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International Journal of Biological Macromolecules
24 (1999) 37–41

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

Quantitative analysis for the cellulose Iα crystalline phase in developing wood cell walls

Yutaka Kataoka, Tetsuo Kondo *

Forestry and Forest Products Research Institute (FFPRI), P.O. Box 16, Tsukuba Norin, Ibaraki, 305-8687 Japan

Received 4 February 1998; received in revised form 20 July 1998; accepted 23 July 1998

Abstract

FT-IR and X-ray analyses were employed to determine the relative ratio of cellulose I_α and I_β crystalline phases present in each developmental stage of coniferous tracheid cell wall formation. The IR spectra showed that initially the I_α phase occupies 50% of the crystalline regions in the primary cell wall cellulose and this value drops to 20% after ceasing of the cell enlarging growth for the formation of the secondary wall cellulose (the remaining regions are composed of the I_β phase). Although it is reasonable that the content for I_β, which is stress-reduced crystalline form, was higher in the secondary wall formation (Kataoka Y, and Kondo T. Macromolecules 1996;29:6356–6358) it is more interesting that during the crystallization of stress-induced I_α cellulose for the primary wall the stress-reduced I_β is also possible to be crystallized in an alternative way. This means that throughout the period the I_α-causing stress may not be necessarily kept loaded. In light of our previously reported hypothesis (Kataoka Y. and Kondo T. Macromolecules 1998;31:760–764) for the formation of I_α phase due to cellular growing stresses in the primary wall cellulose, such an alternating on–off stress effect to account for the occurrence of both I_α and I_β phases might be related to a biological growth system in coniferous wood cells.

Keywords: Cellulose; Wood cell wall; Primary wall; Crystalline phase; Crystallization; Enlarging stress; Microscopic FTIR

1. Introduction

Recently it has been reported that the cellulose crystalline structure in the primary and secondary cell walls changes during coniferous tracheid cell wall formation [1,2]. These results suggest a biogenetic system in which the two allomorphs of cellulose [3] namely the metastable triclinic I_α and the stable monoclinic I_β [4,5] are produced alternatively. The cellulose in the primary cell wall is characterized not only by its abundant metastable I_α form but also by a markedly higher crystallinity and a parallel orientation in the cellular growth direction, in contrast to the I_β-rich cellulose found in the secondary wall. These changes in the crystalline structure indicate that the primary wall cellulose may be crystallized under in vivo stress conditions. Therefore, in our previous papers [1,2] we proposed that the crystallization of the primary cell wall cellulose may be under stress conditions due to cellular enlarging growth. Since both ends of freshly biosynthesized β-glucan molecules are bound to both the TCs (cellulose-synthesizing terminal enzyme complexes) on the plasma membrane and the cell wall, respectively, the suspended molecules should be free and easily drawn during cellular enlarging growth. There may be other effects involved, such as the arrangement of the TCs on the plasma membrane [6–8] or the interactions between nascent cellulose and other polysaccharides [9–12] however, only the drawing force may be effective in simultaneously altering the crystalline form, crystallinity and molecular orientation. In addition, the drawing stresses for the primary wall formation induced by cellular growth can be repeatedly generated and released due to

* Corresponding author. Tel.: +81 298 733211; fax: +81 298 733797; e-mail: kondot@ffpri.affrc.go.jp

0141-8130/99/$ - see front matter
PII S0141-8130(98)00065-8

the fluidity of the cell surface. As such an on–off effect due to drawing stress may be reflected in the allomorphic ratio between the I_α and I_β crystalline forms, if any, it would be predicted by determination of the I_α composition in crystalline regions for both the primary and secondary wall cellulose.

In this study, we want to quantify the crystalline composition of the cell wall cellulose in order to understand the nature of the I_α-causing stress. We monitored the fraction of the I_α phase during wood cell wall formation using a combination of microscopic techniques with FT-IR and X-ray analyses. The results indicate that the I_α phase in the primary cell wall may be crystallized by discontinuous stress, supporting our hypothesis of an on–off drawing effect as outlined above. We also comment on the enlarging growth system in coniferous wood cells.

2. Experimental section

2.1. Materials

The wood sample was ordinary spring wood xylem from 28 year old *Chamaecyparis obtusa* (Japanese cypress). Sample blocks including differentiating wood xylem, as shown in Fig. 1, were cut from the trunk at a height of ≈ 1.25 m and immediately fixed with 3% glutaraldehyde solution at a pH of 6.8 in a phosphate buffer. In the blocks, developing axial tracheid cells and their cell walls were lined up in the radial direction to the wood annual rings in order of maturity starting from the cambial zone to the mature xylem. In general, the tracheid cell wall develops in four stages (P, $P + S_1$, $P + S_1 + S_2$, $P + S_1 + S_2 + S_3$) as follows: the primary wall (P) is formed first during radial cell enlarging growth and then, after cessation of the enlargement, the outer (S_1), middle (S_2), and inner (S_3) layers of the secondary cell wall are successively deposited onto the preformed wall [13]. Then, 30 μm thick radial sections were cut from the sample blocks with a pre-frozen microtome for FT-IR measurements. These sections were used to examine the cellulose growth in each developmental stage by focusing on their radial face using a microscopic accessory [1,2] as illustrated Fig. 1A. We have, therefore, assumed that the cell walls radial to annual rings (radial walls in Fig. 1) are main contributors to the IR spectra, while those walls parallel to the rings (tangential walls in Fig. 1), which were too thick for the IR beam to penetrate, were examined by X-ray analyses. To analyze cellulose in both radial and tangential walls, we also took tangential sections from each developmental stage, as shown in Fig. 1B, for X-ray measurements. Both types of sections were thoroughly disencrusted by employing multiple combinations of two purification methods [14,15] to completely remove pectin, polysaccharides, lignin, and other non-cellulosic substances as described previously [1,2].

2.2. Infrared spectroscopy with a microscopic attachment

FT-IR spectra were obtained using a Perkin-Elmer spectrometer (Spectrum 2000) equipped with a microscopic attachment (Autoimage system). Spectra were collected on small areas (200×25 μm^2) of the radial sections as shown in Fig. 1. The spectrum for each stage of cell wall development (P, $P + S_1$, $P + S_1 + S_2$, $P + S_1 + S_2 + S_3$) was recorded as an average of 32 scans at a resolution of 2 cm^{-1} in the range from 4000 to 600 cm^{-1} with a MCT detector. To determine the I_α fraction in the whole cellulose, the characteristic IR absorption bands [6] at 750 cm^{-1} for I_α and at 710 cm^{-1} for I_β were deconvoluted by using a Gaussian-Lorenzian curve fitting analysis as outlined in a previous report [16]. The IR index for the I_α fraction (f_{IR}) [10] was calculated, using the band areas for I_α (A_{750}) and I_β (A_{710}), as $f_{IR} = A_{750}/(A_{750} + A_{710})$. The corresponding I_α fraction, which is usually determined

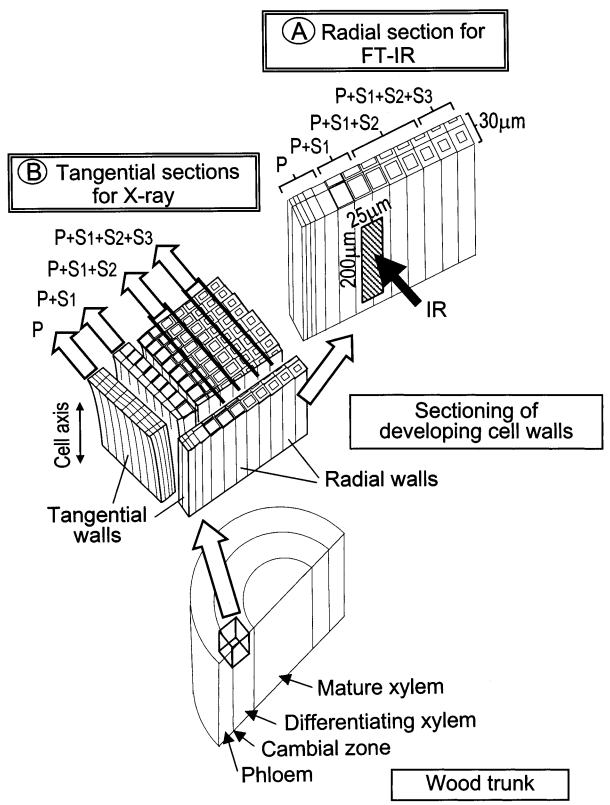

Fig. 1. Schematic showing of sample preparation for FT-IR and X-ray measurements to examine the crystalline cellulose from each developmental stage of coniferous tracheid cell wall formation: P, the primary cell wall formation accompanied by cellular enlarging growth; S_1, S_2, and S_3, deposition of the outer, middle, and inner layers of the secondary wall onto the pre-formed wall.

by solid state CP-MAS ^{13}C NMR measurements (f_{NMR}) [10] was then derived from the equation, $f_{NMR} = 2.55 \times f_{IR} - 0.32$. All spectra had absorbances ranging from 0.30 to 0.022 (or 50–95% in transmittance mode). It was also confirmed that the thickness or shape of the samples did not have any affect on the intensity ratio for the IR absorptions of the two crystalline forms (750/710 cm^{-1}).

2.3. X-ray measurements

Approximately 0.7 mg of cellulose were collected from each developmental stage and placed in a 2 mm diameter sample holder. An X-ray diffractmeter (RIGAKU RINT 2500F) equipped with a transmission type goniometer using nickel-filtered Cu–K_α radiation at 40 kV and 100 mA was employed to record diffraction curves. The goniometer was scanned stepwise every 0.10°, from 10 to 40° in the 2θ range, where a 40 sec radiation exposure was acquired. The resulting diffraction patterns exhibited four typical diffraction peaks ($d1$–$d4$) appearing in the region from 10 to 25°. These peaks were deconvoluted from a background scattering (BG) by using a Pseudo-Voigt function (a combination of Gaussian and Lorenzian functions) curve fitting analysis [17]. The interplanar spacing data (d-spacings) for $d1$, $d2$, and $d3$ are reported to reflect the fractional ratio existing between cellulose I_α and I_β, since each peak contains a combination from the I_α and I_β diffractions whose d-spacings are slightly different [17]. However, because of overlap with the BG, the d-spacings for the $d2$ and $d3$ peaks were difficult to determine accurately, in this paper we will concentrate on the $d1$-spacing which is a combination of the (100) plane of I_α with a 0.613 nm spacing (14.45° in 2θ) and the ($1\bar{1}0$) plane of I_β with a 0.603 nm spacing (14.69° in 2θ) [17].

3. Results and discussion

FT-IR measurements Typical FT-IR spectra for the primary (P) and mature cell wall cellulose ($P + S_1 + S_2 + S_3$) are shown in Fig. 2, together with corresponding curve-fitting results. From the primary to the secondary wall, the IR absorption band at 750 cm^{-1} attributed to the I_α form decreases when compared to the I_β band at 710 cm^{-1}. The band at 710 cm^{-1}, which in previous studies [1,2] was simply a shoulder peak, was seen clearly even in the I_α-rich primary wall using the new FT-IR spectrometer in this study. Therefore, this curve-fitting method employed in this work provided a means by which the changing fraction ratio ($I_\alpha/I_\alpha + I_\beta$) between the two allomorphs of cellulose [10] during cell wall formation could be followed. Fig. 3 shows the change in the IR index for the I_α fraction (left axis) with the corresponding I_α fraction determined

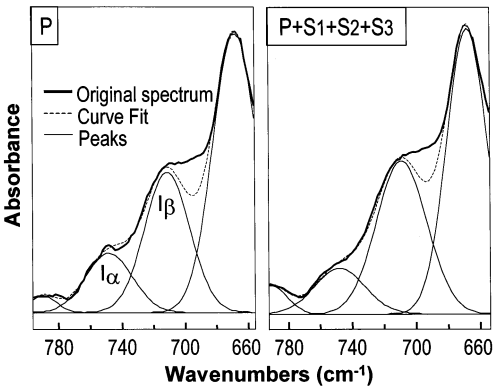

Fig. 2. FT-IR spectra of cellulose in the region from 800 to 650 cm^{-1} for developing Japanese cypress tracheid walls composed only of the primary wall (P) and after deposition of the secondary wall layers ($P + S_1 + S_2 + S_3$), together with the deconvoluted absorption peaks attributed to I_α (750 cm^{-1}) and I_β (710 cm^{-1}) crystalline forms of cellulose.

from solid state [13] C NMR measurements (right axis). The results show that the cellulose making up the primary cell wall (P) is initially 50% I_α phase, but that after the start of secondary wall deposition ($P + S_1$, $P + S_1 + S_2$, $P + S_1 + S_2 + S_3$) the I_α fraction in the cellulose drops to only about 20%. These data indicate that not only the fraction of the I_α phase decreases from the primary to the secondary wall but also that the I_β phase crystallizes even in the primary wall. This leads to the conclusion that the stresses responsible for I_α deposition in the primary cell wall are not kept loaded during crystallization so that the I_α phase could be crystallized. The in vivo stresses may occur in an on–off fashion during certain alternating intervals leading to deposition of an almost equal amount of the I_α and I_β phases in the primary wall during crystallization. On the other hand, the secondary cell wall cellulose which has fewer opportunities to be stressed, seems to favor formation of the stable I_β phase and was found to occupy 80% of the mature cell wall cellulose.

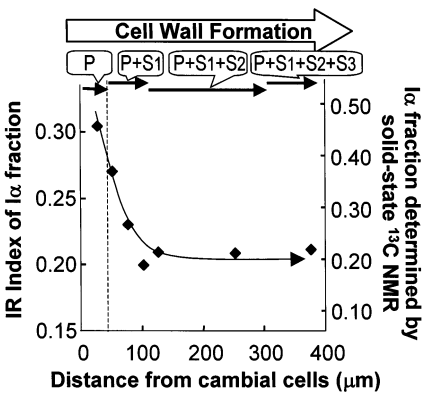

Fig. 3. Change in the IR index for the I_a fraction ratio ($I_\alpha/I_\alpha + I_\beta$) (left axis) and corresponding I_α fraction determined by solid state CP-MAS [13] C NMR [10] (right axis) during tracheid cell wall formation.

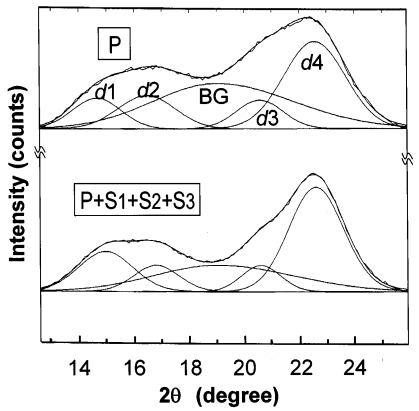

Fig. 4. X-ray diffraction curves for the primary (P) and the mature cell wall ($P + S_1 + S_2 + S_3$) cellulose from 12 to 26° in the 2θ angle. The four typical diffraction peaks from $d1$ to $d4$ in this region were corrected for background scattering (BG).

3.1. X-ray diffraction

The decrease in the I_α fraction from the primary to the secondary wall was also confirmed by X-ray measurements. In Fig. 4, X-ray diffraction curves for the primary (P) and the mature cell wall ($P + S_1 + S_2 + S_3$) cellulose are shown, together with the four diffraction peaks ($d1-d4$) corrected for BG as described in a previous paper [17]. In particular, the interplanar $d1$ spacing change from the primary to the mature cell wall is shown in Fig. 5. As mentioned in Section 2, the $d1$-spacing is believed to reflect the mixed contributions of both the I_α (0.613 nm) and I_β (0.603 nm) phases in the cellulose. Thus, the $d1$-spacing allows us to observe the relative change in the I_α/I_β phase composition without requiring any further peak deconvolution to determine their exact composition. In fact, the $d1$-spacing value was over 0.610 nm during the primary cell wall formation but it started to drop to about 0.605 nm with the deposition of the secondary cell walls. These X-ray diffraction results confirm that the I_α fraction decreases

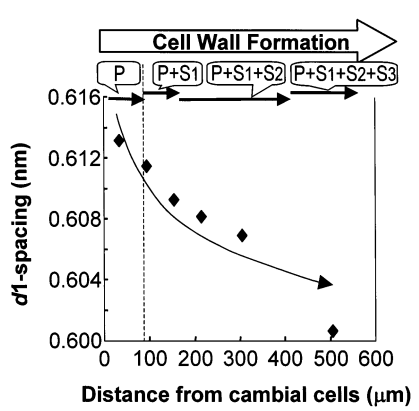

Fig. 5. Change in the $d1$-spacing of cellulose during tracheid cell wall formation.

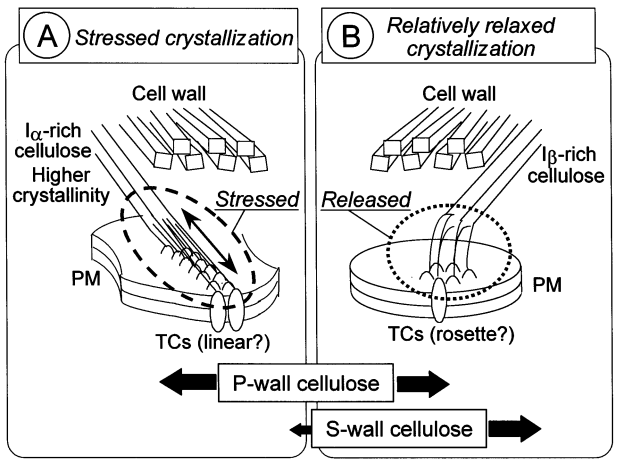

Fig. 6. Schematic representations for stressed (A) and relatively relaxed (B) crystallization forms for cellulose I_α and I_β, respectively, which may occur between the terminal enzyme complexes (TCs) on the plasma membrane (PM) and the preformed cell wall. The primary (P) cell wall cellulose may be stressed (A) and relaxed (B) repeatedly during crystallization, whereas the secondary (S) wall cellulose mainly crystallizes in a relatively relaxed environment (B).

as the formation of the cell walls proceeds. In addition, the decrease in the $d1$-spacing was mirrored in the FT-IR fraction index for the I_α phase in Fig. 3. While the X-ray method measured the I_α and I_β fractions in both the radial (walls radial to the annual rings as shown in Fig. 1) and tangential walls (walls parallel to the annual rings) concurrently, the IR spectra provided information only on the fractions in the radial walls. Therefore, the agreement in the X-ray and IR results demonstrates a uniformity in the cellulose crystalline structure between both cell walls. This means that the in vivo stress operating within the primary cell wall cellulose may be effective in shaping the enlarging cell surface regardless of the cell face being acted upon.

3.2. Model for the crystallization of tracheid cellulose

In the present study we have monitored the changing crystalline composition of the I_α and I_β phases at each stage from the primary to the secondary cell wall. We have provided evidence that the I_α phase fraction diminishes from 50 to 20%, indicating a decrease in in vivo stresses which promote the crystallization of the I_α phase. Moreover, the presence of both the I_α and I_β phases in the primary wall suggests a discontinuity in the stresses responsible for the occurrence of the I_α phase. We would, therefore, like to propose a crystallization mechanism for tracheid cellulose which can be controlled by the on–off effect of in vivo stresses described above to support our previously proposed models [1,2]. As illustrated in Fig. 6, freshly biosynthesized β-glucan chains of cellulose can be stressed along the molecular chains because the terminal end groups

are bound to both the cellulose-synthesizing enzyme complexes (TCs) on the plasma membrane (PM) and the just formed β-glucan chain in the cell wall, respectively. In particular for primary cell wall formation, there may be much stress frequently exerted by the growing cells which extend the plasma membrane to stretch the primary wall (Fig. 6A). Accordingly, the nascent primary wall cellulose may be stressed into crystallizing in the metastable I_α form. In contrast, considering the fluidity in the environment, such stressed crystallization can be somewhat partially relieved to resume I_β deposition (Fig. 6B) for a certain period until a stress exceeds some threshold intensity causing deposition of the I_α phase again. This on–off drawing stress may result in the I_α and the I_β forms being crystallized in a 50:50% ratio in the primary cell wall. In addition, the drawing of nascent molecules can result in orienting them parallel to the cellular enlarging direction [2] resulting in higher crystallinity in the primary cell wall cellulose. This phenomenon is similar to the situation encountered in the drawing of synthetic polymers. Moreover, the idea of an on–off stress controlling crystallization can also explain the biogenesis of why the I_α and I_β domains exist consecutively along one crystal of algal *Microdictyon* cellulose [18].

After cellular enlarging growth ceases, the secondary cell wall cellulose can largely crystallize in a relatively relaxed environment (Fig. 6B) which favors the crystallization of the I_β phase. However, the I_α phase was, in fact, crystallize in the secondary wall, as was shown in above section, suggesting that even nascent cellulose in the secondary cell wall can be stressed when it crystallizes and assembles into microfibrils although the stress may be much less than that for the primary wall. That is to say, there may be some occasionally occurring stresses at the cell surface even after cellular growth. Finally, we believe that the allomorphic composition of tracheid cellulose is determined by discontinuities in in vivo stresses during cell wall deposition. In our model, stress for I_α crystallization which is due to growing cells can be partially relieved by fluidity in the environment and the reduced stress atmosphere may facilitate I_β formation. This mechanism differs significantly from the hypothesis [6–8] that the TCs arrangement on the plasma membrane may be responsible for the stress-induced I_α growth. We would rather imagine that the TCs bound to nascent cellulose molecules could be rearranged on the fluid plasma membrane by a drawing process. This arrangement may be a reflection of the system stresses, but it does not seem to determine the crystalline form of cellulose deposited.

It is also interesting to note that the on–off stresses for the primary wall formation described above might show that tracheid cell enlargement is a discontinuous process. In addition, although tracheid cells seem to elongate along their radial walls, our result suggests that there may be in vivo stresses which effect growth in both the radial and tangential directions.

4. Conclusion

Continuous monitoring of the change in cellulose crystalline composition during wood cell wall formation lead to a novel concept of on–off stressed crystallization, which may be induced by cellular enlarging growth for the primary wall formation. Some other biological insights into the cellular enlarging system have also been proposed.

Acknowledgements

We thank the Perkin Elmer Japan for allowing us to use their FT-IR spectrometer (Spectrum 2000) and microscopic attachment (Autoimage system). Editing of the text by Dr R. S. Werbowyj at the Pulp and Paper Research Institute of Canada (PPRIC) is also acknowledged.

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