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Carbohydrate Research 341 (2006) 2677–2687
Carbohydrate RESEARCH

Physicochemical characterization of cellulose from perennial ryegrass leaves (*Lolium perenne*)

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Received 23 May 2006; received in revised form 3 July 2006; accepted 13 July 2006
Available online 24 August 2006

Abstract—In this study, we investigated the physicochemical properties of the cellulosic preparations obtained from both untreated perennial ryegrass leaves and de-juiced leaves. It was found that treatment at 22 °C with 18% NaOH and 18% KOH for 2 h, and 10% NaOH and 10% KOH for 16 h yielded 28.2%, 28.8%, 22.7%, 23.4%, respectively, of 'cellulose' residue from untreated ryegrass leaves and 35.7%, 36.8%, 32.8% and 34.6%, respectively, from the de-juiced leaves. For each cellulosic fraction, the glucose content was 71.6%, 69.6%, 67.8%, 66.7%, 69.7%, 68.6%, 63.9% and 61.7%, respectively. The structure of the cellulose samples was examined using FTIR and CP/MAS ^{13}C NMR spectroscopy and X-ray diffraction. The cellulosic preparations were free of bound lignin except for noticeable amounts of residual hemicelluloses (28.4–38.3%), and had intrinsic viscosities between 275.1 and 361.0 mL/g, along with molecular weights from 144,130 to 194,930 g/mol. This study found that the cellulose samples isolated from both de-juiced ryegrass leaves and the untreated leaves had a much lower percent crystallinity (33.0–38.6%) than that from wood-based fibres (60–70%) and had much shorter fibres (0.35–0.49 mm) than those of either cereal straws, bagasse or wood. In addition, a partial disruption of the hydrogen bonds and microfibrils may occur during the de-juicing process by mechanical activity, which results in a decreased cellulose crystallinity and fibre length. These findings are significant in relation to hydrolysing ryegrass cellulose for bio-ethanol production.

Keywords: Perennial ryegrass; Cellulose; Fibre length; Crystallinity; Hemicelluloses

1. Introduction

Perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*) are the major ryegrasses in the UK and Europe, which occupy about 70% of the agricultural areas. Tall fescue ryegrass (*Festuca arundinacea*), meadow fescue ryegrass (*Festuca pratensis*) and cocksfoot ryegrass (*Dactylis glomerata*) make up the remainder.[1] Several processes and products have been reported utilizing ryegrass as a raw material for industry. These include pulp and paper production and products of fermentation, such as high value-added xylitol, a naturally occurring sweetener.[2] More importantly, these materials are also useful for bio-ethanol production after pretreatment and enzymatic hydrolysis of the cellulose and hemicelluloses to monosaccharides.[3,4]

Carbohydrates are the main constituents of ryegrass, and structural polysaccharides like cellulose and

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doi:10.1016/j.carres.2006.07.008

hemicelluloses, such as xylans, are predominant. Xylans consist of a main chain of β-(1→4)-linked xylopyranose residues with branches of arabinose and/or uronic acid residues.[5] Cellulose is a linear polymer of 'anhydroglucose' units with β-(1→4) linkages. It crystallizes through hydrogen bonding between the chains and has cellulobiose as the repeat unit. The crystal structure of cellulose in higher plants is that of cellulose Iβ[6,7] in which the sheet stacks have an alternating shear.[8,9] In crystalline cellulose, each cellulose chain approximates a flat ribbon, with alternate glucose units facing in opposite directions.[10] The crystalline regions are interrupted every 60 nm with noncrystalline, amorphous regions. Crystalline regions may contain occasional kinks or folds in the polymer chain, called 'defects'. The type and the number of defects vary with the type of cellulose.[11,12] All the cellulose chains lie parallel, hydrogen-bonded edge to edge. The sheets of chains so formed are stacked on top of one another with staggering along the microfibril. The weak C–H···O hydrogen bonding is thought to be the force that holds sheets of chains together in stacks.[8,13] Evidently, the crystalline structure of cellulose affects physical and mechanical properties of cellulose fibres. An increasing ratio of crystalline to amorphous regions results in the increases in the rigidity of the cellulose fibres, but decreases their flexibility.[14] In addition, the crystallinity of cellulose has an important role on the accessibility of both chemical reagents and enzymes,[15] suggesting that noncrystalline forms of cellulose will be chemically more reactive.

Cellulose is the most abundant renewable carbon source available and can, in part, potentially meet our future needs for chemicals and energy if cellulose can be efficiently isolated and converted to monomeric sugars. In this study, α-cellulose was isolated from both de-juiced leaves and untreated perennial ryegrass leaves (*L. perenne*). The cellulosic preparations isolated were subjected to analysis of their content of associated hemicelluloses, lignin, viscosity, molecular weight, thermal stability and fibre length. Cellulose structures were characterized by Fourier-transform infrared (FTIR), X-ray diffraction (XRD) and cross polarization/magic-angle spinning ^{13}C solid-state nuclear magnetic resonance (CP/MAS NMR) spectroscopy.

2. Results and discussion

2.1. Yield of cellulose

To obtain the pure cellulose, a prior treatment by extraction with organic solvents is required to remove the non-cell-wall components such as wax and chlorophyll. It was found that the pre-treatment of untreated ryegrass leaves and the de-juiced leaves with 2:1 chloroform–ethanol under the conditions used, removed almost of the chlorophyll, wax and other extractives with a combined yield of 5.3% dry matter from untreated ryegrass leaves and 4.4% dry matter from the de-juiced leaves. Treatment with hot water for 4 h at 80 °C then released 24.0% dry weight from untreated ryegrass leaves and 8.5% from the de-juiced leaves, which contained 35.0% and 40.0% water-soluble hemicelluloses and 10.4% and 9.5% lignin, together with some amounts of protein, ash and starch from untreated ryegrass leaves and the de-juiced leaves, respectively.

Further treatment of the holocellulose obtained from untreated ryegrass leaves and the de-juiced leaves with 18% NaOH for 2 h, 18% KOH for 2 h, 10% NaOH for 16 h and 10% KOH for 16 h at 22 °C yielded 17.6% and 25.2%, 17.1% and 24.2%, 22.6% and 29.2% and 21.9% and 23.3% of the hemicelluloses (percent dry matter), respectively (data not shown in Table 1). Consequently, the yield of 'cellulose' residue was 28.2%, 28.8%, 22.7%, 23.4%, respectively, of the dry matter from untreated ryegrass leaves and 35.7%,

Table 1. Yield and extraction conditions of cellulose obtained from untreated ryegrass leaves and the de-juiced leaves

Fraction no.	Extractant	Temperature (°C)/time (h)	Dry material (g)/extractant (mL)	Yield (% dry grass)
C_{1a}[a]	18% NaOH	22/2	3.0/60	28.2
C_{2a}[b]	18% KOH	22/2	3.0/60	28.8
C_{3a}[c]	10% NaOH	22/16	3.0/60	22.7
C_{4a}[d]	10% KOH	22/16	3.0/60	23.4
C_{1b}[a]	18% NaOH	22/2	3.0/60	35.7
C_{2b}[b]	18% KOH	22/2	3.0/60	36.8
C_{3b}[c]	10% NaOH	22/16	3.0/60	32.8
C_{4b}[d]	10% KOH	22/16	3.0/60	34.6

[a] C_{1a} and C_{1b} represent the cellulosic preparations of untreated ryegrass leaves and the de-juiced leaves, respectively, obtained by extraction of the holocellulose with 18% NaOH for 2 h at 22 °C.

[b] C_{2a} and C_{2b} represent the cellulosic preparations of untreated ryegrass leaves and the de-juiced leaves, respectively, obtained by extraction of the holocellulose with 18% KOH for 2 h at 22 °C.

[c] C_{3a} and C_{3b} represent the cellulosic preparations of untreated ryegrass leaves and the de-juiced leaves, respectively, obtained by extraction of the holocellulose with 10% NaOH for 16 h at 22 °C.

[d] C_{4a} and C_{4b} represent he cellulosic preparations of untreated ryegrass leaves and the de-juiced leaves, respectively, obtained by extraction of the holocellulose with 10% KOH for 16 h at 22 °C.

36.8%, 32.8% and 34.6%, respectively from the de-juiced leaves as 18% NaOH, 18% KOH, 10% NaOH and 10% KOH were used under the extraction conditions given. A higher yield of cellulose (7.5–11.2%) from the de-juiced ryegrass leaves was probably due to the substantial release of non-cell-wall polysaccharides and lignin, as well as such components as protein, starch and ash during the de-juicing process, which resulted in the de-juiced ryegrass leaves becoming enriched in polysaccharides. In addition, as shown in Table 1, decreasing alkali strength from 18% NaOH to 18% KOH led to a slight increase in the yield of cellulose by 0.6% from untreated ryegrass leaves and 1.1% from the de-juiced leaves. Similar results showing an increase in cellulose were found by decreasing the alkali strength from 10% NaOH to 10% KOH, which raised the yield of 'cellulose' residue by 0.7% from untreated ryegrass leaves and 1.8% from the de-juiced leaves. In addition, by increasing the duration of extraction from 2 to 16 h, but decreasing the alkali concentration from 18% to 10% NaOH and from 18% to 10% KOH, gave a decreased yield from 28.2% to 22.7% and 28.8% to 23.4%, respectively, from untreated ryegrass leaves. This phenomenon was particularly true when the de-juiced leaves were used as starting materials. The reason for this lower yield of α-cellulose is probably that more hemicelluloses were removed during the treatment of the holocellulose with a stronger alkali such as 18% NaOH for 2 h or 10% NaOH for 16 h at 22 °C.

2.2. Composition of neutral sugars and uronic acids

The analyses of the monosaccharides present in the liquors obtained in the quantitative acid hydrolysis of the eight cellulosic preparations showed that cellulose accounted for 61.7–71.6% of the glucose content (Table 2). A slightly lower content of glucose (61.7–67.8%) in the cellulosic preparations of C_{3a}, C_{4a}, C_{3b} and C_{4b} obtained by extraction with 10% alkali for 16 h from both untreated ryegrass leaves and the de-juiced leaves, corresponded to their lower yields of cellulose as compared to that of the cellulosic samples of C_{1a}, C_{2a}, C_{1b} and C_{2b} (68.6–71.6% glucose) isolated with 18% alkali for a shorter period of 2 h. This indicates that treatment of the holocellulose with 10% alkali for a longer duration also results in some degradation of cellulosic polymers except for substantial dissolution of the hemicelluloses. Interestingly, as estimated from the content of glucose, the four cellulosic preparations obtained from untreated ryegrass leaves showed a slightly higher purity than that of the corresponding other four cellulosic fractions obtained from the de-juiced leaves, which reversed their yields of cellulose. On the other hand, as can be seen in Table 2, the cellulose samples still contained noticeable amounts of non-cellulosic sugars such as xylose (19.0–27.1%), arabinose (5.4–8.0%) and uronic acids, mainly 4-*O*-methyl-D-glucuronic acid (3.8–5.8%). Galactose (1.3–2.9%), mannose (0.5–2.0%) and rhamnose (0.3–1.8%) were also identified in minor quantities as neutral sugar constituents of the cellulose samples. The resistance to extraction with alkali revealed that the hemicelluloses in the cell walls of ryegrass are not only associated with the surface of cellulose but are also not limited at the outer fibre surface. Another likely explanation for the sorption of hemicelluloses on the cellulose framework is through hydrogen bonds, which can retain the hemicelluloses on the network of the fibrils during the alkali extraction.[16] The current observation is consistent with the studies on fractionation of hemicellulosic fragments and their interaction with cellulose in various plant cell walls as reported by Vincken et al.[17] The authors demonstrated that hemicelluloses either bind to the surface of cellulose microfibrils or cross-link the adjacent microfibrils. This network is embedded in a matrix in which various types of non-covalent cross-links, such as hydrogen bonds, between hemicelluloses and cellulose have been claimed.[18]

2.3. Intrinsic viscosity [η], DP and M_w

The viscosity-average DP (degrees of polymerization), P, of a cellulose sample is conveniently estimated from the intrinsic viscosity of its solution in 0.5 M cupric-ethylenediamine hydroxide by applying the equation $P^{0.90} = 1.65[\eta]/\text{mL g}^{-1}$. The molecular weight of the cellulose was estimated by multiplying by 162, the molar mass of 'anhydroglucose'. Table 3 gives the intrinsic viscosity (η), the viscosity-average DP (P) and molecular weight (M_w) of the eight cellulosic preparations.

Table 2. The content of neutral sugars (relative % dry 'cellulose' residues) and uronic acid (% dry residue) in the cellulosic preparations

Neutral sugar/uronic acids	Cellulosic preparation[a]							
	C_{1a}	C_{2a}	C_{3a}	C_{4a}	C_{1b}	C_{2b}	C_{3b}	C_{4b}
Arabinose	5.4	6.8	6.3	6.1	6.5	6.2	8.0	7.8
Rhamnose	1.8	1.1	1.2	0.9	0.5	0.4	0.3	0.3
Galactose	1.4	1.6	2.7	2.9	2.9	2.7	1.3	1.6
Glucose	71.6	69.6	67.8	66.7	69.7	68.6	63.9	61.7
Xylose	19.3	21.1	20.5	21.4	19.0	20.2	25.3	27.1
Mannose	0.5	0.8	1.5	2.0	1.4	1.8	1.1	1.5
Uronic acids	5.5	5.1	4.1	3.8	5.8	5.3	4.3	4.0

[a] Corresponding to the cellulosic preparations in Table 1.

Table 3. The intrinsic viscosity (η), viscosity-average DP (degree of polymerization) and molecular weight (M_w) of the isolated cellulosic preparations

	Cellulosic preparation[a]							
	C_{1a}	C_{2a}	C_{3a}	C_{4a}	C_{1b}	C_{2b}	C_{3b}	C_{4b}
Intrinsic viscosity (η, mL/g)[b]	293.0	324.9	341.5	361.0	275.1	296.8	306.2	335.3
P (Viscosity–average DP)[c]	934.9	1070.2	1131.0	1203.3	889.7	967.9	1002.0	1108.3
Molecular weight (M_w)[d]	151,450	173,370	183,220	194,930	144,130	156,800	162,320	179,540

[a] Corresponding to the cellulosic preparations in Table 1.
[b] Determined by British Standard Methods for determination of limiting viscosity number of cellulose in dilute solutions, Part 1. Cupri-ethylenediamine (CED) method.
[c] Calculated by $P^{0.9} = 1.65[\eta]$.
[d] Calculated by $P \times 162$.

Remarkably, as compared to the intrinsic viscosity (η, 488.7–505.2 mL/g) and the molecular weight (M_w, 272,740–282,870 g/mol) of the crude cellulose from wheat straw,[19] the intrinsic viscosity and molecular weight of the eight cellulosic samples obtained in this study from both untreated ryegrass leaves and the de-juiced leaves, were much lower (η, 275.1–361.0 mL/g; M_w, 144,130–194,930 g/mol). A decrease in alkali strength from NaOH to KOH or alkali concentration from 18% to 10%, together with an increasing extraction duration from 2 to 16 h resulted in an increase in M_w from 151,450 (C_{1a}) to 173,370 g/mol (C_{2a}) and 183,220 (C_{3a}) to 194,930 g/mol (C_{4a}) obtained from untreated ryegrass leaves. Similar increasing trends were observed between the cellulosic preparations isolated from the de-juiced leaves. This implies that a degradation of cellulose occurred during the treatment of delignified ryegrass leaves with a higher concentration of alkali such as 18% NaOH or 18% KOH, in particular, with a stronger alkali, for example, 18% NaOH. More importantly, as the data show in Table 3, the four cellulosic preparations obtained from untreated ryegrass leaves (M_w, 151,450–194,930 g/mol) had higher molecular weights than those of the corresponding four cellulosic samples obtained from the de-juiced leaves (M_w, 144,130–179,540 g/mol) under the same extraction conditions, suggesting that a degradation of macromolecular cellulose also occurred during the de-juicing process. This degradation was presumed due to the mechanical disruption of crystallinity by breaking hydrogen bonds in α-cellulose, opening up the structure and making more β-(1→4)-glycosidic bonds readily accessible to the alkali.

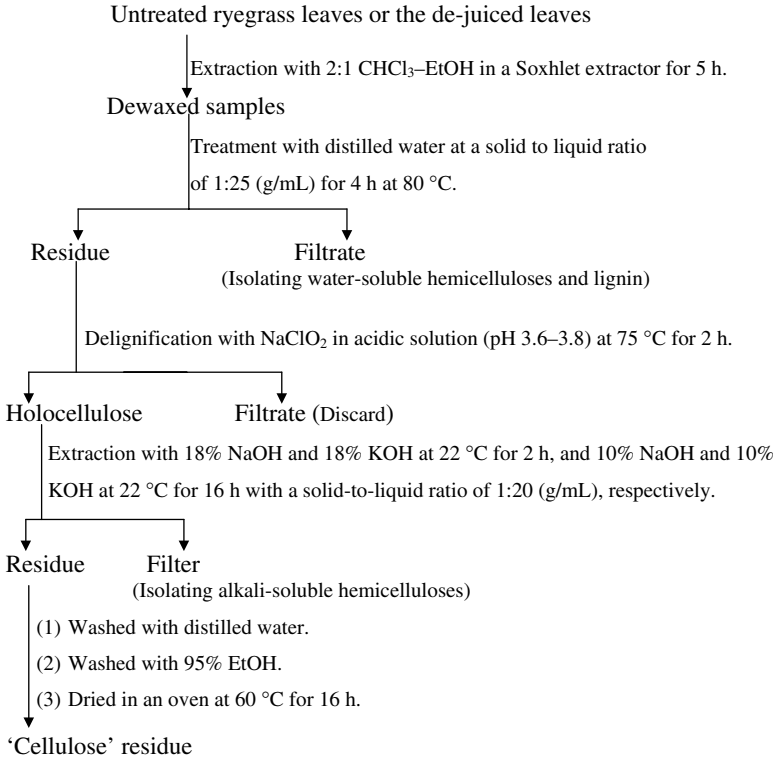

Figure 1. Scheme for isolation of cellulose from untreated ryegrass leaves and the de-juiced leaves.

2.4. FTIR spectra

Figure 2 shows the FTIR spectra of cellulosic preparations C_{1a} (spectrum a), C_{2a} (spectrum b), C_{3a} (spectrum c) and C_{4a} (spectrum d) obtained from untreated ryegrass leaves. The absorption at 3417 cm^{-1} relates to the stretching of H-bonded OH groups, and one at 2916 cm^{-1} to the C–H stretching.[20] The band at 1634 cm^{-1} is attributed to the bending mode of the absorbed water.[19] The band at 1460 cm^{-1} in spectrum a and 1433 cm^{-1} in spectra b–d is assigned to the symmetric CH_2 bending and the C–H bending occurs at 1378 cm^{-1}.[21] The absorption at 1208 cm^{-1} belongs to the C–O–H in-plane bending at C-6.[22] Two absorption bands at 1161 and 897 cm^{-1} arise from C–O–C stretching at the β-(1→4)-glycosidic linkages.[23] The in-plane ring stretching gives a shoulder at 1114 cm^{-1}. Strong peaks at 1064 and 1030 cm^{-1} are indicative of C–O stretching at C-3, C–C stretching and C–O stretching at C-6.[22] A small peak at 678 cm^{-1} corresponds to the out-of-plane bending of COH.[24] Similarly, the bands at 3413, 2924, 1429, 1378, 1168, 1106, 1064, 1030, 897 and 677 cm^{-1} in the spectra of cellulosic samples obtained from the de-juiced leaves (Fig. 3), are associated

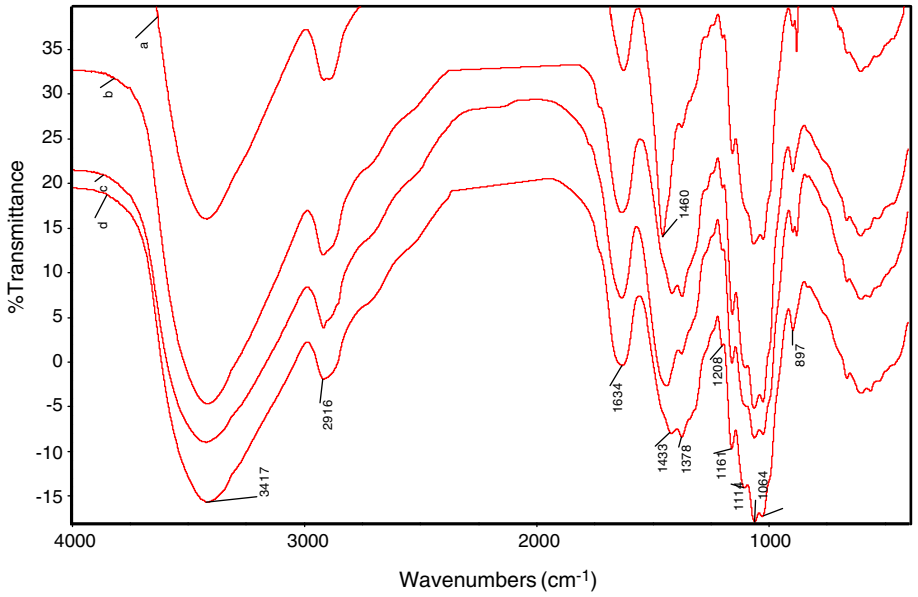

Figure 2. FTIR spectra of cellulosic preparations C_{1a} (spectrum a), C_{2a} (spectrum b), C_{3a} (spectrum c) and C_{4a} (spectrum d).

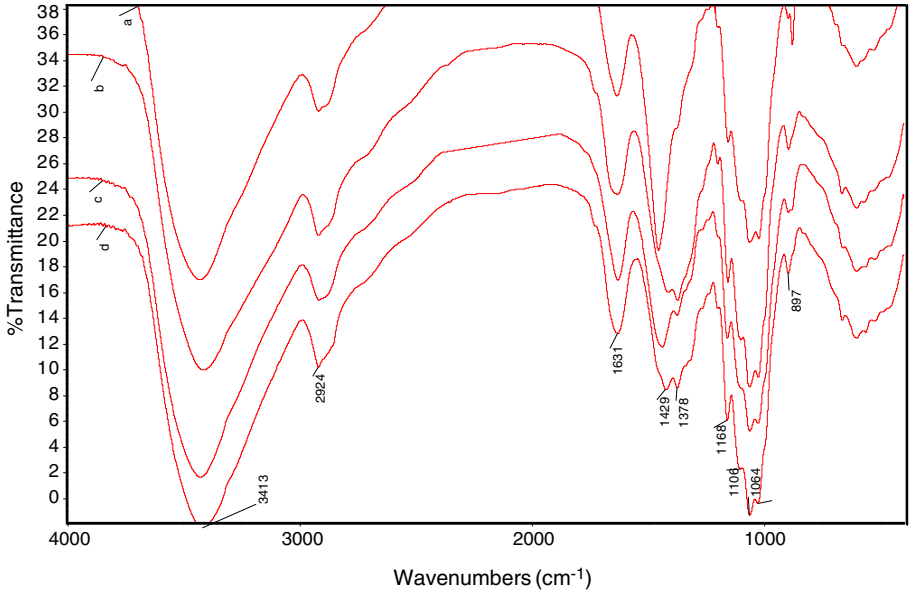

Figure 3. FTIR spectra of cellulosic preparations C_{1b} (spectrum a), C_{2b} (spectrum b), C_{3b} (spectrum c) and C_{4b} (spectrum d).

with the typical values of cellulose. Interestingly, the disappearance of an absorption band at 1520 cm^{-1} for aromatic ring vibrations in lignins revealed that all of the cellulosic preparations are free of bound lignin. This indicated that the first treatment with chlorite, followed by extraction with sodium or potassium hydroxide under the conditions given, completely removed the lignin molecules from the cell walls of both untreated ryegrass leaves and the de-juiced leaves.

2.5. CP/MAS ^{13}C NMR spectra

Figure 4 shows the CP/MAS ^{13}C NMR spectra of the cellulosic preparations C_{3a} (spectrum a) and C_{1b} (spectrum b). Even though CP/MAS NMR spectroscopy has been widely used to study cellulose structures, the underlying reasons for the shape of C-4 resonance (80–90 ppm) remain controversial. Two peaks are identified in most cellulose samples. The assignment of the peak between 85 and 90 ppm to the crystalline region is well accepted. The peak between 80 and 85 ppm is usually assigned to either to the crystal surface or the disordered component in cellulose, which may also comprise a mixture of the two,[25] since native cellulose always include some non-crystalline regions.[11,26] According to the literature,[27] the signals from 60 to 70 ppm are attributed to C-6, from 70 to 80 ppm to C-2, C-3 and C-5, from 80 to 90 ppm to C-4 and from 98 to 110 ppm to C-1. The signals of the xylans should be at 104 ppm (C-1), 82 ppm (C-4), 73–79 ppm (C-2, C-3) and 63 ppm (C-5), but these lines are overlapped by the strong cellulose signals.[28] The signal at 87 ppm originates from C-4 of the highly ordered cellulose of the crystallite interior, whereas the signal at 82 ppm is assigned to the C-4 of disordered cellulose. A similar trend can be seen in signals assigned to C-6 in crystalline cellulose (64 ppm) and on crystal surface or disordered cellulose (62 ppm), although these two signals are not as well resolved. Interestingly, a relatively lower intensity at 82 ppm for disordered cellulose in the spectrum of cellulosic sample C_{1b}, isolated with 18% NaOH for 2 h from the de-juiced leaves as compared to that of the cellulosic samples C_{3a}, which was extracted with 10% NaOH for 16 h from untreated ryegrass leaves, suggested that the cellulosic preparation C_{1b} had a somewhat higher degree of crystallinity than cellulosic sample C_{3a}, which paralleled their content of glucose, which corresponded to the purity of the cellulose. The reason for this higher content of glucose, which paralleled the higher degree of crystallinity, was that larger amounts of amorphous hemicelluloses were removed in the case of alkaline extraction, which increased the relative degree of crystallinity of the cellulose. In addition, the absence of the signals at 56 and 110–160 ppm relating to methoxyl and aromatic groups of lignin implied again that the cellulosic preparations were free of the associated lignin.

2.6. Crystallinity

Common methods for the characterization of crystalline cellulose structure are based on X-ray, FTIR and ^{13}C NMR spectroscopy.[23] Among them, wide-angle X-ray diffraction gives the most direct results such as the crystallinity. In this study, the sample crystallinity (g crystalline cellulose/100 g dry cellulose sample) is defined as the ratio of the amount of crystalline cellulose (cellulose Iβ) to the total amounts of sample material, including crystalline and amorphous cellulose, residual hemicelluloses and pectin, etc. In the case of cellulose, the crystallites are very small, typically 20–50 Å in diameter, which causes considerable peak broadening and serious peak overlap. On the other hand, X-ray diffraction is based on the width of peaks representing directions perpendicular to the fibre axis (indices: 1 1 0, 1 $\bar{1}$ 0 and 2 0 0), while the crystallite length can be determined based on the

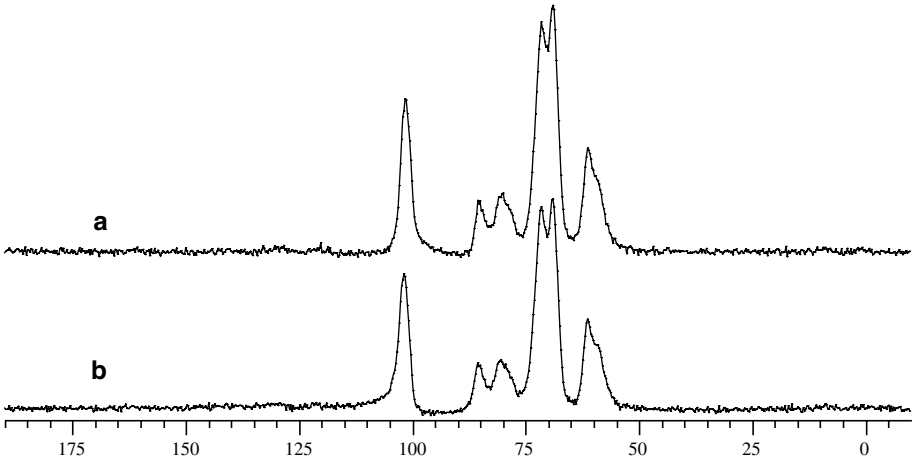

Figure 4. CP/MAS ^{13}C solid-state NMR spectra of C_{3a} (spectrum a) and C_{1b} (spectrum b).

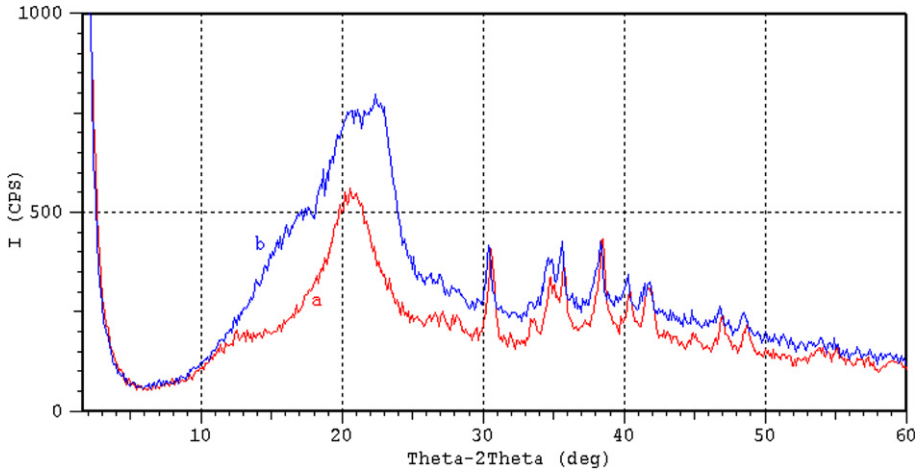

Figure 5. X-ray diffractogram of cellulosic preparations C_{1a} (a) and C_{3a} (b).

width of peaks representing directions parallel with the fibre axis (index: 004).[7] Figure 5 shows the wide-angle X-ray diffraction patterns for cellulosic preparations C_{1a} (a) and C_{3a} (b). The strongest peak, at $2\theta = 21.3$–$22.6°$ (curve a) and $2\theta = 21.3°$ (curve b), originates from the cellulose crystalline plane 002.[29] Clearly, the intensities of peaks from other crystal planes also decreased from the diffractograms a to b, indicating that the cellulosic preparation C_{1a}, isolated with 18% NaOH for 2 h, had a higher degree of crystallinity than that of the cellulosic sample C_{3a}, which was extracted with 10% NaOH for 16 h at 22 °C from untreated ryegrass leaves. The cellulosic preparations of C_{1a}, C_{2a}, C_{3a}, C_{4a}, C_{1b}, C_{2b}, C_{3b} and C_{4b}, were found to have 38.6%, 37.3%, 36.0%, 35.1%, 36.9%, 35.7%, 33.8% and 33.0% crystalline cellulose, which is lower than that of flax, cotton and kenaf that have crystallinities of about 70%, 65% and 60%, respectively.[30] In comparison, the cellulose crystallinity of wood-based materials was thereby found to be on the same level (60–70%), when determined by X-ray diffraction and by ^{13}C NMR spectroscopy, and the pulping process had only a slight effect on the cellulose crystallinity. These data indicate that the cellulose from both untreated ryegrass leaves and the de-juiced leaves have a much lower cellulose crystallinity (33.0–38.6%) or both de-juicing and alkaline extractions during the cellulose isolation decreased the cellulose crystallinity, compared to the other plant fibres, since the cellulose crystallinity is 90–100 g/100 g cellulose in plant-based fibres and 60–70 g/100 g cellulose in wood-based fibres.[30]

In addition, as expected, the degree of crystallinity of the cellulosic preparations parallels the content of glucose as shown in Table 2. Since peeling and degradation reactions are known in cellulose under alkaline treatment conditions, less ordered cellulose fractions and non-cellulosic polysaccharides such as hemicelluloses and pectins were removed by these reactions. Thus, the amounts of crystalline cellulose increased in the 'cellulose' residue samples. Furthermore, the crystallinity of cellulose in the samples isolated using 18% alkali for a shorter period (2 h) was higher than that of the other cellulosic preparations extracted with 10% alkali for a longer duration (16 h) under the same treatment temperature of 22 °C. This can be explained in that extraction with a higher concentration of alkali released more amorphous hemicelluloses; thus the crystallinity of cellulose in these samples was found to be higher. However, it should be noted that small amounts of cellulose were degraded in the hemicellulosic extraction step with 10% alkali for a longer period of 16 h, and therefore resulted in a lower yield of cellulose. At the same time, there was also the possibility that random cleavage of the cellulose occurred in the accessible chains within the crystalline domains. The chains in the outer portions of the crystallites could cleave randomly and protrude from the crystalline domain. The accessible chains could then be considered amorphous because they are part of the crystallite. A significant increase in total amorphous character of the cellulose would also decrease the relative degree of crystallinity.[11,12] More importantly, a relatively higher degree of crystallinity in the cellulosic preparations obtained from untreated ryegrass leaves than that of the corresponding cellulosic samples isolated from the de-juiced leaves, was probably due to the partial disruption of the hydrogen bonds during the de-juicing process by mechanical activities, which makes many more β-(1→4)-glycosidic bonds accessible to alkali reactants and thus decreases the cellulose crystallinity.

2.7. Fibre length

To date, there has been no comprehensive study of ryegrass cellulose quality traits such as fibre length, intrinsic viscosity and molecular size. In this study, we present a comparison of ryegrass fibre length with wood including

Table 4. Cellulose extraction conditions and length weighted average fibre length (mm)

Cellulose sample	Extractant	Temperature (°C)/times (h)	Dry material (g)/extractant (mL)	Mean fibre length
Grass leaves (C_{1a})	18% NaOH	22/2	3.0/60	0.44
Grass leaves (C_{1b})	18% NaOH	22/2	3.0/60	0.38
Wheat straw	18% NaOH	22/2	3.0/60	0.93
Barley straw	18% NaOH	22/2	3.0/60	0.95
Rye straw	18% NaOH	22/2	3.0/60	1.01
Rice straw	18% NaOH	22/2	3.0/60	0.77
Bagasse	18% NaOH	22/2	3.0/60	1.21
Spruce wood	18% NaOH	22/2	3.0/60	2.43
Birch wood	18% NaOH	22/2	3.0/60	1.82

hardwood and softwood, cereal straws and sugarcane bagasse and the values are given in Table 4. The results show that the fibre length of the eight cellulosic preparations ranged from 0.35 to 0.49 mm, indicating that the ryegrass leaves had much shorter fibres than the cereal straws (0.77–1.01 mm), bagasse (1.21 mm) and wood (1.82–2.43 mm) in the same isolating method. In addition, the fibre length of the cellulosic sample obtained from untreated ryegrass leaves (C_{1a}, 0.44 mm) was higher than that of the cellulosic preparation isolated from the de-juiced leaves (C_{1a}, 0.38 mm) under similar extraction conditions. These data suggest that some microfibres could be disrupted by the mechanical activities on the fibres during the de-juicing process. To further confirm this short fibre of the ryegrass leaves and to investigate the effect of extraction procedures on the fibre length, fibre extraction and removal of non-cellulosic components were carried out using the method of Franklin,[31] after suitable modification. Both untreated ryegrass leaves and the de-juiced leaves were treated in tubes containing 50:50 (v/v) hydrogen peroxide (25%) and glacial acetic acid for 6–12 h in a boiling water bath until they were pure white. The liquid was carefully decanted off, and excess acid was removed by five rinses with distilled water. The tubes were then shaken until a homogeneous fibre suspension formed. Fibre length was then measured using a Kajaani FS-200 instrument, which gave values of 0.47 mm for untreated ryegrass fibres and 0.41 mm for the de-juiced leaves. This implies that cellulosic extractions procedures have no significant influence on fibre length.

2.8. Thermal analysis

The thermogravimetric analysis (TGA) and differential thermal analysis (DTA) curves (Fig. 6) of cellulosic samples C_{2a} (curve a) and C_{4a} (curve b) showed an initial shoulder peak between 205 and 225 °C, which is due to a mass loss of approximately 10% for decomposing the residual hemicelluloses. The major second decomposition peak at about 225–310 °C relates to thermal depolymerization of residual hemicelluloses and cellulose (mass loss 36–40%). The third stage of weight loss ranging from 310 to 600 °C (mass loss 13–15%) is attributed to the further breakage of the cellulose and the inorganic compounds. As shown in Figure 6, the two cellulosic samples of C_{2a} and C_{4a} had a residual weight of 38 and 35 wt % at 600 °C. In an inert atmosphere, the end-products of the decomposition of cellulose are carbonaceous residues.[32] As inorganic compounds are required during periods of ryegrass growth, these compounds which are present in the samples show up as ash.[33]

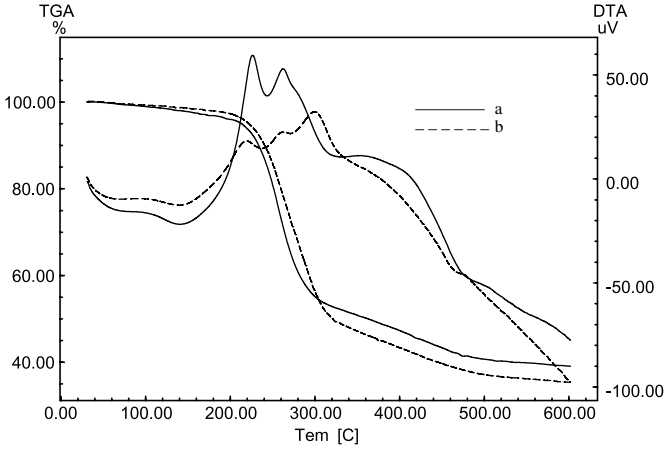

Figure 6. Thermogram of cellulosic preparations C_{2a} (a) and C_{4a} (b).

3. Conclusions

The yield of cellulose obtained from the de-juiced ryegrass leaves (32.8–36.8% dry wt) was higher than that of the cellulose isolated from untreated leaves (22.7–28.8% dry wt) under the same extraction conditions. However, cellulosic preparations obtained from untreated ryegrass leaves had a higher purity, intrinsic viscosity, molecular weight, crystallinity and fibre length. Mechanical de-juicing could disrupt some microfibres, which resulted in much shorter fibres, even though these were shorter than the fibres isolated from cereal straws, bagasse and wood using the same extraction methods. No significant influences of the solvent extraction procedures on the fibre length were found. The degree of crystallinity of the cellulosic preparations paralleled the purity of cellulose samples, glucose content and had a much lower cellulose crystallinity (33.0–38.6%), compared to the other plant fibres of wood, flax, cotton and kenaf.[30] In particular, a partial disruption of the hydrogen bonds may occur during the de-juicing process by mechanical activities, which made many more β-(1→4)-glycosidic bonds accessible to the alkali reactants and thus decreased cellulose crystallinity. More importantly, the decreased crystalline forms of the cellulose will be more accessible to enzymes and can, therefore, be more efficiently converted to monomeric sugars suitable for bioconversion to fuel ethanol.

4. Experimental

4.1. Materials

Perennial ryegrass leaves (*L. perenne*) used in this experiment, cut in mid-August 2005 at the age of 2 months, were kindly supplied by Biochem Wales Ltd., and were approximately 10 cm in height. The ryegrass leaves were de-juiced with a BC 45 twin screw extruder (Clextral) at room temperature. The juice was collected, and the refined ryegrass leaves were gathered at the barrel outlet. Both untreated ryegrass leaves and the de-juiced leaves were dried in an oven at 60 °C for 16 h and then ground by hand before use.

4.2. Isolation of cellulose

A scheme for isolation of cellulose from both untreated ryegrass leaves and the de-juiced leaves is shown in Figure 1. Both untreated ryegrass leaves and the de-juiced leaves were first dewaxed with 2:1 $CHCl_3$–EtOH in a Soxhlet extractor for 5 h. The wax-free samples (20 g) were then soaked in 500 mL of distilled water. The dispersions were stirred for 4 h at 80 °C to remove the water-soluble polysaccharides and lignin. The residue was then delignified with 6.3% sodium chlorite at pH 3.6–3.8, adjusted with 10% HOAc, at 75 °C for 2 h.[34] The hemicelluloses were removed from the two holocellulose samples, obtained from both untreated ryegrass leaves and the de-juiced leaves, with 18% NaOH and 18% KOH at 22 °C for 2 h, and 10% NaOH and 10% KOH at 22 °C for 16 h with a holocellulose to extractant ratio of 1:20 (g/mL), respectively. The residue was filtered off through a 45-μm nylon cloth and washed thoroughly with water and 95% EtOH until the filtrate was neutral. Finally, the cellulose preparations (residue) were dried in an oven at 60 °C for 16 h. Note that the 'cellulose' residue isolated from untreated ryegrass leaves with 18% NaOH and 18% KOH at 22 °C for 2 h, and 10% NaOH and 10% KOH at 22 °C for 16 h were labelled as cellulosic fractions C_{1a}, C_{2a}, C_{3a} and C_{4a}, respectively, while the residue after extraction with the corresponding alkali under the same conditions but from the de-juiced leaves were labelled as cellulosic preparations C_{1b}, C_{2b}, C_{3b} and C_{4b}, respectively. To reduce errors and confirm the results, each experiment was repeated in triplicate under the same conditions. Yields of the celluloses are given on a dry-weight basis relative to the starting untreated ryegrass leaves and the de-juiced leaves, respectively. The standard errors (SE) or standard deviations (SD) were observed to be lower than 6%.

4.3. Physicochemical characterization of cellulose

The monosaccharides in the cellulosic preparations were obtained by hydrolysis with 72% H_2SO_4 for 45 min at room temperature, followed by dilution to 1 M for 2.5 h at 100 °C. The neutral sugars liberated were determined by high-performance anion-exchange chromatography (HPAEC) with pulsed amperoteric detection using a Dionex GP50 gradient pump, an ED50 electrochemical detector, an AS50 autosampler and a Carbopac™ PA1 column. Samples injected into the system were eluted with 0.004 M NaOH (carbonate free and purged with helium) with post-column addition of 0.3 M NaOH at a rate of 1 mL/min. Run time was 45 min, followed by an 8-min elution with 0.5 M NaOH to wash the column and then a 15-min elution with 0.004 M NaOH to re-equilibrate the column. The analysis was quantified against two separate standard solutions using Chromeleon™ computer software. Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay.[35] The average degrees of polymerization (DP) and molecular weight of the cellulose preparations were determined by British Standard Methods for determination of limiting viscosity number of cellulose in dilute solutions, Part 1. Cupri-ethylenediamine (CED) method (BS 6306: Part 1: 1982). The viscosity-average DP (degree of polymerization) (P) of the cellulosic preparation was estimated from their intrinsic viscosity [η] in cupri-ethylenediamine hydroxide (cuene) solution using the following equation:[36]

$$P^{0.90} = 1.65[\eta]/\text{mL g}^{-1},$$

where P is an indeterminate average DP. The molecular weight of the cellulosic preparations was then calculated from their P by multiplying by 162, the molecular weight of an 'anhydroglucose' unit. The hydrolysis and analyses were conducted in duplicate, and the values of individual monosaccharide residues and the molecular weights were less than 5.5% and 6.3%, respectively.

FTIR spectra of the cellulosic samples were obtained on an FTIR spectrophotometer (Thermo Nicolet 510, USA) using a KBr disc containing 1% finely ground samples. They were recorded in the absorption mode in the range 4000–400 cm^{-1} with an accumulation of 32 scans and a resolution of 2 cm^{-1}. Cross polarization and magic-angle spinning ^{13}C solid-state NMR (CP/MAS) experiments were performed on a Bruker DRX-400 spectrometer at 75.5 MHz and 25 °C. The speed of rotation was 5 kHz, and the proton 90° pulse was 6 μs. The delay time after the acquisition of the signal was 2 s.

Crystalline structures of the cellulose samples (listed in Table 1) were analyzed by wide-angle X-ray diffraction on an XRD-6000 instrument (Shimidzu, Japan) with 5°/min scan speed. The cellulose preparations were laid on a glass sample holder and analyzed under plateau conditions. Ni-filtered Cu Kα radiation ($\chi = 1.54$ Å) was generated at a voltage of 40 kV and current of 40 mA was used. The X-ray diffractograms were recorded from 5° to 60° 2θ (Brangg angle) by a goniometer at a scanning speed of 0.02°/s. To determine the % crystallinity, the total diffracted area and the area under the crystalline peaks were determined by integration after correcting the data for absorption. The ratio of the crystalline area to that of the total diffracted area is taken as the % crystallinity.[30] Each sample was prepared and measured twice. The fibre sheets were dried at 105 °C after the X-ray diffraction experiments in order to determine the sample crystallinity based on the dry matter content.

Length-weighted average fibre length of the cellulosic samples was measured using a Kajaani FS-200 instrument. For comparison purposes, cereal straws of wheat, barley, rye and rice, obtained on the farm of The North-Western University of Agricultural and Forest Sciences and Technology (Yangling, China), sugarcane bagasse, collected from Guanzhou Sugar Mill (Guanzhou, China), and 8-year aged wood samples of spruce (softwood) and birch (hardwood), a gift from Mr. Gwynn Lloyd Jones, University of Wales (Bangor, UK) were also treated similarly by the same method as for ryegrass leaves with little modification. The chipped wood samples, straws and bagasse were ground to pass a 3.0-mm mesh screen. The dried powder was then dewaxed and delignified under the same conditions used for ryegrass leaves. The cellulose was then isolated from the holocellulose with 18% NaOH at 22 °C for 2 h.

Thermal analysis of the cellulosic samples was performed using thermogravimetric analysis (TGA) and differential thermal analysis (DTA) on a simultaneous thermal analyzer (SDT Q600, TA Instrument). The apparatus was continually flushed with nitrogen. The sample weighed between 9 and 12 mg and was heated from room temperature to 600 °C at a rate of 10 °C/min.

Acknowledgements

The authors wish to express their gratitude for the financial support from the Welsh Development Agency, National Natural Science Foundation of China (No. 30430550), Guangdong Natural Science Foundation (No. 05103548), and Ministry of Education, China.

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