Journal of Structural Biology 176 (2011) 83–90

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Electron diffraction and high-resolution imaging on highly-crystalline β-chitin microfibril

Yu Ogawa [a], Satoshi Kimura [a,b], Masahisa Wada [a,b,*]

[a] Department of Biomaterials Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Department of Plant and Environmental New Resources, College of Life Sciences, Kyung Hee University, 1 Seocheon-dong, Giheung-ku, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

ARTICLE INFO

Article history:
Received 18 April 2011
Received in revised form 5 July 2011
Accepted 5 July 2011
Available online 13 July 2011

Keywords:
β-Chitin microfibril
Thalassiosira
Lamellibrachia
Electron diffraction
High-resolution electron microscopy
Chitin biosynthesis

ABSTRACT

The ultrastructure of β-chitin microfibrils from a centric diatom, *Thalassiosira*, and a tubeworm, *Lamellibrachia*, was studied using electron diffraction and high-resolution electron microscopy. Electron microdiffraction diagrams corresponding to each projection of the β-chitin crystals were obtained, and all the data support the structure model of anhydrous β-chitin crystals proposed by X-ray diffraction experiments. From high-resolution electron microscopy on ultrathin sections, the cross-sectional shapes of the microfibrils from *Thalassiosira* and *Lamellibrachia* were observed as a rectangular and parallelogram, respectively. The lattice fringes corresponding to the (0 1 0) plane of anhydrous β-chitin crystals were clearly observed in both cross-sections. Based on these observations, we have constructed a molecular packing model for β-chitin microfibrils.

1. Introduction

Structural polysaccharides, such as cellulose and chitin, are the major biomass on Earth and have great potential as a renewable resource. In particular, chitin is almost untapped in this respect and is expected to be used as a novel resource. The chemical structure of chitin is a linear polymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc), which is almost the same as cellulose, except that the hydroxyl group at the C2 position of the glucopyranoside ring is replaced by an acetamide group. Chitin occurs in nature in a fibrous crystalline form, i.e. microfibrils. Through infrared spectroscopy and X-ray diffraction analyses, two crystal polymorphs have been identified, namely α- and β-chitin (Rudall, 1963; Blackwell, 1969).

The major polymorph, α-chitin exists as a part of insect integuments, crustacean shells and tendons as well as the spines of *Phaeocystis*, a type of Haptophyceae, and the grasping spines of arrow worms (Rudall, 1963; Atkins et al., 1979; Chrétiennot-Dinet et al., 1997). α-Chitin can also be obtained artificially using several methods, such as regeneration from a solution of chitin and high-concentration acid or alkaline treatments of β-chitin (Perrson et al., 1992; Saito et al., 2000; Noishiki et al., 2003). Reports on X-ray and electron diffraction studies of this polymorph indicate that the crystal structure of α-chitin adopts a two-chains orthorhombic unit cell with space group $P2_12_12_1$ (Minke and Blackwell, 1978; Saito et al., 1995; Sikorski et al., 2009), which means the molecular chains in α-chitin are arranged in an antiparallel manner. Thus, in this structure, the molecular sheets formed by aggregation of glucopyranoside rings are aligned with their polarities different between adjacent sheets, and hydrogen bonds stabilize the structure within and between these sheets. Because of this structural feature, α-chitin has a higher thermodynamic stability than β-chitin does (Wada and Saito, 2001; Ogawa et al., 2011).

The other polymorph, β-chitin, is a rarer form that is only found in some aquatic organisms, such as squids, diatoms and tubeworms. β-Chitin is known to have an anhydrous form, and at least two hydrate forms (Blackwell, 1969; Gardner and Blackwell, 1975). Recently, the crystal structure of anhydrous β-chitin was reinvestigated using an X-ray fiber diffraction analysis employing high-resolution intensity data obtained from highly crystalline β-chitin from a centric diatom, *Thalassiosira* (Nishiyama et al., 2011). According to the proposed model, the structure is characterized as a one-chain monoclinic unit cell with space group $P2_1$ with unit cell parameters of $a = 4.82$ Å, $b = 9.25$ Å, $c = 10.38$ Å and $\gamma = 97.2°$, indicating a parallel packing of the molecular chains, unlike α-chitin. In the structure of anhydrous β-chitin, the molecular sheets have the same polarity as all the others in the crystal, and hydrogen bonds exist only within these molecular sheets, which should lead to anhydrous β-chitin having lower stability. The crystal structure of β-chitin hydrates is not understood, and

* Corresponding author at: Department of Biomaterials Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81 3 5841 2677.
E-mail address: awadam@mail.ecc.u-tokyo.ac.jp (M. Wada).

1047-8477/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2011.07.001

only the unit cell parameters of the dihydrate form have been reported as a one-chain monoclinic unit cell with space group $P2_1$ with a = 4.80 Å, b = 11.15 Å, c = 10.44 Å, and γ = 96.39° (Kobayashi et al., 2010). According to this analysis, the dihydrate form must have a parallel chain arrangement, the same as in the anhydrous form; it is also considered to contain two water molecules for each GlcNAc residue.

As mentioned above, the crystal structure, or atomic arrangements in the unit cells of chitin polymorphs, has mainly been revealed using X-ray diffraction studies. However, it is not fully understood how the molecules are arranged as a whole in the individual chitin microfibrils, although such information is strongly related to the mechanical and surface properties, as well as to the biosynthetic mechanisms. This knowledge gap may result from the fact that X-ray diffraction data generally provide macroscopic or average information from a large number of microfibrils, rather than from a single microfibril. Thus, a more microscopic analysis is necessary to gain better understanding of the ultrastructure of chitin microfibrils.

In the fields of crystallography and morphology of cellulose, electron microscopy has had an important role for several decades. In particular, the visualization of the crystalline lattice of cross-sections of native cellulose microfibrils reveals the molecular arrangement in the individual microfibrils that related their crystal structure to their shapes (Sugiyama et al., 1985; Helbert et al., 1998a,b). Based on these observations, some hypotheses about the surface structure and biosynthetic mechanisms have been formulated, and these have been supported by other studies, such as AFM observations and computer simulation studies (Cousins and Brown, 1995; Helbert et al., 2000). This approach should also be effective for chitin microfibrils, but only a few papers have reported on the electron microscopy of the cross-sections of chitin microfibrils. Previously, we reported on the lattice imaging of highly crystalline α-chitin microfibrils and proposed a model of the molecular arrangement in the cross-sections of chitin microfibrils (Ogawa et al., 2010). For the β-polymorph, electron microscopy on cross-sections was also carried out on β-chitin microfibrils from tubes of pogonophorans, *Tevnia* and *Riftia* (Gaill et al., 1992; Shillito et al., 1997). However, these observations were taken at relatively low resolution and did not lead to the construction of a model of the molecular arrangement. Therefore, in this study, high-resolution electron microscopy and electron-diffraction studies were carried out on cross-sections of highly crystalline β-chitin microfibrils obtained from two different sources. The first source was the spines of a centric diatom, *Thalassiosira*, which exist as pure chitin microfibrils outside the cell (Herth et al., 1979; Revol and Chanzy, 1986; Noishiki et al., 2004). The second source was a component of the tubes of a vestimentiferan, a type of hydrothermal vent worm, *Lamellibrachia*. In these tubes, the β-chitin microfibrils are arranged with a protein matrix (Kobayashi et al., 2010; Imai et al., 2003). Using these observations, we were able to construct a molecular packing model of the β-chitin microfibrils and compare the differences in the packing between the two samples. We also discuss the relationship between their structure and properties as well as the mechanism of chitin biosynthesis, especially the crystallization of chitin molecules.

2. Materials and methods

2.1. Sample and purification

2.1.1. Thalassiosira

A centric diatom, *Thalassiosira weissflogii* (CCMP-1051), obtained from the Center for Culture of Marine Phytoplankton (CCMP; Maine, USA), was cultured in an L1 medium at 20 °C under aeration with a 12 h light to 12 h dark cycle of 100 μmol photons m^{-2} s^{-1} for about one month. The culture suspension was lightly homogenized using a kitchen mixer for several seconds to dislodge any chitin spines from the diatom bodies. The homogenate was centrifuged at 2000*g* to sediment the cells while the chitin spines remained in the supernatant liquid. Then, the supernatant liquid was centrifuged at about 18,000*g* to collect the chitin as a pellet. The precipitate was treated with 1 N KOH (room temperature, overnight) and 0.3% $NaClO_2$ (buffered to pH = 4.8, 80 °C, 3 h), with centrifugal rinsing with water after each step. These treatments were successively repeated three times (Noishiki et al., 2004). The purified chitin was kept in a water suspension until use.

2.1.2. Lamellibrachia

Satsuma tubeworms (*Lamellibrachia satsuma*) were collected from Kagoshima Bay, Japan, at a depth of about 100 m using a remotely operated vehicle, Hyper-Dolphin (JAMSTEC, Japan). The bodies of the tubeworms were removed by washing the tubes with water. The tubes were purified using a repetitive treatment employing 5% KOH and 0.3% $NaClO_2$ solutions, as described previously (Kobayashi et al., 2010). The purified samples were delaminated under a stereoscopic microscope and mounted on carbon-coated grids for electron microscopy.

2.2. Scanning electron microscopy

For SEM observations, *T. weissflogii* and the unpurified tube of *L. satsuma* were fixed in a 1% solution dissolved in 75% seawater (room temperature, 4 °C, 1 h), dehydrated with a graded ethanol series and *t*-butyl alcohol, and freeze-dried. The samples were placed on sample holders and coated with amorphous osmium. Scanning electron microscopy was performed with a Hitachi S-4800 SEM (Hitachi, Japan) operated under an accelerating voltage of 1.5 kV under the secondary electron mode.

2.3. Embedding and ultrathin sectioning

2.3.1. T. weissflogii

To observe the cross-sectional microfibrils, an oriented fiber of β-chitin microfibrils from *T. weissflogii* was prepared using the method of Blackwell (Blackwell, 1969). In brief, a 1 mg/L chitin suspension was mixed with the same volume of a 1% fibrinogen solution containing 3% NaCl. The mixture was supplemented by several drops of concentrated aqueous thrombin solution to form a gel. The gel sheet was cut into strips and slowly stretched uniaxially to about five times its length, followed by air-drying. This oriented fiber was dehydrated by directly immersing it in propylene oxide, and then the sample was embedded in an epoxy resin mixture (Luft, 1961). The hardened resin block was set on an ultramicrotome (Ultracut UCT, Leica, Germany) equipped with a 45° diamond knife (Diatome, USA) with a clearance angle of 6°. Ultrathin sections (<50 nm thick) were obtained and mounted on a Triafol microgrid.

2.3.2. L. satsuma

The unpurified dry tubes were dehydrated using a series of graded ethanol and propylene oxide, and the samples were then embedded in an epoxy resin mixture and sectioned in the same way as for *T. weissflogii*. For conventional observations, the cross-sections were stained with a 1% osmium oxide solution (overnight, 4 °C), a 2% uranyl acetate solution (30 s) and Reynolds lead citrate (3 min) (Reynolds, 1963). Unstained cross-sections were also prepared for diffraction contrast. For lattice imaging, cross-sections of the purified tubes were prepared using the same method as for the unpurified tubes to increase the probability of acquiring lattice images.