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Technical Note

High-throughput determination of thioglycolic acid lignin from rice

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Abstract For research and development of plants suitable for forage and biofuel production, the development of a high-throughput system of lignin determination, especially for herbaceous species, is important. However, currently available methods for lignin determination are not suitable for high-throughput analysis of herbaceous samples. In this paper, we describe a straightforward, high-throughput method for lignin determination as thioglycolic acid lignin from rice straw using disposable plastic microtubes, TissueLyser, and a UV microplate reader. We successfully plotted calibration curves derived from the readings of a spectrophotometer and a microplate reader using thioglycolic acid lignin prepared from bamboo milled wood lignin as a lignin standard. Based on the calibration curve, we could determine the lignin content in various organs from dried rice straw and rice seedlings. We confirmed that 20 mg of dried material from rice plants is adequate for stable determination of the lignin content. Using this method, 100 independent samples from rice straw can be analyzed in three days per person.

Key words: High-throughput, lignin, thioglycolic acid, rice.

Lignin is one of the most abundant natural polymers produced by plants. Lignin is a complex polymer formed by the oxidative radical coupling of 4-hydroxycinnamyl alcohols (monolignols), although there are many examples showing that other phenolics besides monolignols can be incorporated into lignin (Sarkanen and Hergert 1971; Lu and Ralph 1999; Ralph et al. 2008).

Currently available methods for determining lignin content have their origins in wood chemistry (Hatfield and Fukushima 2005). Therefore, the original methods have been optimized for analyzing the secondary xylem of trees and are generally unsuitable for herbaceous samples. Recently, lignin determination for various samples other than wood has attracted much attention to aid molecular breeding of plants suitable for forage and biofuel production. In addition, a straightforward, high-throughput method is generally desired because, in most cases, many transgenic and/or mutant lines produced by molecular breeding must be evaluated (Yamada et al. 2006). Although the original methods for determining lignin of woody species have been modified for herbaceous samples in many reports (Hatfield and Fukushima 2005), the techniques are low-throughput and employ complicated experimental steps.

Here we report a straightforward, high-throughput method for lignin determination in rice straw as thioglycolic acid lignin (TGAL). Using this method, we measured lignin content in various organs from rice and confirmed the utility of the technique.

Rice (*Oryza sativa* cv. Nipponbare) straw, grown in a paddy field in Azuchi Town, Shiga, Japan, were gifted by the Shiga Prefecture Agricultural Technology Promotion Center. Three independent rice straws were separated into seven parts (leaf blade, leaf sheath, culm, panicle, rachis, hull, and seed without hull). In addition, rice cv. Nipponbare seedlings were grown in a growth chamber under a long-day photoperiod (16 h light, 8 h dark) at 25°C. The aerial parts of three seedlings were harvested when the leaf (leaf blade and sheath) length was 15, 20, 25, and 30 cm. The harvested samples were dried at 60°C for 16 h.

Each sample was first chopped with scissors into about 5–10 mm long and transferred to a stainless steel grinding jar with grinding balls (Qiagen). The sample was pulverized with a TissueLyser (Qiagen) for 2.5 min

Abbreviations: TGAL, thioglycolic acid lignin
This article can be found at http://www.jspcmb.jp/

at 26 Hz at room temperature (RT). The powdered samples (each ca. 20 mg) were transferred to dried 2 ml polypropylene microcentrifuge tubes with screw caps (Sarstedt), and the tubes were dried in an oven at 60°C for 1 h. After cooling and weighing, the samples were extracted once with 2 ml water, centrifuged for 10 min at 16,100 *g* at RT, and the supernatant was discarded. Next, the pellet was extracted with 1.8 ml methanol at 60°C for 20 min and centrifuged at 16,100 *g* for 10 min at RT. The supernatant was discarded and the methanol extraction was repeated. The pellet was dried *in vacuo* and weighed, and 1 ml 3 N HCl and 0.1 ml thioglycolic acid (Nacalai Tesque) were added. The screw caps were tightly closed and the samples were heated at 80°C for 3 h. After centrifugation at 16,100 *g* for 10 min at RT, the supernatant was removed and the pellet was vortexed for 30 s in 1 ml distilled water. After centrifugation at 16,100 *g* for 10 min at RT, the supernatant was discarded and the pellet was resuspended in 1 ml 1 N NaOH then shaken vertically at 80 rpm for 16 h. The tubes were centrifuged at 16,100 *g* for 10 min at RT, and the supernatant (1 ml) was transferred to fresh 1.5 ml tubes and acidified with 0.2 ml concentrated HCl. After chilling the tubes at 4°C for 4 h, the tubes were centrifuged at 16,100 *g* for 10 min at RT. The supernatant was removed and pellet was dissolved in 1 ml 1 N NaOH. After fifty-fold dilution with 1 N NaOH, the solution was submitted to spectrophotometric measurement.

Ultraviolet (UV) spectra of a 1 N NaOH solution of TGAL prepared from bamboo (*Phyllostachys heterocycla*) milled wood lignin (Nakatsubo et al. 1972; Nakamura and Higuchi 1978) and from rice were recorded in a 10 mm quartz cell with a UV-1600PC spectrophotometer (Shimadzu). For plotting calibration curves, 1–5 mg dried bamboo milled wood lignin was weighed and the TGAL solution was prepared as above. The solution was diluted 50-fold with 1 N NaOH and absorbance at 280 nm was recorded in a 10 mm quartz cell with a UV-1600PC spectrophotometer and in a UV-Star 96-well plastic microplate (Greiner) containing 200 μl of the diluted TGAL solution in each well on a SH1000Lab microplate reader (Corona Electronics). Lignin concentration was determined by dividing the amount of lignin calculated based on the calibration curve by the dry weight of the sample after the methanol extraction.

Figure 1 shows UV spectra of the 1 N NaOH solution of TGAL prepared from bamboo milled wood lignin and from rice straw. The spectra were similar and a characteristic local maximum at about 280 nm in both bamboo and rice TGAL was observed. Therefore, we used bamboo TGAL prepared from bamboo milled wood lignin as a lignin standard.

Figure 2 shows a calibration plot based on the absorbance at 280 nm of the 50-fold diluted TGAL solution prepared from bamboo milled wood lignin recorded in a 10 mm cell and a UV-Star 96-well plastic microplate. The equation was $y=127.12x$ and $y=233.42x$, respectively, where x is the absorbance at 280 nm and y is the concentration of bamboo TGAL. The absorbance at 280 nm of the same samples was lower than that recorded on a microplate reader, because the light path length is shorter in a microplate. R^2 values for a 10 mm cell and a UV-Star 96-well plastic microplate were 0.9754 and 0.9717, which indicates good linearity.

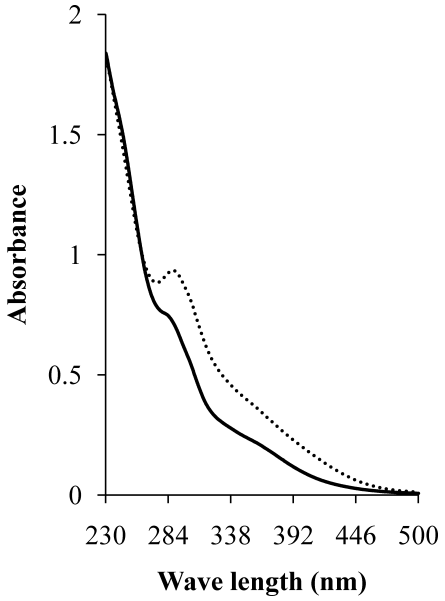

Figure 1. Ultraviolet spectra of thioglycolic acid lignin prepared from bamboo (solid line) and Nipponbare rice straw (dotted line) measured with a UV-1600PC spectrophotometer (Shimadzu).

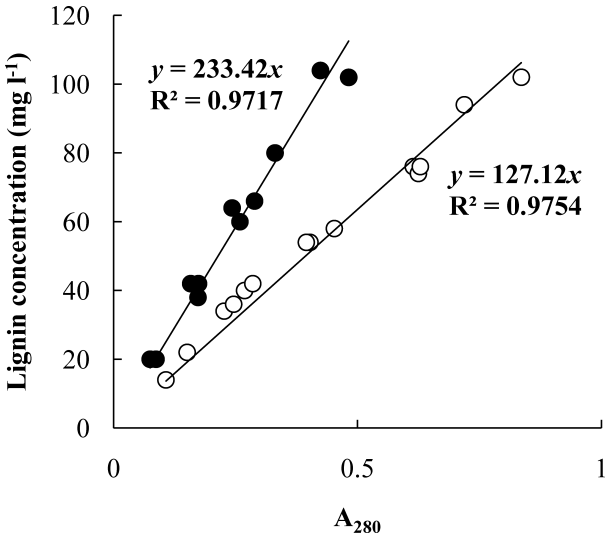

Figure 2. Calibration curves derived by using bamboo milled wood lignin as a standard. Absorbance was measured at 280 nm (A_{280}) in a 10 mm quartz cell with a UV-1600PC spectrophotometer (Shimadzu; open circle) or in a 96-well plastic microplate with a SH1000Lab microplate reader (Corona Electronics; solid circle).

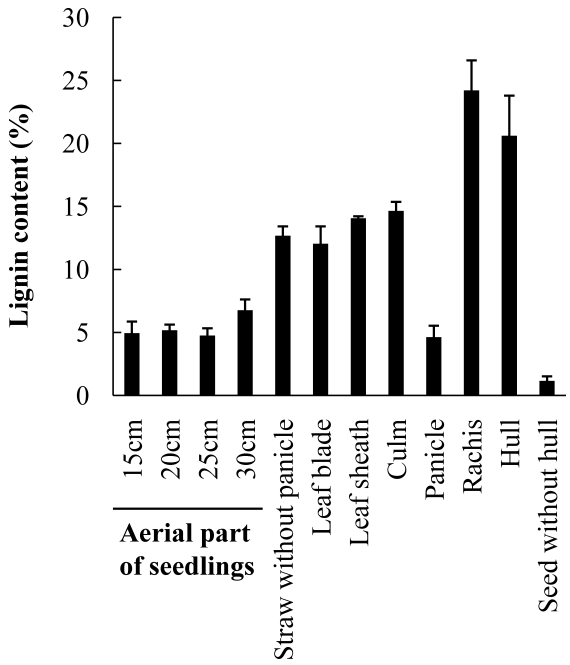

Figure 3. Percentage of lignin content in various organs from dried rice straw and rice seedlings. Bars represent the mean of triplicate measurements. Error bars represent the SD.

We next calculated the lignin content of several organ samples from dried rice straw and rice seedlings based on the calibration curves. In the rice straw, the lignin content was highest in the rachis (24.2±2.4%) and lowest in the seed without hull (1.15±0.35%) (Figure 3). The total lignin content in rice straw without panicle was 12.7±0.74%. The low lignin content in the panicle (4.62±0.9%) and the seed without hull may be ascribed to the large amount of starch and proteins present in seeds. The lignin content of the hull is high (20.6±3.1%), but the amount was smaller than that reported in Zhang et al. (2006), probably because silica contaminated the acid-insoluble fraction of Klason lignin prepared by Zhang et al. The aerial parts of seedlings with leaves 15, 20, and 25 cm long contained only about 5% lignin and the lignin content slightly increased to 6.7% in 30-cm-long leaves (Figure 3). These results are consistent with the finding that lignin content does not increase in the leaf and stem of immature oat and wheat plants and lignin starts to increase as the plants approach maturity (Sarkanen and Hergert 1971).

Several methods for determining lignin content of herbaceous samples have been reported (Hatfield and Fukushima 2005). For example, in the Klason method, samples are treated with 72% sulfuric acid at RT and subsequently hydrolyzed in 3% sulfuric acid at 100°C. Total lignin was calculated from the weight of the undissolved residue in the reaction mixture as acid-insoluble lignin and the absorbance at 280 nm of the solution as acid-soluble lignin. However, the Klason method requires relatively large samples (>200 mg) and is not suitable for herbaceous samples containing proteins and polyphenols, which result in overestimation of total lignin (Lai and Sarkanen 1971).

The acetyl bromide method developed by Johnson et al. (1961) is another lignin determination method frequently used. Plant material (5–35 mg; Goldschmid 1971) is treated with 25% acetyl bromide in acetic acid. Although the required sample amount is less than that required by the Klason method, the reaction is still harsh and needs large reaction vessels (>10 ml) made of glassware.

In contrast, the TGAL method can be processed under milder conditions (dilute HCl containing thioglycolate) in disposable plastic microcentrifuge tubes (Campbell and Ellis 1992). The TGAL method is suitable for herbaceous samples that contain large amounts of polyphenols and proteins because of the rarity of these contaminants. The IR spectrum of TGAL indicates the absence of condensation reactions (Lai and Sarkanen 1971). Furthermore, determination of lignin contents can be obtained from a small sample (10–15 mg; Hatfield and Fukushima 2006).

Although the lignin content can be compared successfully among rice samples in this study, *p*-coumaric and ferulic acids, which comprise about a few percent of cell wall in Graminaceae plants and covalently bind to lignin through ester and ether linkages (Iiyama et al. 1994), may not be included in the lignin content calculated by the TGAL method. It was reported that the reaction at reflux temperature for 2 h in a dioxane and 2 N HCl mixture (9 : 1) and the alkaline treatment with 1 N NaOH at R.T. result in the hydrolysis of ether and ester linkage of *p*-coumaric and ferulic acids in rice lignin (Sun et al. 2001). Therefore, the TGAL method including the TGAL preparation by 3 N HCl and thioglycolic acid and the TGAL extraction by 1 N NaOH presumably hydrolyzes the ester- and ether-linkages with hydroxycinnamic acids. The quantitative determination of hydroxycinnamic acids released by the TGAL method may be assessed by another way (Sun et al. 2001).

In conclusion, we confirmed that 20 mg of dried material from rice plants is adequate for stable determination of the lignin content. Coupled with a TissueLyser and a UV microplate reader, we could analyze 100 samples in 3 days per person, which is ten-fold faster than the method reported by Campbell and Ellis (1992). Our modified TGAL method is applicable to samples from herbaceous plants other than rice by using the appropriate lignin standard for calibration.

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