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Isolation of structurally distinct lignin–carbohydrate fractions from maize stem by sequential alkaline extractions and endoglucanase treatment

Mika Henrikki Sipponen [a,b,*], Catherine Lapierre [b], Valérie Méchin [b], Stéphanie Baumberger [b]

[a] Aalto University, School of Chemical Technology, Department of Biotechnology and Chemical Technology, Espoo, Finland
[b] Institut Jean-Pierre Bourgin, UMR 1318 INRA/AgroParisTech, Pôle SCSM, 78000 Versailles, France

HIGHLIGHTS

- Maize stem was treated with alkali before and after endoglucanase hydrolysis.
- Two soluble lignin–carbohydrate (LC) fractions were quantitatively recovered.
- LC1 and LC2 contained 39% and 8% of total lignin and 24% and 2.1% of ferulic acid.
- Thioacidolysis, acid/alkaline hydrolysis, FTIR were used to study each fraction.
- The procedure revealed important heterogeneity regarding structure of grass lignins.

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ABSTRACT

Sequential fractionation of extractive-free maize stems was carried out using two mild alkaline extractions (0.5 and 2 M NaOH, 20 °C, 24 h) before and after endoglucanase treatment. This procedure provided two lignin–carbohydrate fractions (LC1 and LC2) recovered after each alkali treatment. LC1 and LC2 contained 39% and 8% of the total lignin amount, respectively. These two fractions contained structurally distinct lignin molecules. While the content of resistant interunit bonds in lignin was 77% in LC1, it was increased up to 98% in LC2. Not unexpectedly, both alkali-soluble fractions contained substantial amount of *p*-coumaric and ferulic acids ether-linked to lignins. These results outline heterogeneity of maize stem lignins related to fractionation of grass materials.

1. Introduction

Materials and energy based on renewable resources are sought to gradually replace those obtained from fossil sources and relying largely on crude oil refining. This change is driven both in the EU and in the US by socio-economic factors (Domac et al., 2005) as well as policy decisions (Charles et al., 2007) to increase the proportion of renewable components in transportation and energy sectors. Grasses represent a substantial reservoir of renewable lignocellulosic biomass that may be sustainably collected together with other agricultural residues as feedstock for so-called biorefineries with the scope of complete utilization of the cell wall components including lignins and hemicelluloses.

Grass lignins are made of syringyl (S) and guaiacyl (G) units together with lower amounts of *p*-hydroxyphenyl (H) units. These units are linked by labile β-O-4 bonds as well as by resistant interunit bonds, referred to as condensed bonds (Adler, 1977). Compared to non-grass cell walls, the organization of grass cell walls is more complex due to presence of ferulic acid (FA) that cross-links arabinoxylans (AX) and lignins (Grabber et al., 2000). Two other structural specificities of grass lignins are their acylation by *p*-coumaric acid (CA), mainly on S lignin units as reviewed in Ralph (2010) and high content of free phenolic groups as reviewed by Lapierre (2010). The latter structural trait makes half of total grass lignins easily soluble in alkali at room temperature (Lapierre et al., 1989), a property that is not shared by wood lignins (Beckman et al., 1923).

* Corresponding author at: Aalto University, School of Chemical Technology, Department of Biotechnology and Chemical Technology, P.O. Box 16100, FI-00076 Aalto, Espoo, Finland. Tel.: +358 947022547; fax: +358 9462373.
E-mail address: mika.sipponen@aalto.fi (M.H. Sipponen).

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Maize (*Zea mays* L.) is a major crop that has frequently served as a model for fractionation and pretreatment of agricultural residues (corn stover and straw). Lignins make approximately one-fifth of mature maize stems and should be addressed as valuable components rather than as low-value byproducts. Therefore, isolation of lignins should be carefully considered in the fractionation processes of lignocellulose biorefineries as lignin polymers isolated by mild procedures may be better suited for value-added applications such as enhancers of hydrophobic properties of renewable fibers (Sipponen et al., 2010). Isolation of carbohydrate-free technical lignins without severe structural changes and/or costly purification schemes remains a challenge as native lignins are covalently linked to hemicelluloses (Gellerstedt and Henriksson, 2008). These covalent bonds are formed in the process of lignin polymerization when quinone methide intermediates spontaneously react with nucleophilic groups such as the carboxylic groups of uronic acids, the alcoholic groups of sugars or their hemiketal groups. Such reactions lead to ester, ether or glycosidic bonds between sugars and lignin units (Fengel and Wegener, 1983). Therefore, rather than attempting to isolate carbohydrate-free lignins, an alternative approach would be fractionating grass cell walls under mild conditions, which may allow co-solubilization of lignins together with carbohydrate and recovery of these lignin–carbohydrate fractions (LC) in which the structure of native lignins may be better retained.

For many decades, mild alkaline treatment of straw has been used to increase its feeding value (Jackson, 1977). Alkaline pretreatments seem also well suited to improve saccharification of grass materials in biotechnological conversion processes (Li et al., 2012). Further, endoglucanases (EGs) (EC 3.2.1.4) cleave specifically cellulose, and may thus allow isolation of a portion of lignins otherwise structurally restricted from dissolution in alkali. Previously, endoglucanase hydrolysis of cellulose has been reported prior to isolation of Lignin–Carbohydrate Complexes (LCCs) from wood and pulp materials (Lawoko et al., 2006). Furthermore, EG treatment in series with alkaline extraction has been described for upgrading cellulose pulp into viscose (Ibarra et al., 2010). However, studies on utilization of EGs as tools in bioconversion of grass materials are scarce. In this study, a sequential alkaline extraction of maize stem material with the aid of EG treatment is presented in order to isolate, under mild conditions, lignin–hemicellulose fractions for structural analysis. Evidence is provided that two alkali-soluble LC fractions with quite distinct structural properties can be isolated from extractive-free and mature maize stems.

2. Methods

Maize F2 (INRA) line was grown on experimental field conditions in Lusignan (France), harvested at silage stage, and stems without leaves ground to pass a 1 mm-screen exhaustively extracted by water and then 96% ethanol in a Soxhlet apparatus. The extractive-free (EFR) maize stem (MS) was recovered with a 54% yield (weight percentage on a dry matter basis). The endo-1,4-β-D-glucanase Novozym 476 was obtained from Novozymes (Denmark). This commercial preparation contained 6.7 mg mL^{-1} protein, assayed using the method of Bradford (1976).

2.1. Determination of enzyme activities

Endoglucanase activity was determined according to Ghose (1987) using 1.5% (w/v) carboxymethyl cellulose (CMC) Na-salt, low viscosity (Sigma) as substrate. The assay for endoxylanase activity was adapted from Nakamura et al. (1993) using 1.5% (w/v) beechwood xylan (Sigma–Aldrich) or larchwood xylan (Ega-Chemie) suspension as substrate in 50 mM Na-citrate buffer (pH 4.8) at 50 °C for 10 min. The amount of reducing sugars formed in the endoxylanase and endoglucanase assays were determined by the DNSA method (Miller, 1959) using xylose and glucose standards, respectively. One international unit of activity (IU) refers to the amount of the enzyme that releases 1 μmol of xylose or glucose in 1 min under the assay conditions.

2.2. Isolation of alkali-soluble LCs

The LCs were isolated according to the scheme in Fig. 1. Maize EFR (17.5 g) was extracted with 360 mL of 0.5 M aqueous NaOH under N_2 in a glass bottle at 20 °C for 24 h. The solid residue was separated from the alkaline extract by centrifugation, washed repeatedly with deionized water until neutral pH and lyophilized giving residue R1. The wash-supernatants were combined with the primary alkaline extract, and the solution was clarified by filtration (Whatman No. 4) before adjustment to pH 2 by 6 M HCl. The suspension was kept at +4 °C in dark for 16 h before the precipitate was separated by centrifugation, suspension washed three times with deionized water acidified by HCl (pH 2), and lyophilized giving LC1. R1 (2% suspension w/v) was hydrolyzed with 52.8 IU g^{-1} (activity measured on CMC) of endo-1,4-β-D-glucanase preparation "Novozym 476" (Novozymes, Denmark) in 0.1 M Na-acetate buffer (pH 5) for 90 h at 45 °C. Sodium azide (0.025% w/v) was used to prevent microbial contamination. The hydrolyzed solids were separated by centrifugation, washed copiously with deionized water, and lyophilized giving residue R2. Supernatant of the enzymatic hydrolysate was analyzed for its monosaccharide content before and after hydrolysis in 4% (w/w) H_2SO_4 at 121 °C for 1 h. R2 was extracted with 2 M NaOH (4% w/v suspension) for 24 h at 20 °C under N_2, and LC2 as well as residue R3 recovered as described above for the first alkaline extraction. Laboratory grinder (IKA) equipped with a blunt-edged blade was used to disintegrate the compact freeze-dried residues. For calculation of mass balances, the lyophilisates were weighed immediately after freeze-drying and were considered as moisture-free. Dry matter contents of the lignocellulosic fractions were determined gravimetrically in parallel with each extraction step by drying separate samples overnight at 105 °C.

2.3. Analytical procedures

2.3.1. Lignin determination and carbohydrate analysis

Lignin content was determined gravimetrically from the maize EFR and from the corresponding LC and residue samples according to the Klason protocol adapted from Dence (1992). Structural carbohydrates in residue samples were analyzed based on published

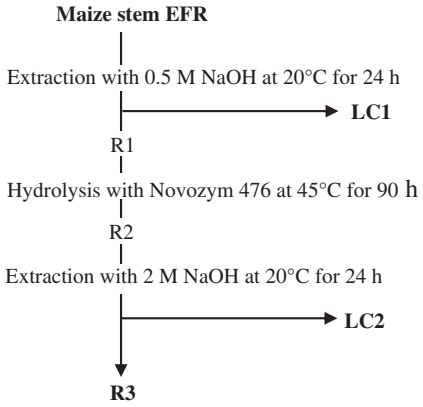

Fig. 1. Scheme for fractionation of maize stem EFR into lignin–carbohydrate fractions LC1, LC2 and alkali-insoluble residues R1–R3.