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FT-IR study of the *Chara corallina* cell wall under deformation

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Abstract—Fourier-transform infrared (FT-IR) microspectroscopy was used to investigate both the chemical composition of, and the effects of an applied strain on, the structure of the *Chara corallina* cell wall. The inner layers of the cell wall are known to have a transverse cellulose orientation with a gradient through the thickness to longitudinal orientation in the older layers. In both the native state and following the removal of various biopolymers by a sequential extraction infrared dichroism was used to examine the orientation of different biopolymers in cell-wall samples subjected to longitudinal strain. In the *Chara* system, cellulose microfibrils were found to be aligned predominantly transverse to the long axis of the cell and became orientated increasingly transversely as longitudinal strain increased. Simultaneously, the pectic polysaccharide matrix underwent molecular orientation parallel to the direction of strain. Following extraction in CDTA, microfibrils were orientated transversely to the strain direction, and again the degree of transverse orientation increased with increasing strain. However, the pectic polysaccharides of the matrix were not detected in the dichroic difference spectra. After a full sequential extraction, the cellulose microfibrils, now with greatly reduced crystallinity, were detected in a longitudinal direction and they became orientated increasingly parallel to the direction of strain as it increased.

Keywords: Cell wall; *Chara corallina*; FT-IR microspectroscopy; Dichroic difference; Orientation

1. Introduction

FT-IR spectroscopy is a powerful method for the characterisation of biological materials. It provides information about the structure, chemical microenvironment of the constituent biomolecules, their conformation and, when polarised radiation is used, the orientation of biopolymers, which include cell-wall polysaccharides.[1] The coupling of a microscope attachment to an FT-IR spectrometer has enabled spectra to be obtained from single cell walls, and this technique called FT-IR microspectroscopy, has been used to follow changes in both composition and molecular interactions during the sequential extraction of onion parenchyma cell walls[2] and other higher plant cell walls.[3]

FT-IR spectroscopy using polarised radiation can be used to investigate the orientation of biomolecules in an orientated polymer system. Molecular orientations have been observed in the cell walls of elongated carrot,[4] tobacco[5] and onion cells.[6] FT-IR has also demonstrable potential for investigating what happens to a cell-wall biopolymer subjected to mechanical stress. This holds promise as a new way to investigate the functional architecture of the cell wall.[6,7]

In this paper FT-IR microspectroscopy was used to follow changes in the FT-IR spectra of *Chara* cell walls at different stages during a sequential extraction of biopolymers. Analysis of characteristic IR bands allowed an assessment of which polysaccharides were being removed from the cell wall during each extraction step.

Molecular alignment resulting from imposed strain on native and extracted *Chara* cell walls was investigated. Cell-wall sample strips (native and extracted) were stretched longitudinally to the cell axis, and polarised FT-IR spectra were collected for each stage of

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the stretching process. The dichroic difference spectra[7] were compared at each strain in order to assess the orientation of some of the polymers in the native and extracted cell-wall samples.

2. Experimental

2.1. Plant materials

C. corallina was grown in 20 L glass tanks according to the method described.[8] Branches of algae were obtained from the tanks on the day of use and stored in distilled water.

2.2. Sample preparation

Single internode cells were excised from the axis using a razor blade and placed in a Petri dish containing distilled water. Using a binocular light microscope, a hypodermic needle was inserted into the tube of the cell wall then withdrawn slowly using the sharp tip as a knife to form a cut along the length of the cell. The tube was then opened up to provide a rectangular strip of cell wall, approximately 50 mm × 2.5 mm, which was rinsed thoroughly with distilled water to remove all cell contents. Storage was in distilled water until required for infrared analysis, when the excess water from the surface of the strips was dried using soft paper tissue and measurements taken while the sample was still moist.

2.3. Sequential extraction of the cell wall

Opened *Chara* internode cell walls were extracted sequentially using the method described.[9] Following each extraction stage cell-wall strips were removed, neutralised and stored in distilled water before preparation for infrared analysis as above.

2.4. FT-IR microspectroscopy

FT-IR microspectroscopy of the *Chara* cell wall was conducted using a Bio-Rad FTS 175C FT-IR spectrophotometer (Cambridge, MA) in conjunction with a UMA-500 microscope attachment. A KRS5 (TlBr and TlI mixed crystal) wire-grid polariser (Graseby Specac, Orpington, UK) was used to determine molecular orientation within the cell walls. All data were collected using the Win-IR software (Bio-Rad) version 4.0.

The unstretched individual cell-wall samples were measured on a 1 mm thick, 13 mm diameter BaF_2 disc (Crystan Ltd, Poole, UK) with a microscope aperture of approximately 100 × 100 μm. Spectra were recorded at a resolution of 8 cm^{-1} with 64 interferogram scans being co-added before Fourier transformation. At least five cell-wall samples were scanned for each stage of the sequential extraction and five spectra were obtained from each sample strip.

2.5. FT-IR spectroscopy of the cell wall under applied stress

The ends of the cell-wall strips were attached to the clamps of a custom made stretching cell using double-sided adhesive tape (Fig. 1). The stretching cell, with the cell-wall sample, was transferred to a Minimat miniature materials tester (Rheometric Scientific Ltd, Epsom, UK). Here the strips were stretched longitudinally in 0.1 mm intervals, from an initial length of 12.7 mm (the distance between the rigid stretching cell clamps) until breakage occurred. Polarised FT-IR spectra were recorded for each 0.1 mm longitudinal deformation step in each sample. At least five replicate cell-wall samples, unextracted, following CDTA extraction and following a full sequential extraction, were individually stretched and measured in this way.

The dichroic difference, ΔA, was measured by subtracting the spectrum collected with the polarisation perpendicular ($A_\perp$) to the direction of polarisation from the spectrum collected with the polarisation parallel ($A_\parallel$) to the direction of the polarisation: $\Delta A = A_\parallel - A_\perp$. The polarisation was set such that the parallel polarisation was parallel to the long axis of the cell. The dichroic difference is considered more sensitive to orientation than the dichroic ratio (defined as $A_\parallel/A_\perp$), especially when the level of orientation within the sample is low.[7,10] Within a dichroic difference, positive signals denote frequencies of dipole moments orientated parallel (longitudinal) to the stretch direction, whereas negative values denote orientation perpendicular (transverse) to the stretch direction. The polariser had been standardised using polyethylene. The dichroic difference of the glycosidic link, (C_1–O–C_4), band at about 1162 cm^{-1} was plotted against the strain experienced by the cell-wall samples for each stretching step, where strain is defined as the change in sample length/initial sample length ($\Delta l/l$).

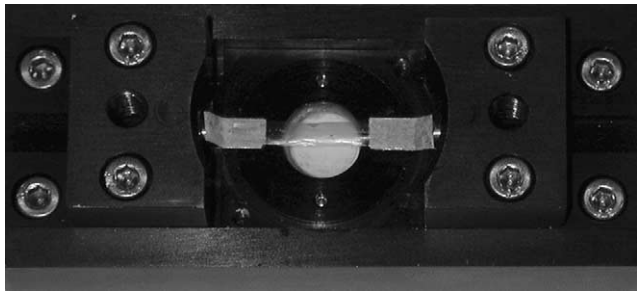

Figure 1. Custom-made stretching cell with cell-wall sample.

2.6. X-ray crystallography

X-ray diffraction patterns were obtained for the unextracted and fully extracted *Chara* cell wall using X-ray crystallography as described for carrot.[11] Measurements were made under conditions of ambient temperature (24 °C) and relative humidity (44%) and the instrumental background was determined by scanning the empty sample holder under the same conditions used for the samples. The percentage crystallinity of the samples was calculated from the ratio of the measured area under the diffraction peaks to the total measured area under the whole diffraction pattern.[12]

3. Results and discussion

3.1. The *C. corallina* cell wall

3.1.1. The untreated cell wall. The FT-IR spectrum of the *C. corallina* cell wall in the native state is shown in Figure 2. FT-IR spectra recorded at different positions within each sample were essentially identical indicating little intra-sample compositional variation. The spectra obtained from moist cell walls were comparable with those recorded by Homblé et al.[13] for the dried cell walls of *C. corallina*, and for the dried cell wall of the alga *Nitella*.[14,15] The main spectral features of *C. corallina* cell wall exhibited some similarity with those recorded for other cell walls, that is, onion epidermis,[6,16] tomato pericarp,[16,17] carrot epidermal cell walls[16] and elongated carrot suspension cells.[4] This confirmed that the algal cell wall was similar in chemical composition to that of higher plants as stated by Cleland.[18]

Band assignments for spectra of cellulose and pectin in the 1800–800 cm^{-1} region were based on literature data.[7,16,19–22] Referring to the spectra of onion cell walls, the glycosidic link for cellulose was at 1158 cm^{-1}, the backbone vibrations of polygalacturonic acid (PGA) at 1107, 1054, 1014 and 959 cm^{-1}. The band at 1745 cm^{-1} resulted from pectin ester groups, those at 1605 and 1419 cm^{-1} resulted from carboxylate groups, and band shoulders from xyloglucan (XG), RG I, and galactans were observed between 1075 and 1065 cm^{-1}.[6]

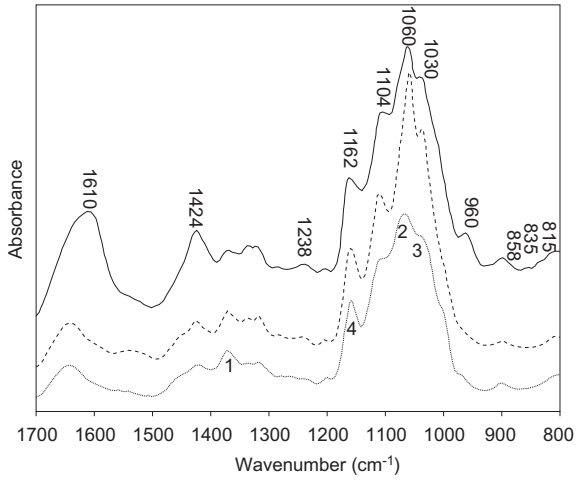

Figure 2. FT-IR microscope absorbance spectra of the *C. corallina* cell wall, in the native state (—), following extraction in CDTA (- - -), and following a full sequential extraction (···).

The spectral patterns of the *Chara* cell wall were clearly similar to those of pure cellulose.[17,23,24] Cellulose, which occupies a large proportion of the cell wall, is highly crystalline compared to the remaining cell-wall components and therefore dominates the spectrum.[7] The pectins and hemicelluloses within the wall affect the spectral shape only in fine detail as their peaks have a lower intensity, a more diffuse spectral shape and are strongly overlapped by the cellulose bands. There were no bands at 1745 and 1444 cm^{-1}, showing that PGA is not esterified in the *Chara* cell wall and that the pectin involved is in the form of pectate only (with bands at 1610, 1424 and 960 cm^{-1}).[21] This is consistent with earlier studies on the polysaccharide content of the *Nitella* cell wall,[25] which stated that the pectins within the Characean cell wall were non-esterified. The spectra for onion cells, on the other hand, did show bands for pectin ester.[26]

3.1.2. CDTA-extracted cell walls. Cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetate (CDTA) (0.05 M, pH 6.5) is a chelating agent that solubilises pectic polysaccharides, that are held adjacent to each other within the cell wall by Ca^{2+} bridges.[27] The spectra obtained for the cell wall following extraction in CDTA, were dominated by absorption bands associated with cellulose, for example, 1162, 1060 and 1030 cm^{-1}. Numerous bands vanished from the IR spectra recorded for cell walls extracted in CDTA, compared to those for the native cell wall (Fig. 2). These bands can be assigned to the components of the pectic polysaccharides. The absorption bands at 1610 and 960 cm^{-1} were reduced in the spectral fingerprint and there was a large reduction in the band at 1424 cm^{-1}, due to the removal of carboxylate groups, and shifts in the 1104 and 1238 cm^{-1} bands towards 1110 and 1245 cm^{-1}, respectively, all indicating a loss of pectins. The differences in the spectra confirmed the removal of some pectin by CDTA, in agreement with data from the carbohydrate analysis of CDTA-extracted cell walls (Table 1), which showed, for example, the galactose was reduced from 2.0 to 1.5 mol%.[9]

By subtracting the spectrum for the CDTA-extracted cell wall, from that obtained for the native cell wall, a spectrum of the components removed by CDTA was produced, and is shown in Figure 3. The subtraction spectrum corresponded very well with the spectrum for PGA,[22] with IR bands at 1608, 1425, 1095 and 1009 cm^{-1}, denoted by **p**. The bands at 1137 and

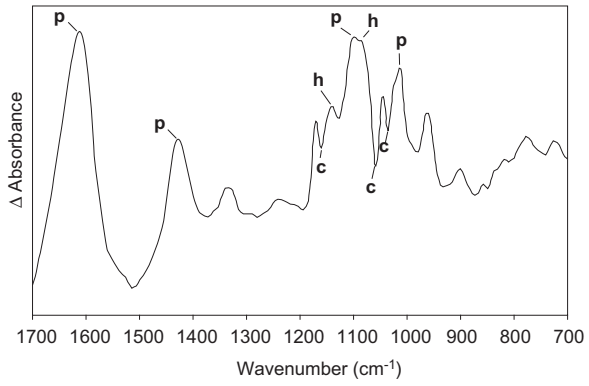

Figure 3. FT-IR subtraction spectra (native *Chara* cell wall—CDTA-extracted cell wall), △ absorbance. Pectin (**p**), cellulose (**c**) and hemicellulose (**h**) bands shown.

1080 cm^{-1}, denoted by **h**, corresponded to hemicelluloses,[22] such as xyloglucan, which were removed from the cell wall in small amounts by CDTA. The negative bands after subtraction denoted by **c**, at 1162, 1060 and 1030 cm^{-1}, corresponded to cellulose, due to the increased overall percentage of cellulose after CDTA extraction, in agreement with chemical analysis.[9]

3.1.3. Na_2CO_3 and KOH extraction of the cell wall. No significant difference was observed in the spectra obtained for the *Chara* cell wall following the Na_2CO_3 (at 1 and 20 °C), 0.5 M KOH and 1 M KOH extraction stages of the sequential extraction (therefore not shown), apart from further slight reductions in the pectic polysaccharide absorption bands. This was expected following previous results on carbohydrate content,[9] where the chemical composition of the cell wall showed very little change during these stages of the sequential extraction procedure.

3.1.4. Full sequential extraction of the cell wall. The infrared spectrum of the *Chara* cell wall following a full sequential extraction including 4 M KOH is shown in Figure 2. The pattern of dominant bands at 1371, 1065 and 1038 cm^{-1}, denoted by **1**, **2** and **3** (see Fig. 2) is not typical for cellulose. However carbohydrate analysis[9] showed that the total glucose content increased from 50 to 80 mol% (Table 1) suggesting that the glucan structures involving cellulose are the main residual units after a full extraction of the *Chara* tissue. The cellulose bands at 1060 and 1030 cm^{-1} might be present, however, but are overlapped with bands of altered pattern implying a decrease in cellulose content or cellulose crystallinity, but this cannot be proved or otherwise using IR spectra alone, and therefore X-ray analysis was invoked (see below). Thus the final spectral shape suggests a change in cellulose structure and is characteristic of hemicelluloses, in particular glucomannans,[22] while the glycosidic link shift to a lower frequency of 1158 cm^{-1}, **4** (Fig. 2) indicates that cellulose and glucomannan were overlapping. Support for the above from analysis of the uppermost spectrum in Figure 2 showed three weak bands beyond 900 cm^{-1} of which two at 858, 835 cm^{-1} are from pectin and one at 815 cm^{-1} from glucomannan.[22] The two pectin bands were missing from the spectra of extracted material (lower two spectra).

It was determined previously, during carbohydrate analysis,[9] that xyloglucan hemicelluloses were removed from the cell wall during the 4 M KOH extraction stage (Table 1). This could not be confirmed, as xyloglucan bands were not visible in the absorbance spectra at any stage of the extraction due to strong overlapping by cellulose bands. However, there was no evidence during carbohydrate analysis to suggest that glucomannans were being removed from the wall (Table 1), and the spectra confirm that they remained present. Cell-wall analysis of the alga *Nitella*[28] has also shown two distinct hemicelluloses with the glucomannan more tightly bound to cellulose than the xyloglucan, and with some mannose-rich hemicellulose not being removed by 4 M KOH. The presence of mannans has also been reported to contribute to the change of cellulose crystallinity.[29]

3.2. X-ray crystallography

A weak crystalline diffraction pattern was achieved. The main peak at 22.7° 2θ corresponded to highly organised, crystalline cellulose I,[20] and the three sharp peaks at 29°, 36° and 39° 2θ, were due to the presence of inorganic salts, such as exogenous calcium carbonate, due to the bulk nature of the sample. Probine and Preston[30,31] found that the cell wall of *Nitella* also contained weakly crystalline cellulose I and Koyama et al.[32] described the cellulose I_α in *Chara*. The total crystallinity index of the whole native *Chara* cell wall was 5–6%. This was slightly

Table 1. Carbohydrate compositions before and after sequential extraction[a]

Extracted wall (number of replicates)	Cell wall carbohydrates (mol%) (standard deviation)							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Urn
Control (10)	1.89 (0.04)	2.77 (1.15)	1.88 (0.09)	3.89 (0.11)	4.51 (0.31)	2.03 (0.28)	49.61 (1.10)	33.42 (1.09)
CDTA (10)	1.18 (0.12)	3.87 (0.31)	2.36 (0.33)	5.17 (0.18)	6.16 (0.30)	1.47 (0.18)	71.70 (2.26)	7.72 (1.33)
4 M KOH (10)	0.51 (0.06)	4.11 (0.22)	1.99 (0.13)	3.95 (0.15)	6.48 (0.06)	0.70 (0.10)	79.81 (0.09)	2.46 (0.11)

Adapted from Table 2 of Toole, G. A.; Smith, A. C.; Waldron, K. W. The effect of physical and chemical treatment on the mechanical properties of the cell wall of the alga *Chara corallina, Planta*, **2002**, *214*, 468–475. © 2002 Springer-Verlag. All rights reserved.[9]

[a]Abbreviations: Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; Urn, uronic acid.

lower but comparable to a crystallinity index of 10–13% for carrot cell-wall residue using the same methodology.[11]

The X-ray diffraction pattern obtained for the *Chara* cell wall following a full sequential extraction, showed a smooth curve with no diffraction peaks for either cellulose or hemicellulose indicating that the sample was completely amorphous, and thus in agreement with the spectra shown in Figure 2. Previous work[11] showed that the crystallinity index for cotton lint cellulose dropped from 43% to 29% following treatment in 4 M KOH, indicating that strong alkaline treatment decreases the crystallinity of cellulose.

3.3. Determination of structural orientation within the cell wall

3.3.1. The untreated cell wall. The dichroic differences for the longitudinally stretched, native *Chara* cell wall are shown in Figure 4a. The antisymmetric stretching mode of the cellulosic glycosidic link (C_1–O–C_4) was used as a measure of predominant microfibrillar orientation during longitudinal stress, as it is co-aligned with the molecular backbone of the microfibril. It appeared at 1169 cm^{-1} in the dichroic difference for *Chara* compared to 1162 cm^{-1} band position in nonpolarised cellulose.[20] Such a band shift may result from band position difference between the two polarised spectra of the stretched material. The uppermost spectrum (Fig. 4a) showed a negative band at 1169 cm^{-1}. Therefore the initial, predominant orientation of the cellulose microfibrils within the *Chara* cell wall was transverse. This is consistent with the results of Morikawa et al.[14] using pre-stretched *Nitella* cell walls. As the cell-wall sample was stretched, 0.1 mm at a time, the intensity of the band at 1169 cm^{-1} for each dichroic difference increased negatively. Due to stretching, the microfibrillar component of the cell wall became increasingly transversely orientated, in relation to the direction of stretch and the length of the cell-wall strip.

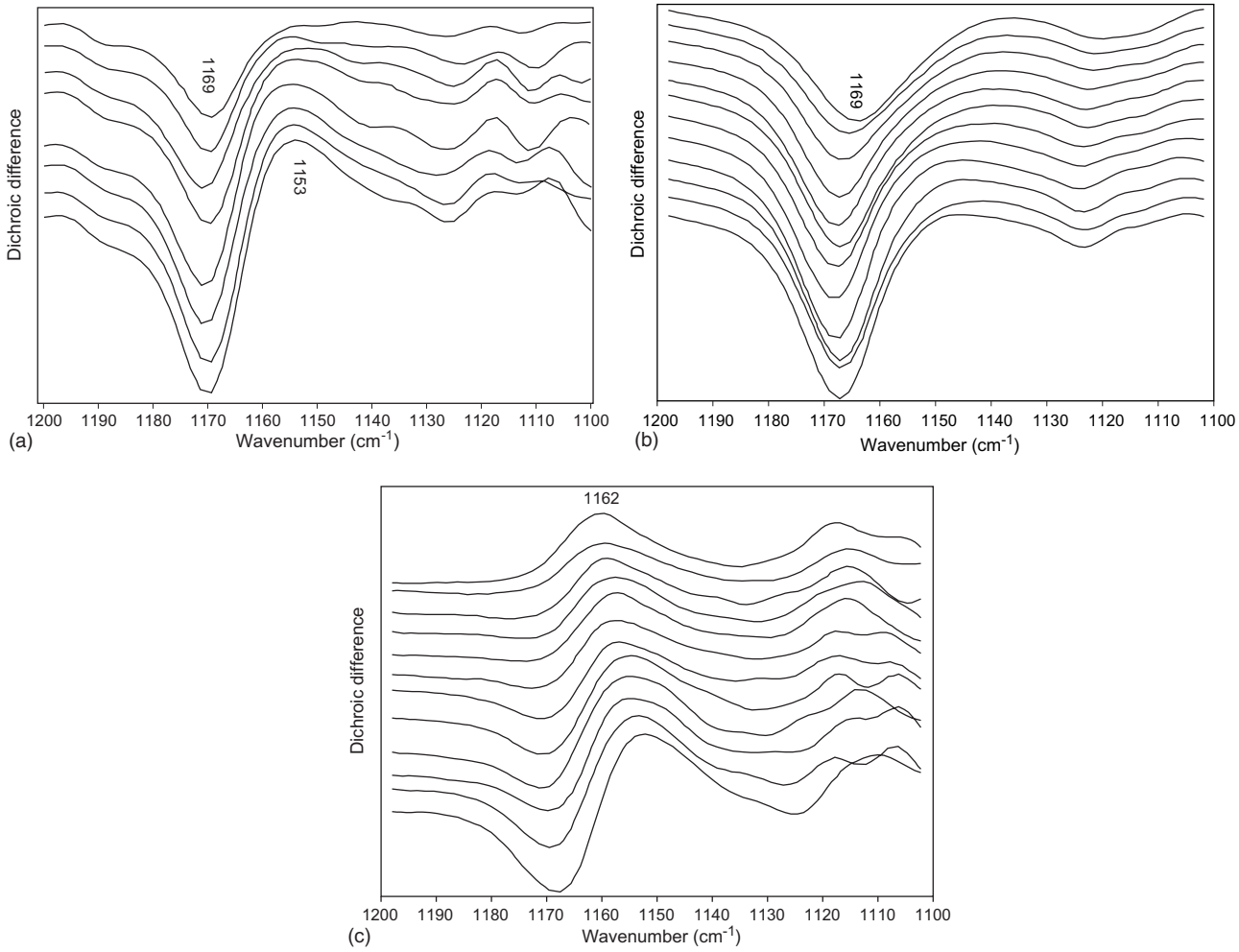

Figure 4. Dichroic difference spectra ($A_{\parallel} - A_{\perp}$), for (a) the untreated *Chara* cell wall, (b) the *Chara* cell wall following extraction in CDTA, (c) the *Chara* cell wall following a full sequential extraction. The cell wall was stretched by 0.1 mm, for each spectrum, starting from the uppermost to the lowermost.

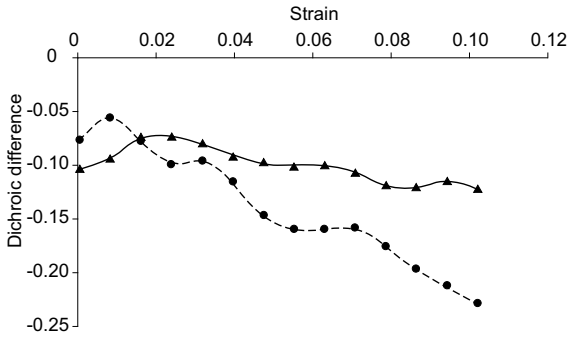

Figure 5. Dichroic difference, $(A_{\parallel} - A_{\perp})$, of the cellulose glycosidic link band, at 1169 cm^{-1}, as a function of the strain imposed on (a) the unextracted cell-wall sample strip (▲), (b) the CDTA-extracted cell-wall sample strip (●).

A plot of dichroic difference for the glycosidic link band, at 1169 cm^{-1} as a function of the strain $(\Delta l/l)$ applied to the cell-wall strip, shows it becoming more negative (Fig. 5a). As the cell-wall samples were extended, the absorption of the band at 1153 cm^{-1} due to PGAs[22] also changed. Although this band was not visible in the original spectra of the cell wall, due to overlapping by stronger bands, it was clearly revealed in the dichroic difference spectra, especially as the strain was increased (Fig. 4a). The IR band at 1153 cm^{-1} had almost zero intensity before extension (uppermost spectrum), indicating very little orientation of the PGAs without the application of stress. As the extension increased the peak became increasingly positive indicating increasingly parallel orientation of the PGAs, which lie between the microfibrillar layers,[33] into the direction of extension following each strain increment.

These changes may be understood with reference to earlier studies on algae. Green[34] proposed that cell-wall microfibrils, in *Nitella*, were deposited transversely to the axis of elongation and that these transverse microfibrils would be subjected to passive reorientation during growth, such that outer wall regions would possess microfibrils that were aligned longitudinally in response to the strain, in agreement with the multinet growth hypothesis of Roelofsen and Houwink.[35] Morikawa et al.[14] concluded that the cellulose chains were preferentially orientated in an almost transverse direction to the cell axis, during early infrared studies on *Nitella*, in agreement with results obtained by X-ray diffraction.[29] Richmond[36] stated that the inner 25% of the cell wall, where the transversely orientated microfibrils were yet to be subjected to passive reorientation, was the principal stress bearing, and growth regulating, region of the wall. In cylindrical cells such as the *Chara* internode, with predominantly transverse microfibrils, the stress in the longitudinal direction is borne principally by noncrystalline components.[37]

In the *Chara* system, the microfibrils that were aligned transverse to the length of the cell became increasingly transverse in orientation. Simultaneously, the pectic polysaccharide molecules between the cellulose microfibrils were reorientated parallel to the stretch direction.

3.3.2. CDTA-extracted cell wall. The dichroic differences for the longitudinally stretched, *Chara* cell wall following extraction in CDTA are shown in Figure 4b. As with the unextracted cell wall, the uppermost spectrum had a negative band at 1169 cm^{-1}, indicating a predominantly transverse microfibrillar orientation in the unstressed cell wall, which increased with strain. A plot showing the dichroic difference of the glycosidic link band, at 1169 cm^{-1} (for each dichroic difference spectrum in the stretching series), as a function of the strain (Fig. 5b) again showed increasingly negative values as the strain on the sample increased. Since the CDTA removes pectic polysaccharides from the cell wall the PGA positive absorbance at 1153 cm^{-1} did not show a maximum (Fig. 4b) in comparison to the untreated sample (Fig. 4a).

3.3.3. Fully sequentially extracted cell wall. The dichroic differences following a full sequential extraction (Fig. 4c) show that the glycosidic link band at 1162 cm^{-1} was now positive. This indicated that the cellulose, now with greatly reduced crystallinity, was identified by longitudinally orientated microfibrils, which became increasingly orientated parallel to the direction of strain. As the strain was increased, the band gradually performed a band shift and finally became symmetrically bipolar, an effect arising from the subtraction of two spectra with different polarisations having a small frequency shift between $A_{\parallel}$ and $A_{\perp}$. Stress sensitivity involves a small shift in band position to a lower frequency, along with an asymmetric shape change of the entire band. The results are often interpreted in terms of the distribution of external load, resulting in displacement of the individual bond absorption frequencies.[10] This shift in frequency and the conversion to a bipolar band, was due to molecular deformation of the glycosidic bond, during longitudinal extension of the cell-wall strip and thus of the cellulose microfibrils that were orientated parallel to this extension.

4. Conclusions

FT-IR is able to distinguish between the contributions of the different pectic, hemicellulosic and the dominant cellulosic elements of the cell wall of *C. corallina*, particularly as sequential extraction removes different pectin moieties. However the full extraction to 4 M KOH alters the cellulose-dominant spectrum corresponding to a loss of cellulose crystallinity. These observations are accentuated in the dichroic differences between parallel and perpendicular orientation where the dominant transversely orientated cellulose microfibils are seen in

the native wall and after removal of some pectic species. After full sequential extraction the removal of most of the crystallinity leaves longitudinally orientated cellulose. In all cases strain in the longitudinal direction increases the degree of orientation compared to the unstretched sample, and maintains its direction.

Acknowledgements

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