing out the anion. At present, we do not know why monocarboxylic acids have weaker interactions with chitosan molecules than other acids studied so far, why the removal of former acids is accompanied by water molecules, and why the hydrophobicity of the acid and the humidity of storage accelerate the water removing action of the acids. In order to define the mechanism, the detailed crystal structure of the Type II salt of chitosan must be analyzed, and this study is underway.

**Transformation of chitosan polymorph from hydrated to anhydrous.** Recently, we analyzed the crystal and molecular structures of hydrated "Tendon"[6] and anhydrous "Annealed"[12,13] polymorphs of chitosan where chitosan chains have a similar extended two-fold helix stabilized by O(3)----O(5') intramolecular hydrogen bonds in both crystals. In the hydrated crystal, adjacent antiparallel chitosan chains are bonded to each other by two interchain hydrogen bonds forming a sheet. Furthermore, the

adjacent sheets are bonded by hydrogen bonds involving water molecules.[6] Whereas, in the anhydrous polymorph, intermolecular hydrogen bonds connect adjacent parallel chitosan chains to make a sheet, and adjacent sheets are antiparallel.[12] These facts suggest that a drastic change in the arrangement of chitosan chains occurs during the transformation from the hydrated to the anhydrous crystal.

Two transformation procedures from the hydrated to the anhydrous polymorph of chitosan are illustrated in Figure 3. So far, the anhydrous chitosan has been prepared by annealing the hydrated crystal in the presence of water at a high temperature, such as 240 °C, although the temperature required depends on the molecular weight of chitosan.[11] The requirement for such a high temperature may be due to the drastic change in the chain arrangement of chitosan.[6] This results in a little loss of orientation and a thermal decomposition of sample specimen to some extent, particularly, on the surface. The decomposition is not appropriate for preparing the "Annealed" crystal when a good sample of inert chitosan is required. The present results indicate that the anhydrous polymorph can be easily obtained even at room temperature via Type II salts of monocarboxylic acids without any loss of orientation and thermal decomposition. At first, the hydrated chitosan reacts with a monocarboxylic acid to make a hydrated Type II salt where chitosan chain has an eight-fold helix. After that, during the storage of the salt, the acid evaporates spontaneously out of the chitosan chains accompanied by water molecules, resulting in the occurrence of the anhydrous polymorph of chitosan. Both transformations from hydrated to anhydrous polymorph are irreversible although the Type II salt can be converted to the hydrated Tendon chitosan by neutralization with an aqueous alkali such as sodium hydroxide solution.

The best preparation of the anhydrous polymorph of chitosan from the hydrated crystal seems to be via the propionic acid salt since the salt can be obtained at a low temperature (4 °C) for a few days and the spontaneous

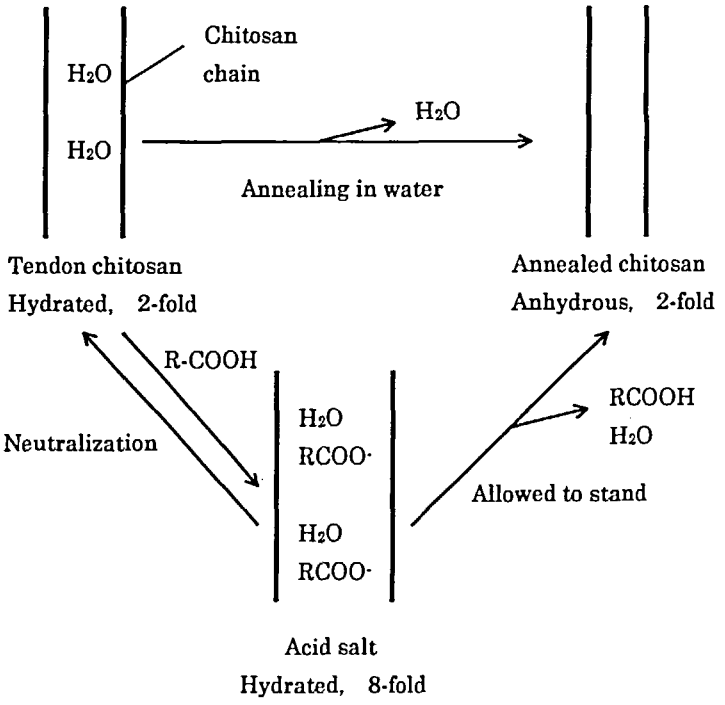


**Figure 3**. Two processes of water removing from the hydrated crystal of chitosan.



removal of the acid accompanying water molecules from the salt requires approximately 3 weeks storage at 100% r.h. and room temperature. The crystallinity of the resultant anhydrous crystal was measured to be 71%.

## EXPERIMENTAL

Tendon chitosan is desirable for getting a good fiber pattern because of its high orientation. In order to prevent loss of molecular orientation it was prepared by heterogeneous *N*-deacetylation of tendon chitin from crab, *Chionecetes opilio* O. Fabricus, with 67% sodium hydroxide solution at 110 °C for 2 h under nitrogen atmosphere. Repeating the reaction twice led to complete *N*-deacetylation of resultant chitosan, as determined by FT-IR spectra and a colloidal titration.[23] The viscosity average molecular weight

of the tendon chitosan was $1.7\times10^6$.[11] The preparation of all chitosan salts of inorganic[16] and organic acids[17-19] other than monocarboxylic acids have been reported previously. Since chitosan dissolves in aqueous monocarboxylic acids leading to a loss of molecular orientation, the tendon chitosan was immersed in a mixture (3:1 v/v) of 2-propanol and an aqueous solution of respective formic, acetic, propionic and butyric acids (concentration of each acid, 4.0 M) at various temperatures for a given period depending on the kind of acid, followed by washing with 75% 2-propanol water solution, 2-propanol and then drying in air.

The X-ray fiber diffraction patterns were recorded using a flat film camera at 75% relative humidity in a helium atmosphere with a Rigaku Geigerflex X-ray diffractometer employing Ni-filtered CuKα radiation generated at 40kV and 15mA. Crystallinity of a chitosan specimen was calculated from WAXD intensity curves of a powder diffraction pattern using a microdensitometer Type 2405 produced by ABE SEKKEI, Tokyo, Japan.



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