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X-ray diffraction and ultrastructural analyses of dye-altered celluloses support van der Waals forces as the initial step in cellulose crystallization

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Previous conflicting diffraction studies on dye altered cellulose have supported two opposing bonding schemes within a glucan minisheet: van der Waals forces and hydrogen bonds. Past molecular mechanics energy analysis has been used as evidence to support the van der Waals forces model. Additional evidence from electron microscopy and X-ray diffraction is presented here. Theoretical extrapolation supports a 3-ply sheet construction of a glucan dye sheets. A 9.4 Å reflection previously reported for glucan dye sheets as evidence for a cellulose I dye complex has been re-interpreted as a cellulose II-Tinopal composite structure where the dye molecules are intercalated between folded glucan chains. Glucan dye sheets and the cellulose II-Tinopal composite are thought to have arisen from glucan chains associated by van der Waals forces.

(Keywords: Tinopal LPW^{TM}; cellulose; cellulose II-Tinopal composite)

INTRODUCTION

The model for non-microfibrillar, dye-complexed cellulose (referred to as glucan dye sheets in this paper) has undergone several refinements since the first report of synthesis of tubes from extended glucan sheets and the inducement of microfibrils from them†. In 1984, Kai disputed the model of Brown *et al.*[1] in favour of a crystalline 'cellulose-brightener complex'. Based on an X-ray diffraction analysis, he proposed that dye molecules intercalate between the $(\bar{1}\,1\,0)^{i}$ plane of the cellulose lattice to form a complex of many stacks of glucan sheets alternating with sheets of dye molecules. In 1985, Kai and Koseki[2] proposed that *in vivo* crystallization of cellulose I occurs when minisheets of glucan chains held together via van der Waals forces stack on top of each other and form hydrogen bonds between the sheets to form cellulose I microfibrils. This proposal was based on a combination of alkali swelling and electron diffraction studies.

Based on an electron diffraction analysis, Haigler and Chanzy in 1988[3] rejected Kai's cellulose-brightener complex model in favour of a modified tubular glucan dye sheet model. The helical orientation of microfibrils induced by acid washing and the ordering of a 3.99 Å reflection in four arcs suggested that the glucan sheet was helically coiled into a tube. The electron beam resistant, single 3.99 Å reflection was interpreted as the spacing between stacks of dye molecules associated with a single or a small packet of glucan sheets. The direction of dye stacking was deduced to be oriented perpendicular to the glucan chain axis, and the researchers suggested that the long axes of the dye molecules probably were parallel to the glucan chain axes.

Using the conventional *a*, *b*, *c* three-dimensional coordinate system of crystallography, the glucan chain axis is often given as parallel to the *c* axis[4,5]. Both the *a* axis and the *b* axis are perpendicular to the *c* axis. Because two different planes may be perpendicular to a third plane in three-dimensional space, the Haigler–Chanzy description was unclear as to which direction the dye stacks were ordered. In recent work on the interruption of chitin synthesis with fluorescent brighteners[6], this interaction, however, was diagramatically illustrated with dye stacks associated via van der Waals forces with small, noncrystalline, packets of *n*-actylglucosamine chains. By analogy, such a model would indicate that the predominant force holding the glucan chains together in a mini-sheet would be hydrogen bonds.

In 1991, Kai and Xu[7] presented new evidence for the original cellulose-brightener complex model[8]. Based on a modified X-ray diffraction analysis, a 9.4 Å reflection in addition to the previously reported 4.0 Å reflection was interpreted as evidence for multiple sheets in a highly crystalline cellulose-dye complex. They assigned the 9.4 Å reflection to the $(\bar{1}\,1\,0)$ plane[8], and implied that the indexing was the same as for unaltered bacterial cellulose. Kai and Xu further suggested that the implicated spacings were modified due to the incorporation of the dye molecules in the spaces between the $(\bar{1}\,1\,0)$ plane. However, because the unit cell dimensions of the cellulose-dye complex could not be determined to verify this supposition, the indexing by Kai and Xu should still remain suspect.

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† While these colleagues indexed their cellulose I diffraction patterns according to Gardener and Blackwell[4], the indexing has been translated according to Woodcock and Sarko[5] in order to maintain a constant system of indexing for this paper.

Thus, the Haigler/Chanzy model for glucan dye sheets[3] describes the interaction as a non-crystalline (but ordered) glucan sheet coated with dye molecules and helically coiled to form a tube. On the other hand, the Kai/Xu model[7] describes the interaction as a crystalline cellulose-dye complex. The differences in these interpretations may have arisen from differences in the synthesis conditions. These various structural interpretations can be evaluated by repeating the different synthesis conditions and observing the products with electron microscopy and X-ray diffraction. Thus, the goals of this study were: (1) to determine the structure of glucan dye sheets using the two different methods of synthesis, and (2) to determine the bonding between glucan chains with regard to the *in vivo* crystallization of cellulose I based on the structure of glucan dye sheets.

EXPERIMENTAL

Preparation of glucan dye sheets–Haigler/Chanzy protocol (HCP)

Glucan dye sheets were synthesized in static culture from resting cell suspensions incubated for 24 h in a volume of 100 ml at a final concentration of 1 mM Tinopal LPW^{TM}, 40 mM glucose, and 50 mM sodium phosphate buffer (pH 7–8) in the dark at room temperature. This concentration of Tinopal LPW^{TM} was chosen in order to maintain a high saturation level of brightener for the entire period of glucan dye sheet production. Resting cells of *A. xylinum* strain AY 201 (ATCC 23769) were prepared by the Celluclast^{TM9} method[10]. A single highly crenate colony (5–9 days) was used to inoculate 100 ml of Schramm and Hestrin (SH) medium[11] containing 150 μl of filter sterilized Celluclast^{TM}. The inoculated solution was incubated for 2 days at 28°C, in the dark on a rotary shaker at 120 rpm. After incubation, the solution was filtered through six layers of cheesecloth to remove undigested cellulose, then centrifuged at 1744 × *g* for 5 min at 4°C. The pellet was washed twice in excess 50 mM sodium phosphate buffer (pH 7–8) to remove any remaining Celluclast^{TM}. The cells were resuspended in approximately 1 ml of 50 mM sodium phosphate buffer (pH 7–8).

Preparation of glucan dye sheets—Kai/Xu protocol (KXP)

Another sample of glucan dye sheets was prepared according to the procedure of Kai and Xu[7]. In their study, Kai and Xu specified strain IFO 13693[7], which is also distributed through the American Type Culture Collection as strain 23769 (AY 201). However, they did not describe the colony morphology used for the inoculum. Because the rough colony variant is the only one which has been identified which synthesizes glucan dye sheets[1], strain AY 201 rc will be used. Ten Roux bottles (150 ml of SH medium[11] per bottle) each were inoculated with *A. xylinum* strain AY 201 rc and incubated for 4 days at 28°C in the dark in static culture. The cellulose pellicles were squeezed to release cells, and the resulting broth was filtered through cheese cloth. The filtrate was centrifuged for 10 min at 6160 × *g* at 4°C. The supernatant was removed, and the pellet was resuspended in excess 50 mM sodium phosphate buffer, pH 7.9. The resuspended solution was centrifuged again at 6160 × *g* at 4°C for 10 min. The pellet was resuspended in a final volume of 30 ml of 50 mM sodium phosphate buffer, pH 7.9. The cell suspension was mixed with 50 ml of 80 mM glucose in 50 mM sodium phosphate buffer, pH 7.9 and 20 ml of 5 mM Tinopal LPW^{TM} (in deionized water). This mixture was incubated in an Erlenmeyer flask wrapped with aluminium foil for 24 h at 28°C on an orbital shaker at 120 rpm. After 24 h, the cell suspension product was centrifuged for 10 min at 6160 × *g* at 4°C. The pellet was resuspended in excess 0.2% NaOH, incubated for 48 h in the dark on an orbital shaker at 90 rpm at room temperature, and washed thoroughly with deionized water to remove the alkali.

Electron microscopy

Before preparation for X-ray diffraction, samples were contacted with formvar coated, 300 mesh copper grids and negatively stained with 2.5% uranyl acetate and observed using a Philips 420 transmission electron microscope (TEM) at 100 kV.

X-ray diffraction analysis

Powder patterns of the various samples were obtained with a Philips PW 1024/30 Debye Scherrer camera using Ni-filtered CuK_α (1.542 Å) radiation at 35 kV and 25 mA. The reflections obtained were compared to those from a control sample of bacterial cellulose synthesized in the absence of Tinopal LPW^{TM} and a control sample of Tinopal LPW^{TM} recrystallized from a 5 mM stock solution by air drying under a black box.

For comparison with the data in Kai and Xu's study[7], a sample of glucan dye sheets—(KXP) was analysed using an X-ray diffractometer with a diffracted beam graphite monochromator with Cu$K_{\alpha 1}$ radiation (1.5405 Å) over a 2θ range of 0–40°. This sample was air dried in layers on a glass slide, following the procedure of Kai and Xu[7]. After analysis, this sample was scraped from the glass slide and packed in a glass tube for powder diffraction analysis as described above.

RESULTS

X-ray powder diffraction for bacterial cellulose (*Figure 1A*) produced reflections at 6.1 Å, 5.3 Å, 3.9 Å, and 2.6 Å, characteristic of the expected cellulose I allomorph. The crystallized Tinopal LPW^{TM} (*Figure 1B*) produced sharp reflections at 2.9 Å and 2.0 Å as well as a very line broadened reflection at 4.0 Å. The sample of glucan dye sheets–(HCP) gave a single 4.0 Å reflection (*Figure 1C*). The sample of glucan dye sheets–(KXP) gave several sharp reflections (*Figure 1D*) at 9.4 Å, 4.7 Å, 4.0 Å, 3.1 Å, 2.6 Å, 2.4 Å, and 1.5 Å as well as what appear to be several low intensity reflections at 3.7 Å, 2.2 Å, 2.1 Å, and 1.2 Å.

The quantity of reflections was not sufficient to determine a unit cell for the material synthesized with the Kai/Xu protocol because the several sharp reflections at 4.7 Å, 3.1 Å, and 2.4 Å are most likely multiple orders of the 9.4 Å spacing because the quotient of 9.4 Å and each of the other reflections yields an integer. The 4.0 Å reflection may be attributed to the stacking of the dye molecules and/or the ordering of glucan chains, and the 2.6 Å reflection probably arises from ordering of the glucan chains. The several low intensity reflections and background scatter indicate that the sample has a definite amorphous component in addition to the crystalline component determined from the several sharp reflections.