Identification of chitin allomorphs in poorly crystalline samples based on the complexation with ethylenediamine

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Abstract

Chitin is a key component of hard parts in many organisms, but the biosynthesis of the two distinctive chitin allomorphs, α- and β-chitin, is not well-understood. The accurate determination of chitin allomorphs in natural biomaterials is vital. Many chitin-secreting living organisms, however, produce poorly crystalline chitin which leads to spectrums with only broad lines and imprecise peak positions under conventional analytical methods such as X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FT-IR), and solid state nuclear magnetic resonance spectroscopy (NMR), resulting in inconclusive identification of chitin allomorphs. Here, we developed a novel method for discerning chitin allomorphs based on their different complexation capacity and guest selectivity, using ethylenediamine (EDA) as a complexing agent. From the peak shift observed in XRD profiles of the chitin/EDA complex, the chitin allomorphs can be clearly discerned. By testing this method on a series of samples with different chitin allomorphs and crystallinity, we show that the sensitivity is sufficiently high to detect the chitin allomorphs even in near-amorphous, very poorly crystalline samples. This is a powerful tool for determining the chitin allomorphs in phylogenetically important chitin-producing organisms and will pave the way to clarify the evolution and mechanism of chitin biosynthesis.

Introduction

Chitin is one of the principal organic skeletal components in invertebrate animals such as arthropods, mollusks, and annelids.[1,2] Chitin is bio-synthesized in the form of microfibrils, where the elongated chitin molecules are packed in highly ordered manner to form nanofibrous structures.[3–7] Chitin crystalline nanofibers exhibit two distinctive forms, one with an antiparallel packing of chitin molecules known as α-chitin,[8,9] and another with a parallel packing known as β-chitin[10–12] – both of which occur in nature and are biosynthesized by organisms.[1,2] Arthropods such as crustaceans produce α-chitin, which is thought to be more abundant naturally compared with β-chitin, which is produced by diatoms,[13] annelid worms[4], and mollusks such as squids[14]. The two forms in chitin are analogous to cellulose, which also forms parallel (cellulose I)[15] and anti-parallel (cellulose II)[16] packing, but in cellulose only the parallel-packed cellulose I occurs naturally[17]. The mechanisms behind natural production of parallel-packed nanofibers is relatively well understood from cellulose research,[18,19] but less is known about the natural production of chitin nanofibers, especially how living organisms make highly crystalline anti-parallel α-chitin.[20]

The precise identification of chitin allomorphs is inevitable and vital in addressing this question. Analytical methods often employed to distinguish them include X-ray diffraction (XRD),[14,21] Fourier-transform infrared spectroscopy (FT-IR),[22,23] and solid state nuclear magnetic resonance spectroscopy (NMR).[24,25] In the cases where the chitin samples are highly crystalline, such as chitin in the tubes of siboglinid worms,[4,26] the peaks in the spectrums are indeed sufficiently sharp and well-defined for locating precise peak positions or peak splitting characteristic to each chitin allomorph. However, poorly crystalline samples, like those with low crystallinity or small crystal size, result in spectrums with only broad lines with imprecise peak position leading to inconclusive identification of chitin allomorphs.[27–29]

The accurate determination of chitin allomorphs in poorly crystalline chitin is the key to understanding their natural biosynthesis, since evolutionarily important chitin-secreting living organisms such as lancelets, tunicates, and mollusks generally produce poorly crystalline chitin.[27,30,31] Here, we propose a novel method for the identification of chitin allomorphs based on the different complexation capacity and guest selectivity of α- and β-chitin, using ethylenediamine (EDA) as a complexing agent.[32–35] These differences between the two chitin allomorphs arise due to the presence of hydrogen bonding between molecular sheets in α-chitin, which is lacking in β-chitin.[32] We first outline the limitations and pitfalls of the conventional methods based on the complexation with water (i.e. hydration). Then, we test and show the effectiveness of this new method in determining chitin allomorphs using samples with various levels of crystallinity.

Materials and methods

Materials

Purified water was used throughout this study (Milli-Q Advantage A10, Merck, Germany). Hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium borohydride ($NaBH_4$), acetic acid (AcOH), chloroform ($CHCl_3$), sodium chlorite ($NaClO_2$), acetic anhydride (Ac_2O), methanol (MeOH), ethanol (EtOH), ethylenediamine (EDA) and chitosan (chitosan 100; degree of acetylation (DA): ~0.2) were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Sample collection and purification

A red king crab (*Paralithodes camtschaticus*) and several individuals of spear squids (*Heterololigo bleekeri*) were purchased in a grocery store. The king crab's exoskeleton and the squids' gladius ('squid pen') were isolated by dissection. The siboglinid tubeworm (*Lamellibrachia satsuma*) was collected from Nikko seamount (23°04.86'N, 142°19.51'E, 458m deep, Dec 2020) by a manipulator on the remotely operated vehicle (ROV) *KM-ROV* during the R/V *Kaimei* cruise KM20-10C leg 2. A Scaly-foot Snail (*Chrysomallon squamiferum*) was collected from Solitaire hydrothermal field (19°33.410 S, 65°50.890 E, 2606 m depth, Feb 2013) by a suction sampler on the deep-submergence vehicle (DSV) *Shinkai 6500* during R/V *Yokosuka* cruise YK13-02, and the scales were dissected from the foot. Planktonic copepods (*Pontella fera*, Calanoida) were collected from the surface water during the R/V *Yokosuka* cruise YK19-11 by a neuston net from the Northwest Pacific approximately 400 km east off Japan (35°07.0'N, 145°00.0'E), identified and sorted out under a dissecting microscope, then air-dried whole. Purification of chitin samples was performed according to the previous studies.[36–39]

Each sample was cut into small pieces with about 1 cm in size (except Scaly-foot Snail's scale and copepods that were purified whole) and soaked in a 2:1 (by volume) mixture of chloroform and acetic acid for 24 h to extract lipids. Demineralization, deproteinization, and decolorization were then carried out using 1 M HCl, 2.5 M aqueous NaOH, and 0.3 w/v% aqueous $NaClO_2$ solution followed by a thorough washing with water, respectively. Each purification step was repeated 4 times. Purified samples were freeze-dried, except the squid pen that was subjected to air-dry at room temperature from water and EtOH, oven-dry at 60 from water, and freeze-dry from water. A *N*-acetylated chitosan sample was prepared by the acetylation of chitosan in acidic alcohol solution.[40,41] Briefly, chitosan powder (chitosan 100, 1.5 g) was dissolved in 6 mL of AcOH/54 mL of water, followed by dilution with 240 mL of MeOH. The chitosan solution was then cooled to −10°C, and the desired amount of Ac_2O (molar ratio Ac_2O/NH_2: 2.1) was added. After complete mixing at −10 °C, the mixture was poured into cylindrical molds (bore size: 14 mm) at room temperature and left for 10 h to ensure complete gelation. After thorough washing with water, the *N*-acetylated chitosan sample was oven-dried at 105°C. To observe the

transformation from β-chitin to α-chitin, the purified squid pen was treated with 7, 7.5, and 8 M HCl for 0.5 h. After thorough washing with water, the HCl treated squid pen samples were oven-dried at 105°C. To observe the transformation from β-chitin to chitosan, the purified *L. satsuma* was treated with 12.5 M aqueous sodium hydroxide containing a small amount of $NaBH_4$ to prevent depolymerization, at 90°C for 0.5, 3, and 6 h. After thorough washing with water, the NaOH treated *L. satsuma* samples were oven-dried at 105°C.

X-ray diffraction analysis

Wide-angle X-ray diffraction experiment was performed on Nanopix (Rigaku Japan) at 40 kV and 30 mA with monochromatized and collimated Cu Kα radiation (λ = 1.548 Å). The capillary-sealed dry and wet specimen (saturated complex with water or EDA prepared by the immersion in water or EDA at room temperature for several hours[33,42]) was subjected to X-ray diffraction measurements by the transmitting beam, where the camera length (sample-to-CCD distance: 82.37 mm) was calibrated with Si powder (d = 0.31355 nm). The peak top position was determined from the position with the highest intensity in the peak. The diffraction peaks were fitted with a pseudo-voigt function, and the crystal size, *D*, perpendicular to the diffraction planes, (020) plane in α-chitin and (010) plane in β-chitin, was evaluated using Scherrer's expression:

$$D = \frac{0.9 \times \lambda}{\beta \times \cos\theta}$$

where θ is the diffraction angle, λ is the wavelength of X-ray and β is the peak width at half of the maximum intensity.

Results and discussion

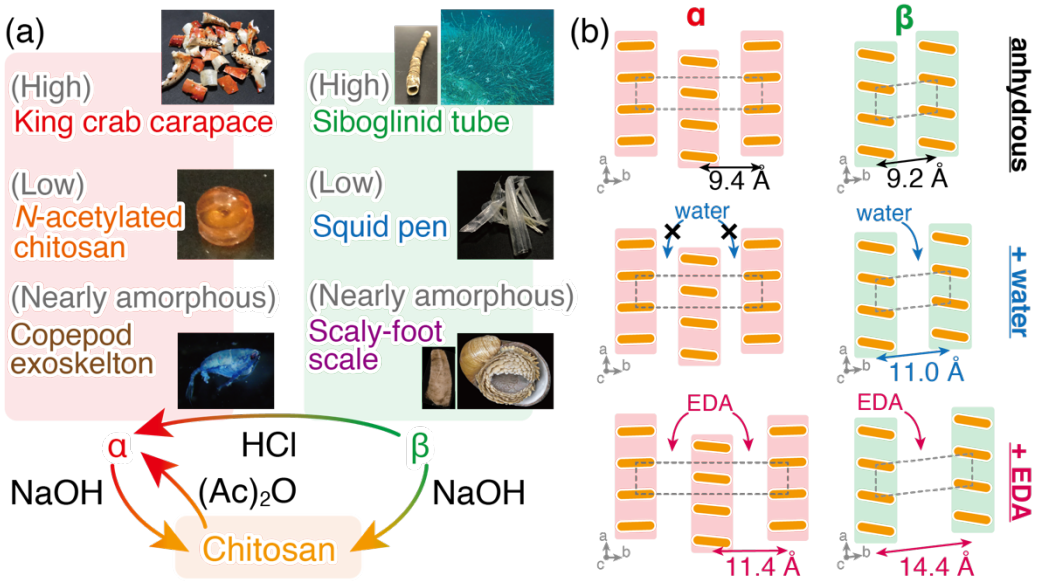

Figure 1. Schematic illustration of (a) the chitin samples used in this study and (b) cross-sectional crystal structure of native, dihydrate, and EDA complex of α- and β- chitin. Dotted rectangles represent unit cell of crystal structure of native, dihydrate, and EDA complex of α- and β-chitin.[9,32,33,43,44]

An overview of the chitin samples with known allomorphs used in the present study is shown in **Fig 1a**. Native chitin samples are characterized by three parameters: i) allomorphs, α and β, ii) crystallinity, and iii) degree of *N*-acetylation. The β allomorph can be transformed into α-chitin by acid treatment[21] or regeneration (dissolution and coagulation).[45] Due to the higher stability of the antiparallel α-chitin compared to the parallel β-chitin, this transformation is irreversible and α-chitin never reverts to β-chitin. Chitin allomorphs are naturally produced with a wide range of crystallinity, depending on the organisms of origin.[1,46,47] The degree of *N*-acetylation is another important parameter that varies among the organism-biosynthesized natural chitins and affects the quality of chitin allomorphs.[1,46–48] The monomeric unit of chitin is *N*-acetyl-*D*-glucosamine and the acetyl groups can therefore be removed by alkaline hydrolysis. Through deacetylation, chitin allomorphs lose crystallinity and become a rather amorphous substance denoted as chitosan. Through the *N*-acetylation of chitosan by acetic anhydride in acidic alcohol solutions, chitosan can be reverted to chitin and the resulting *N*-acetylated chitosan is identical to α-chitin with poor crystallinity.[41] For a comprehensive test across an array of possible chitins, here we used α-chitin from the highly crystalline king crab carapace, the poorly crystalline *N*-acetylated chitosan, and β-chitin from the highly crystalline siboglinid worm tubes, the poorly crystalline squid pen. In

addition, we also examined chitosan as the representative of samples with a low degree of acetylation.

The identification of chitin allomorphs was conducted based on the complexations of chitin, schematized in **Fig. 1b**. In both α- and β-chitin, the molecular sheets of chitin molecules are the primary building units (**Fig. 1b**). The different distances between these molecular sheets in α- and β-chitin result in characteristic peaks at 2θ = 9.4° and 9.6°, respectively. When hydrated with water, β-chitin takes up water between the molecular sheets and increases the distance between them, leading to a shift of above-mentioned peak to a lower angle, the extent of which depends on the number of the incorporated water molecules: 2θ = 8.6° in the case of monohydrate (one molecule per unit cell) and 2θ = 8.0° in dihydrate (two molecules per unit cell).[10–12,43,44] Since this incorporation of water does not occur in α-chitin, the peak shift induced by hydration has often been used to identify the chitin allomorphs.[49] However, in poorly crystalline samples, the identification based on hydration can be erroneous from a lack of clearly discernable peaks, due to the characteristic peaks being rather close together (detailed in the following section). Therefore, we newly employed ethylenediamine (EDA) as the complexing agent. Although EDA can be incorporated in both α- and β-chitin, the β-chitin/EDA complex has a much larger inter-sheet distance (14.4 Å)[33] compared with those of β-chitin dihydrate (11.0 Å)[10,44] or α-chitin/EDA complex (11.4 Å),[32] making the peak shift easier to discern even in the poorly crystalline samples.

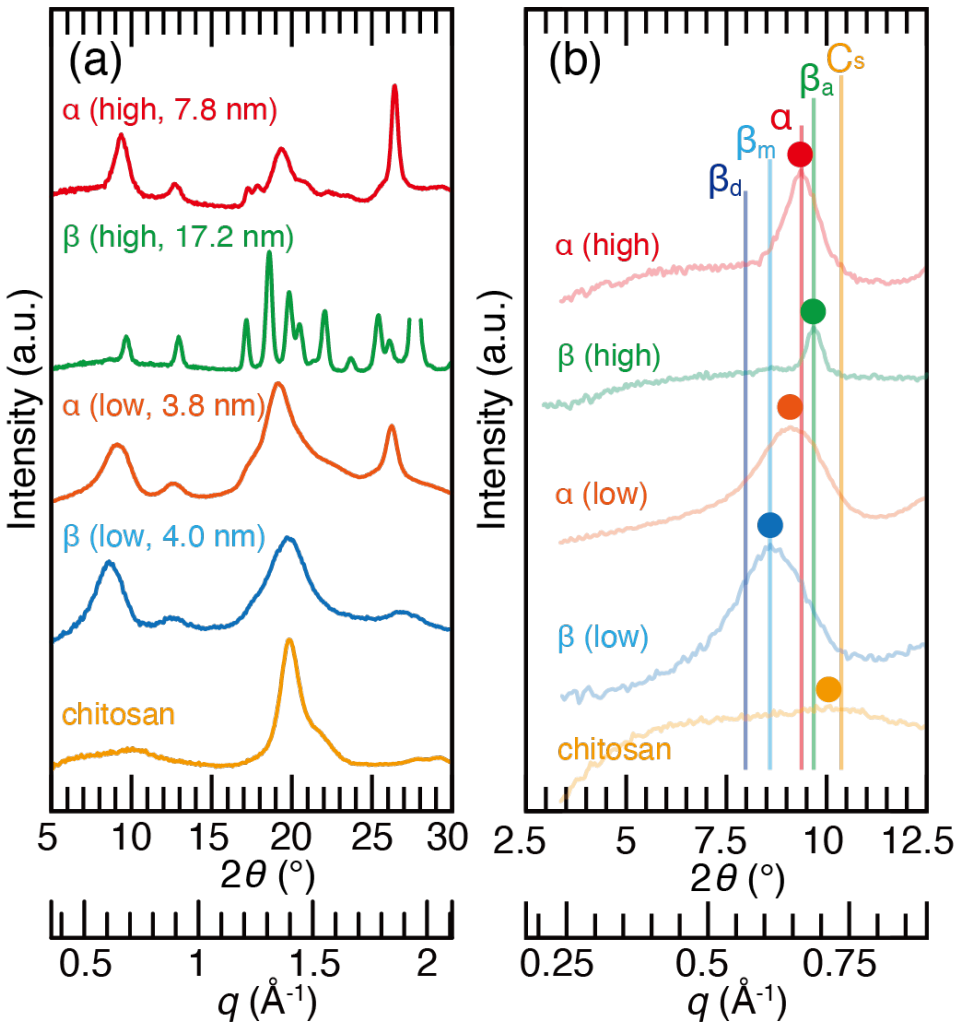

Figure 2. (a) X-ray diffraction profiles of the chitin samples used in this study. From top to bottom, king crab carapace, siboglinid worm tube, *N*-acetylated chitosan, squid pen, and commercial chitosan, were used as the representatives of high ("high" in red) and poorly ("low" in orange) crystalline α-chitin, high ("high" in green) and poorly ("low" in blue) crystalline β-chitin, and samples with low degree of acetylation ("chitosan" in yellow), respectively. The values in the brackets are the crystal size calculated from the peaks at $2\theta = 8.5$ - $9.7°$ (diffraction of (020) planes in α-chitin and (010) planes in β-chitin); (b) Enlarged profiles of **Fig. 2a**. α, β_a, β_m, β_d, and C_s represent the peak positions of α-chitin, anhydrous β-chitin, β-chitin monohydrate, β-chitin dihydrate, and chitosan from the literature data.[9,32,33,43,44,50] Colored dots represent the peak-top position of the profiles.

The representative XRD profiles of the chitin samples are shown in **Fig. 2a**. When the crystallinity is high, one can distinguish α- and β-chitin with ease from the considerable number of sharp peaks. However, the poorly crystalline samples exhibited almost identical XRD profiles.

Commercial chitosan also showed a broad profile, but the absence of the characteristic peak at 2θ = 8.5 - 9.7° is useful in its identification. It should be noted that depending on the preparation condition, chitosan forms hydrates that are rather crystalline, the XRD profile of which is similar to that of β-chitin (detailed in the following section). The detail of characteristic peaks at 2θ = 8.5 - 9.7° is shown in **Fig. 2b**. The peak positions of highly crystalline α- and β-chitin materials matched well with the literature data.[9,43] However, those of poorly crystalline α-chitin were lower than the literature data and located in between α-chitin and β-chitin monohydrate. This is probably due to a property often reported in cellulose, where the peak shifts to a lower angle by $2\theta = \sim 0.2°$ when the crystallinity is low or the crystal size is small.[51] This peak shift can be a cause for misidentifying α-chitin as β-chitin. Another issue was seen in the poorly crystalline β-chitin, the peak of which matched well with that of β-chitin monohydrate, even though the chitin sample used (squid pen) was oven-dried at 60°C.

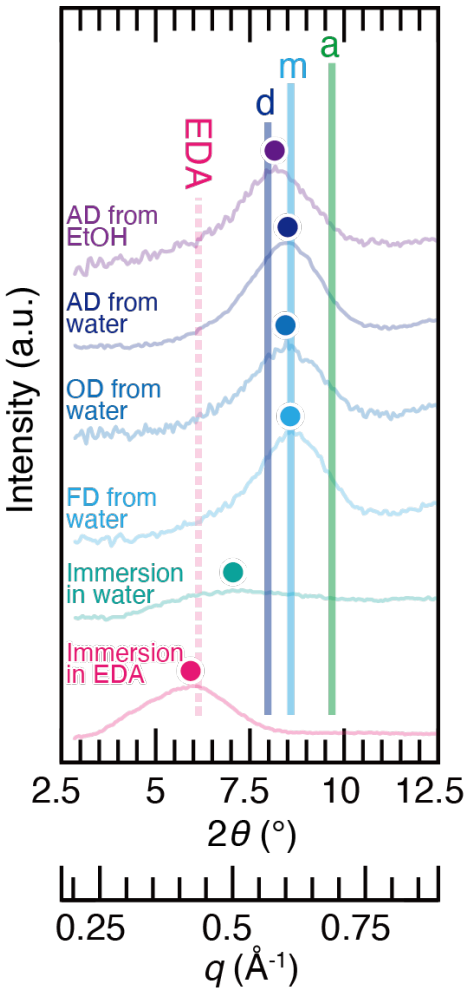

Figure 3. X-ray diffraction profiles of the squid pen prepared under different conditions. AD, OD, and FD represent air-dry, oven-dry at 60°C, and freeze-dry, respectively. a, m, d, and EDA represent the peak positions of anhydrous β-chitin, β-chitin monohydrate, β-chitin dihydrate, and

type II form of β-chitin/EDA complex from the literature data. [33,44,52] Colored dots represent the peak-top position of the profiles.

This recalcitrant nature of β-chitin hydrate can be a cause for misidentification of allomorphs when using the conventional method based on the hydration with water.[49] To demonstrate this, we analyzed poorly crystalline β-chitin samples (squid pen) prepared by a series of moderate drying methods (air-dried from ethanol and water, oven-dried from water at 60°C, and freeze-dried from water) that are often employed in the biomaterial studies (**Fig. 3)**. No drying method gave the peak corresponding to anhydrous β-chitin. It is known that to obtain anhydrous form of β-chitin one needs to dry at above 105°C.[53] Moreover, β-chitin prepared by air-drying from ethanol matched closely with literature values for β-chitin dihydrate. This lower peak position compared to the other drying methods was probably due to the trace of β-chitin/ethanol complex.[54] Therefore, attention needs to be paid in the use of biological samples stored in ethanol, as the peak of air-dried samples after ethanol storage does not shift to a lower angle by the rehydration, and the sample can be misinterpreted as α-chitin. In addition, when the sample is rehydrated by the simple immersion in water, the peak becomes highly blurred, and the peak position is not discernible. In this respect, the use of EDA as a complexing agent is advantageous: the peak of EDA/β-chitin complex stood out even with simple immersion in EDA. This high sensitivity of chitin against EDA makes the preparation of XRD samples and interpretation of the results much more straightforward compared to the hydration method.

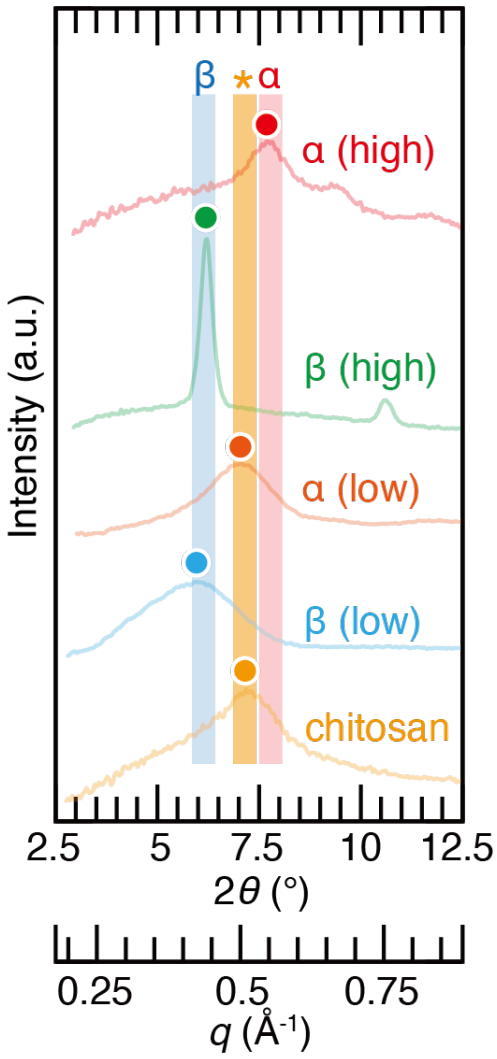

Figure 4. X-ray diffraction profiles of EDA complex of chitin samples used in this study. From top to bottom, king crab carapace, siboglinid worm tube, *N*-acetylated chitosan, squid pen, and commercial chitosan, were used as the representatives of high ("high" in red) and poorly ("low" in orange) crystalline α-chitin, high ("high" in green) and poorly ("low" in blue) crystalline β-chitin, and samples with low degree of acetylation ("chitosan" in yellow), respectively. Colored dots represent the peak-top position of profiles. Shades denoted as α and β are the reported peak position of EDA complex of α- and β-chitin with a margin of potential experimental error of plus or minus $2\theta = 0.25°$.[32,33] Shade marked with asterisk is the tentative peak position of the EDA/chitosan complex, newly reported herein, with a margin of potential experimental error of plus or minus $2\theta = 0.25°$ to the peak position observed in chitosan of **Fig. 4**.

To test the effectiveness of our chitin/EDA complex method, the chitin samples were subjected to complexation with EDA by the simple immersion in EDA as shown in **Fig. 4**. The

EDA complex of both high and poorly crystalline β-chitin fell into the reported peak position of β-chitin/EDA complex[33] with a margin of potential experimental error of plus or minus $2\theta = 0.25°$, which takes into account the potential peak shifts toward a lower angle induced by the low crystallinity or toward a higher angle caused by slightly different camera lengths (distance between sample and detector) among the measurements upon the use of glass capillary with diameter of 2 mm in XRD experiments. While the EDA complex of highly crystalline α-chitin was in accordance with the literature data, those of poorly crystalline α-chitin and chitosan fell into a position tentatively marked with an asterisk on **Fig. 3**. Although the origin of such EDA complex is unknown, we interpret that the poorly crystalline α-chitin and chitosan can incorporate a slightly larger number of EDA molecules between the molecular sheets than the highly crystalline α-chitin. Either way, we show that one can distinguish the chitin allomorphs from the peak top position of the EDA complex by the following three categories: β-chitin when the peak is located between $2\theta = 5.8$ and 6.3°, poorly crystalline α-chitin or chitosan between $2\theta = 6.9$ and 7.4°, and highly crystalline α-chitin between $2\theta = 7.5$ and 8.0°.

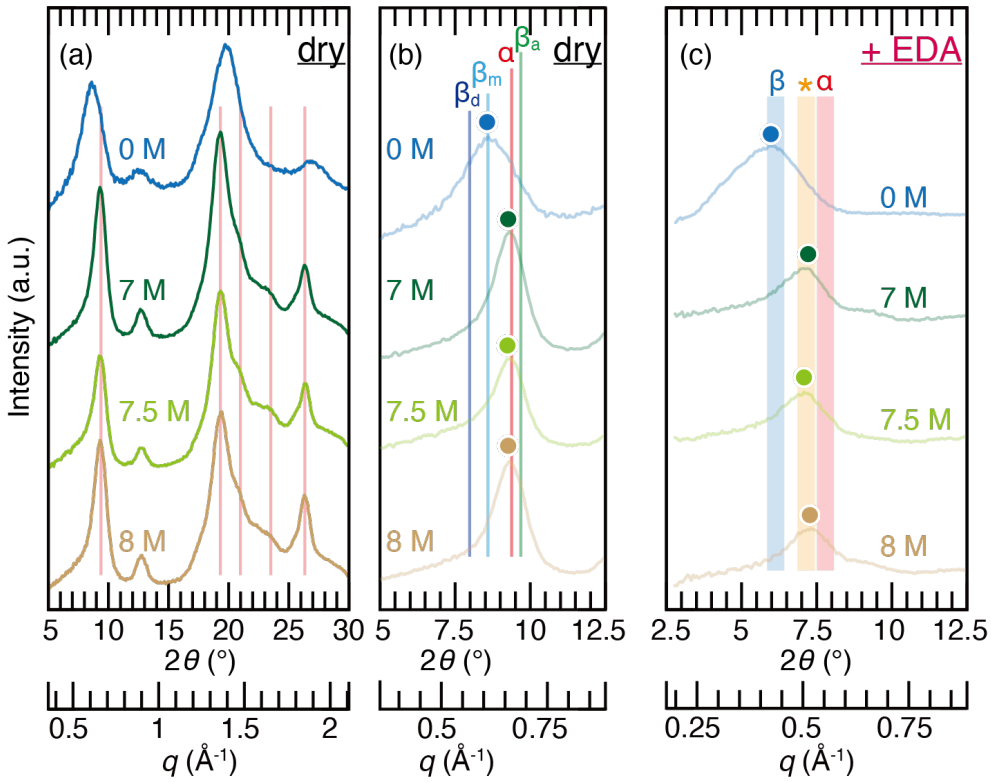

Figure 5. (a) X-ray diffraction profiles of squid pen samples treated with 0 (water), 7, 7.5, and 8 M HCl. Lines in red correspond to the diffractions characteristic of α-chitin; (b) Enlarged profiles of **Fig. 5a**. α, β_a, β_m, and β_d represent the peak positions of α-chitin, anhydrous β-chitin, β-chitin monohydrate, and β-chitin dihydrate from the literature data.[9,43,44] Colored dots represent the peak-top position of the profiles; (c) X-ray diffraction profiles of EDA complex of squid pen

treated with 0 (water), 7, 7.5, and 8 M HCl. Shades denoted as α, *, and β are the same as in **Fig. 4**. Colored dots represent the peak-top position of the profiles.

To further corroborate the three categories, α-chitin prepared from β-chitin by a series of hydrochloric acid treatment was subjected to complexation with EDA. It is known that HCl at 7 M and above strongly swells β-chitin and the transformation to α-chitin occurs upon washing.[49,55] As shown in **Fig. 5a**, β-chitin was successfully transformed to α-chitin. The peaks centered at 2θ = 9.3° were indexed as reflections from the (020) plane of α-chitin (**Fig. 5b**). By using the α-chitin samples, the validity of our EDA complexation method was examined (**Fig. 5c**). Through the complexation with EDA, the peak top position of β-chitin treated with 7, 7.5, and 8 M HCl fell into 2θ =6.9 and 7.4°, indicative of poorly crystalline α-chitin or chitosan. Therefore, the allomorphs were correctly identified with EDA complexation.

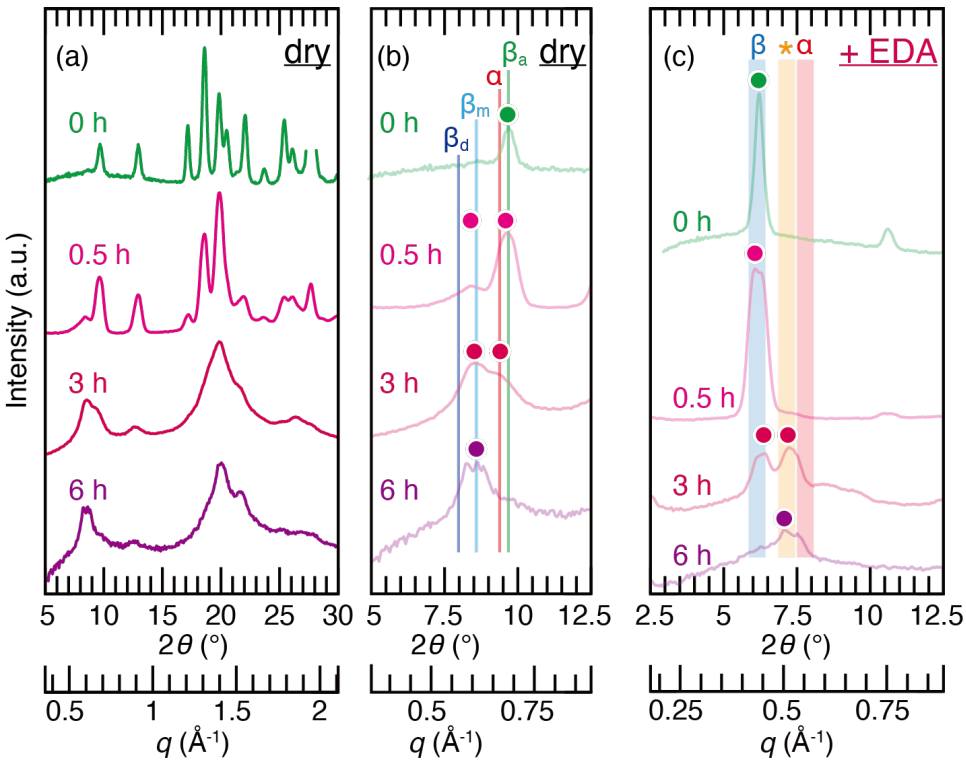

Figure 6. (a) X-ray diffraction profiles of the siboglinid worm tube treated with 12.5 M NaOH for 0 (water), 0.5, 3, and 6 h; (b) Enlarged profiles of **Fig. 6a**. α, β_a, β_m, and β_d represent the peak positions of α-chitin, anhydrous β-chitin, β-chitin monohydrate, and β-chitin dihydrate from the literature data,[9,43,44] respectively. Colored dots represent the peak-top position of profiles; (c) X-ray diffraction profiles of the EDA complex of the siboglinid worm tube treated with 12.5 M NaOH for 0 (water), 0.5, 3, and 6 h. Shades denoted as α, *, and β are the same as in **Fig. 4**. Colored dots represent the peak-top position of profiles.

In addition to the acid treatment, alkaline treatment was conducted in order to examine the transformation from β-chitin to chitosan through deacetylation (**Fig. 6a**). By immersing in 12.5 M aqueous NaOH solution, the β-chitin in siboglinid worm tubes gradually lost its high crystallinity, and a new peak was observed at 2θ = 8.5°, which corresponded to an unknown hydrate form of chitosan.[56] Judging only from **Fig. 6b**, this transformation may be misinterpreted as from anhydrous β-chitin to β-chitin monohydrate. However, the complexation with EDA clearly showed the gradual disappearance of β-chitin and the appearance of chitosan during the course of the alkaline treatment (**Fig. 6c**). A peak from the diffraction of (010) plane of the β-chitin/EDA complex gradually diminished, and a new peak appeared at the 2θ =6.9 and 7.4° position (marked with asterisk in **Fig. 6**) indicative of poorly crystalline α-chitin or chitosan.

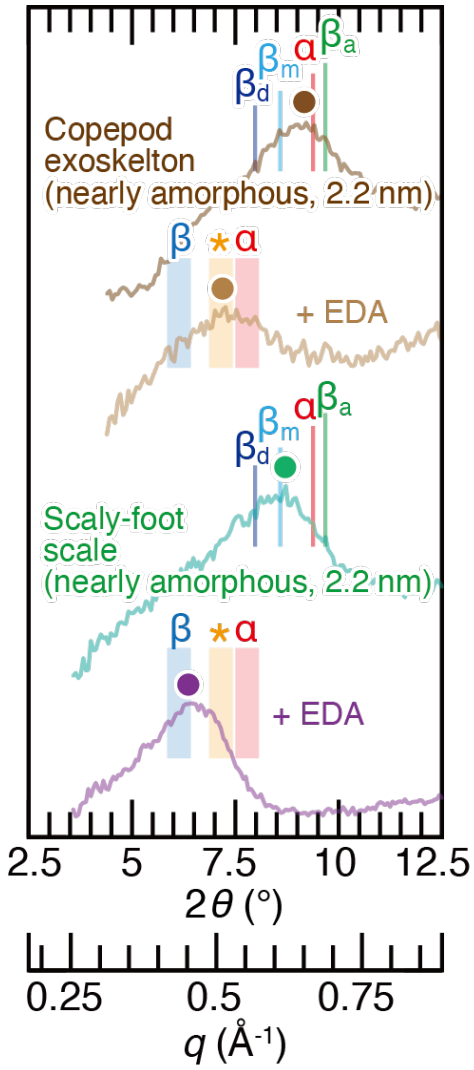

Figure 7. X-ray diffraction profiles of near-amorphous α-chitin copepod exoskeletons, those

complexed with EDA, the near-amorphous β-chitin Scaly-foot Snail scale (sampled position near the distal tip of the scale), and the one complexed with EDA. α, β_a, β_m, and β_d represent the peak positions of α-chitin, anhydrous β-chitin, β-chitin monohydrate, and β-chitin dihydrate from the literature data.[9,43,44] Shades denoted as α, *, and β are the same as in **Fig. 4**. Colored dots represent the peak-top position of profiles. The values in the brackets are the crystal size calculated from the peaks at 2θ = 8.5 - 9.3° (diffraction of (020) planes in α-chitin and diffraction of (010) planes in β-chitin)

The abovementioned results confirmed the validity of identification of chitin allomorphs based on the complexation of chitin with EDA. We then extended this EDA complexation method to chitin samples characterized by nearly-amorphous, very poor crystallinity, including the α-chitin planktic copepod exoskeletons (*Pontella fera*, Calanoida)[57] and the β-chitin scales of the Scaly-foot Snail (*Chrysomallon squamiferum*).[58] For copepods (**Fig. 7)**, the peak top position in the dry state was between α-chitin and β-chitin monohydrate due to the very poor crystallinity. However, EDA complexation revealed that copepod exoskeletons consisted of poorly crystalline α-chitin or chitosan. This is in line with copepods being a group of crustaceans which are known to use α-chitin. For the Scaly-foot Snail, we used the part of its scales close to the distal tip. As the scales show accretionary growth in the longitudinal direction, the distal tip has been exposed longest to the environment and the β-chitin would be more disordered than freshly secreted parts near the base.[58] In **Fig. 7**, the peak position of scale samples complexed with EDA was located at the border of the known peak position for β-chitin, identifying it correctly as β-chitin. These examples exemplify that the method proposed herein is capable of separating the chitin allomorphs even when they are near-amorphous.

Conclusions

We successfully identified chitin allomorphs in both highly and poorly crystalline, even near-amorphous, chitin samples using a newly developed method employing the chitin/EDA complex. The advantage of this highly sensitive method is not only that the results are straightforward to interpret, but also the simple sample preparation process: simply immersing chitin into EDA at room temperature. Our method presents a powerful tool for determining chitin allomorphs, especially in poorly crystalline samples, and will pave the way to building an overarching understanding of chitin biosynthesis along the phylogenetic tree of chitin-producing organisms. A limitation is that poorly crystalline α-chitin is difficult to separate from chitosan when using this method only. For this, additional information such as the degree of acetylation measured by electric titration, FT-IR, or solid-state NMR is necessary.

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