Preparation of Tunicin Cellulose Iβ Samples for X-ray and Neutron Diffraction.

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ABSTRACT

*Methods are described for extracting crystalline cellulose Iβ microfibrils from the cellulosic mantles of tunicin (*Halocynthia roretzi*) and then reconstituting them into highly oriented films that diffract synchrotron X-rays and neutrons to atomic resolution.*

INTRODUCTION

Cellulose is biosynthesized as nanometer thick crystalline microfibrils of *poly*(1-4)-β-D-glucan. These microfibrils consist of two crystal phases, namely Iα and Iβ [1]. Until recently, the accepted crystal structure for naturally occurring cellulose, cellulose I, was based on X-ray data collected from fibres containing both Iα and Iβ phases, without knowledge of this dimorphism [2,3]. In this report we describe advances in sample preparation and characterization methods that have allowed us to produce samples of pure Iβ phase that diffract X-rays and neutrons to atomic resolution.

The sea organism *Halocynthia roretzi*, commonly known as the 'sea squirt' or 'tunicate', because of the presence of a thick leathery mantle or tunic in their mature phase, was used as the source of cellulose. The mantles of tunicate consist of a composite structure of cellulose microfibrils organized in helical order and dispersed in a matrix of protein and acid mucopolysaccharide [4]. The cellulose from tunicate, called tunicin, is known from ^{13}C CP/MAS solid state NMR studies to consist of predominantly the Iβ phase [5,6]. Electron microscopy and diffraction studies have shown that the microfibrils are highly crystalline [7-10], each microfibril corresponding to a distinct single crystal approximately 10 nm in width [4, 9, 11]. Although these microfibrils have dimensions that are amenable to an electron diffraction study [10,11], they are too small for X-ray or neutron diffraction.

METHODS

Preliminary X-ray Measurements

The suitability of tunicate mantles for X-ray diffraction studies was assessed by collecting X-ray data from deproteinized mantles on a Philips 1720 X-ray generator equipped with a Wharus flat film X-ray camera, operated at 30kV and 20 mA with a Ni-filter and CuKα radiation. The resultant patterns contained at least 7 orders of diffraction and indicate that the crystalline cellulose microfibrils in the mantle diffract X-rays to high resolution, Figure 1. However, the smearing of these diffraction orders into powder rings indicates that the microfibrils have random orientation within the mantle. This is in agreement with the observation from electron microscopy studies that the microfibrils appear to be organized in liquid crystalline order [4]. In order to obtain fibre diffraction samples, the methods described below had to be devised for extracting the microfibrils from the mantle and re-orienting them in suitably sized assemblies.

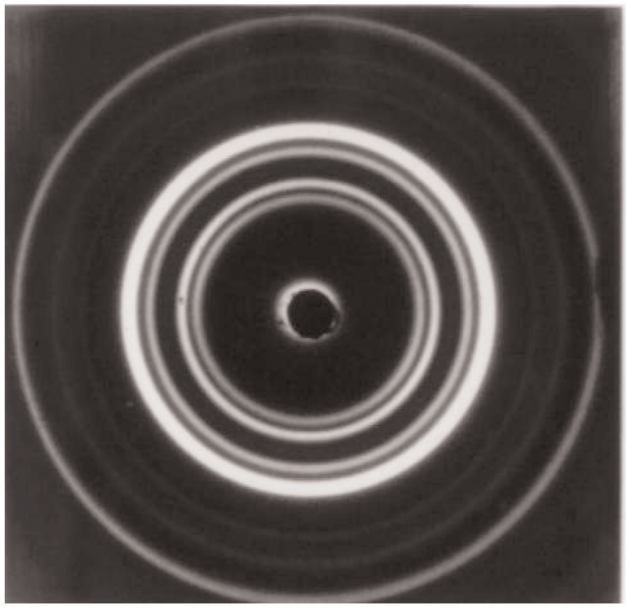

Figure 1. X-ray diffraction collected from the deproteinized mantle of tunicate (*Halocynthia roretzi*).

Extraction and Purification of Cellulose Microfibrils

Halocynthia roretzi were kindly supplied by Prof. Fumitaka Horii, Institute for Chemical Research, Kyoto University. The following procedure was arrived at as a means of extracting and purifying the cellulose without destroying the crystal quality of the microfibrils. The cellulosic mantles were separated from the rest of the organism and then partially hydrolyzed with sulphuric acid and soaked in 5% KOH solution. A white material was obtained after repeatedly treating the hydrolyzate with a 0.3% $NaClO_2$ solution buffered to about pH 4 at 70ºC, and 5% KOH. The white material was cut into pieces of about 5 mm square while still wet. These sections were disintegrated using a double cylinder type homogenizer. The suspended cellulose fragments were collected by centrifugation. The solid content of the suspension was about 5%.

Two parts of 70 % sulphuric acid were added to one part of the wet cellulose sample and stirred vigorously with a Teflon coated propeller at 50ºC overnight. The viscosity decreased drastically during this treatment. The resulting suspension was diluted about 5 times with distilled water and centrifuged at about 2000g. The transparent supernatant was removed by decantation and the cellulose-containing pellet was re-suspended in de-ionized water and centrifuged again. This process was repeated until the supernatant became turbid. At this stage the supernatant is a suspension of cellulose microcrystals stabilized by sulphate surface groups. The turbid supernatant was collected and the centrifugate was further washed with de-ionized water, each time harvesting the turbid supernatant, until only brownish impurities remained at the bottom of the tube. Finally the microcrystals in the turbid supernatant were collected by centrifugation at 16,000g and stored in a refrigerator at 4ºC until further use.

Preparation of Films of Oriented Microfibrils

Films of oriented microfibrils were prepared using a variation of a previously described method [12]. The microcrystalline suspension described above was put into a glass vial and diluted to a solid content of about 1 %. Above a critical concentration, the particles in this colliodal dispersion organize spontaneously into a chiral nematic liquid crystalline phase. A suspension of these particles is shown in Figure 2.

Sulphuric acid was added to the suspension to about 1%. The suspension became very thick and more turbid when sulphuric acid, or for that matter any electrolyte, was added. After agitation with a Teflon coated magnetic stirrer, the viscosity dropped, athough it was still higher than in the electrolyte-free suspension. During stirring, an oriented gel formed on the surface of the vial. At this point the magnetic stirrer was removed from the vial and the gel was disintegrated by rigorous shaking of the vial. With the cap closed, the vial was attached horizontaly to a motor and spun at about 500 rpm around its centre axis. A smooth layer of jelly formed on the inner surface of the vial within an hour, which gradually thickened with time. This layer was removed and then dried, resulting in a film several centimeters long and a few microns thick, which when viewed under a polarizing microscope showed a strong birefringence (Fig. 3).

Assembly of Samples for Neutron and Fibre diffraction Studies

Samples large enough for X-ray or neutron diffraction studies were built by selecting films under a polarization microscope and then assembling these films into thick stacks as described previsouly [13]. Particular care had to be taken in deciding the dimensions of the neutron samples. On the one

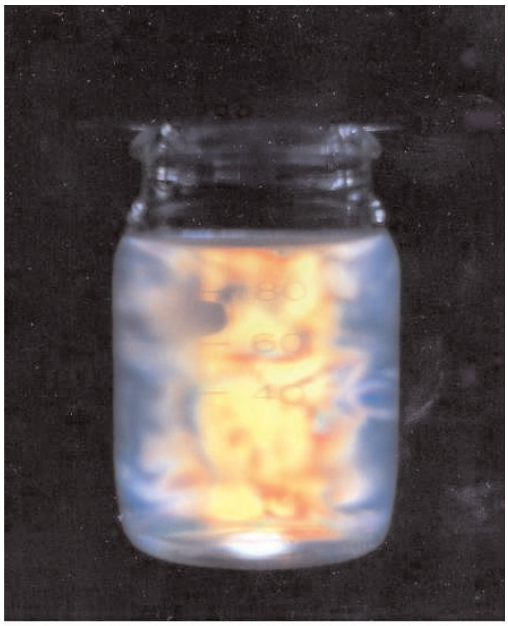

Figure 2. A 0.5% (w/v) supension of purified cellulose microfribrils from the mantle of tunicate (*Halocynthia roretzi*) observed in polarized light between crossed polarizers.

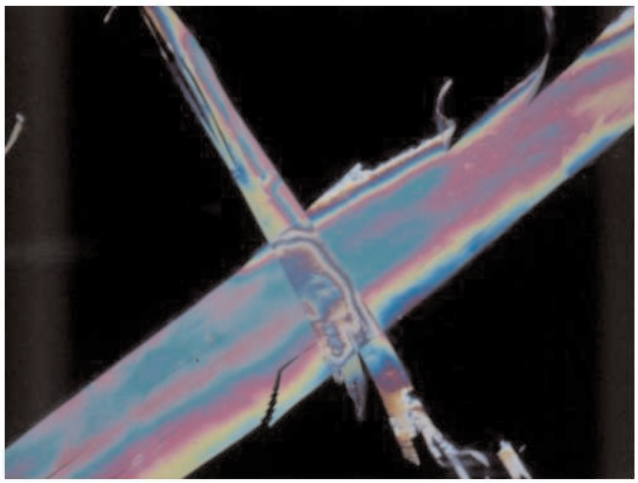

Figure 3. Films of oriented purified cellulose microfibrils extracted from tunicate (*Halocynthia roretzi*) viewed through a polarizing microscope with crossed polarizers (axes vertical and horizontal).

hand, neutron beams are relatively weak in flux so large sample sizes are desirable in order to maximise the number of diffracted neutrons. On the other hand, hydrogen in the sample has a large spin incoherent neutron scattering cross-section that effectively attenuates the diffracted neutron beams and also contributes to a large background signal. The final thickness of the neutron sample, τ , was therefore determined not by the amount of material avaible, but rather by a compromise between the above-mentioned two factors.

The optimum total reflected energy for a cuboid shaped sample occurs when $\mu\tau \sim 1$, where μ is the effective linear absorption coefficient [14]. For cellulose Iβ, μ was calculated to be ~5.3cm-1 and therefore a sample with τ~2mm was used. The neutron experiments also involved collecting data from samples where all OH moeities had been replaced by OD [15]. In this case μ ~3cm^{-1} and a τ~3.5mm was used. For the X-ray experiments much smaller samples with τ~ 200mm were used in order to match the approximate size of the synchrotron X-ray beam. An example of some of the diffraction data recorded from these samples is shown in Figure 4.

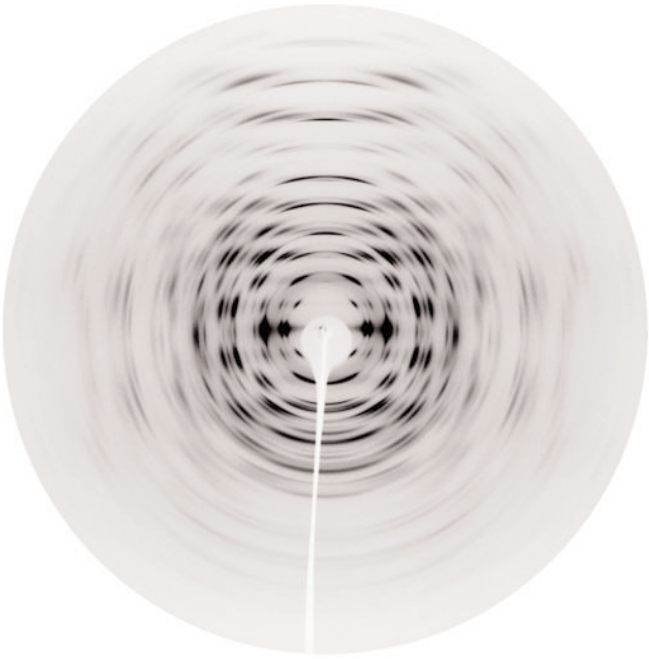

Figure 4. X-ray diffraction data collected from oriented purified cellulose microfibrils extracted from tunicate (*Halocynthia roretzi*). The data were collected using a wavelength of 0.72Å and a MAR image plate on station ID02A at the ESRF, Grenoble. The fibre axis is vertical. Diffraction features are present at scattering angles that correspond to **d**-spacing of less than 1Å.

DISCUSSION

A number of sea organisms contain cellulose microfibrils of high crystal quality but poor alignment. X-ray fibre diffraction patterns to ~2Å resolution were recorded in the 1970's from stretched algal (*Valonia ventricsa*) cell walls [2,3]. In contrast, electron diffraction patterns have been recorded to ~0.7Å resolution from minute fragments of oriented *Valonia ventricsa* cell wall [16]. This observation first suggested to us that if crystals of cellulose could be extracted and then reconstituted into highly oriented fibres it might be possible to

collect X-ray data of similar quality to the electron diffraction data.

The method presented here is derived from ongoing developments in the study of suspensions of colloidal cellulose microcrystals [17-19]. Our method is quite general, although there are usually variations in detail, and has been applied to other cellulose allomorphs. So far these samples have all diffracted synchrotron X-rays, and after hydrothermal deuteration [15], neutrons to atomic resolution (~1Å) [13,15,20,21]. The resolution of these data has led to visualize individual atoms and to identify small variations in conformation. The ability to locate hydrogen atoms has allowed us to identify hydrogen patterns that can be related to the surface properties of cellulose microfibrils. These results have important implications for our understanding of the factors that influence the structure and properties of cellulose.

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