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Note

Microscale thioacidolysis method for the rapid analysis of *β-O*-4 substructures in lignin

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Abstract Thioacidolysis is a method to detect the *β-O*-4 substructures of lignin, and has been employed as a diagnostic test for the presence of lignin. However, the conventional thioacidolysis protocol is low-throughput and is a bottleneck in the characterization of lignins in a large number of samples such as transgenic lines. Recently, a rapid analysis protocol for thioacidolysis was reported. In this study, we modified mainly the work-up process. Our microscale protocol showed higher yields of thioacidolysis products than the conventional protocol.

Key words: High-throughput, lignin, microscale, thioacidolysis, *β-O*-4 substructure

Lignin, which is a major component of the secondary cell wall of vascular plants, is a complex phenylpropanoid polymer and is biosynthesized via the cinnamate/monolignol pathway (Umezawa 2010). Lignins are generally classified into three major groups: guaiacyl (4-hydroxy-3-methoxyphenyl), syringyl (3,5-dimethoxy-4-hydroxyphenyl), and *p*-hydroxyphenyl lignins. Gymnosperm lignin is mainly composed of guaiacyl unit, while angiosperm lignin is composed of guaiacyl and syringyl units. Grass lignins comprise guaiacyl, syringyl, and *p*-hydroxyphenyl units. These lignins fill the spaces between cell wall polysaccharides and confer mechanical strength and imperviousness to the cell wall (Vanholme et al. 2010), while these characteristics of lignins are obstacles for chemical pulping, forage digestion and biofuel production (Vanholme et al. 2008; Wang et al. 2011).

During the last two decades, there has been intense interest in the metabolic engineering of lignin biosynthesis to develop plant materials, which suit the purpose, as well as the elucidation of the functions of the genes involved in lignin biosynthesis (Chiang 2006; Vanholme et al. 2008, 2010; Weng et al. 2008). In each transgenic study, a large number of transgenic plant lines are produced. However, conventional methods for lignin analysis are time-consuming and bottlenecks in characterizing lignins of a large number of transgenic plants. Hence, the establishment of high-throughput analytical methods was highly required.

To characterize lignins, their quantitation and structural analyses are necessary. Recently, we reported a high-throughput thioglycolic acid protocol for measurement of lignin content (Suzuki et al. 2009). We also developed a high-throughput protocol for the basic analysis of lignin aromatic components, the alkaline nitrobenzene oxidation method (Yamamura et al. 2010, 2011). In addition, high-throughput protocols are required for the methods providing degradation products derived specifically from *β-O*-4 lignin substructures, the most characteristic ones in lignin. The methods, which have been viewed as a diagnostic test for the presence of lignins, include acidolysis (Lundquist 1992), thioacidolysis (Lapierre and Monties 1986; Rolando et al. 1992), DFRC (Degradation Followed by Reductive Cleavage) (Lu and Ralph 1997a,b), and TIZ (Tosylation, Iodination, and Zinc-metal treatment) (Katahira et al. 2003) methods.

Among them thioacidolysis is being used most frequently. The method employs an acid-catalyzed reaction, which results in the depolymerization of lignins. The detection of phenyltrithioethylpropane compounds (Figure 1) from the thioacidolysis of plant cell wall material provides unambiguous evidence for the occurrence of *β-O*-4 substructures. Recently, a rapid protocol for the method was reported by Robinson and Mansfield (2009). The protocol was improved in terms of

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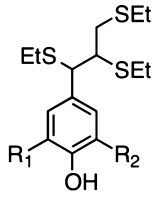
volume scaling, processing vessel, and sample handling, resulting in increase of throughput. Here, we report another microscale protocol of thioacidolysis, mainly improved in the work-up process.

In this study, *Oryza sativa* cv. Nipponbare leaf sheaths and *Acacia mangium* heartwood were used as plant materials. *A. mangium* heartwood meal was a gift from Koshii Wood Industry Co., Ltd. *O. sativa* was grown as follows. Four-week-old seedlings, which were hydroponically-cultivated, were transplanted into plastic pots (1/10000a Wagner's pot ICW-2; ICM, Ibaraki, Japan) containing 1.0 kg of soil (Bonsol No.2 : vermiculite = 2 : 1, v/v) with nitrogen-phosphorus-potassium fertilizer (8%, w/w), and were grown in a greenhouse under natural light conditions. After grain ripening, the plants were harvested and air-dried for 1 month. Leaf sheaths were chopped with scissors into ca. 5 mm long pieces, and were stored in a dry box at room temperature (r.t.) until use.

The sample was pulverized by a TissueLyser with stainless steel grinding jars with balls (Qiagen GmbH, Hilden, Germany) for 3 min at 25 Hz at r.t. The powder obtained was extracted twenty times with methanol at 60°C. Then the powders were further extracted five times with hexane at r.t., and five times with distilled water at 60°C, and then freeze dried. The freeze-dried powder thus obtained, which is referred to as the extract-free sample, was subjected to thioacidolysis by both conventional (Matsui et al. 1994) and microscale protocols (Table 1).



R_1, R_2=H : *p*-Hydroxyphenyltrithioethylpropane
R_1=OMe, R_2=H : Guaiacyltrithioethylpropane
R_1, R_2=OMe : Syringyltrithioethylpropane

Figure 1. Thioacidolysis products.

The conventional protocol (Matsui et al. 1994) was carried out as follows. Briefly, 10 mg of the extract-free sample was placed into a 23-ml glass test tube (catalog number 71-063-012, Asahi Glass Co., Ltd., Kanagawa, Japan). Three milliliters of dioxane/ethanethiol (9 : 1, v/v) containing 92 mM BF_3 etherate (referred to as thioacidolysis solution) and 12 μl of docosane (10 mg ml^{-1} in methanol, internal standard) were added to the tube, and the tube was tightly screw-capped. The reaction mixture in the tube was heated in an oil bath at 100°C for 4 h, then cooled on ice. The reaction was stopped by adding 5 ml of 0.4 N $NaHCO_3$. Next, the solution was adjusted to pH 3–4 by adding 6 N HCl and extracted three times with diethyl ether. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Ten microliters of the solution was dried, and the resulting residue was dissolved in *N*,*O*-bis(trimethylsilyl)-acetamide (BSA) (8 μl). After standing at 60°C for 45 min, an aliquot (0.8 μl) of the solution was subjected to gas chromatography-mass spectrometry (GC-MS) analysis as previously reported (Nakatsubo et al. 2008) using a Shimadzu QP-5050A GC-MS system (Shimadzu Co., Ltd., Kyoto, Japan) with the following condition: Shimadzu HiCap CBP10-M25-025 column (25 m × 0.22 mm); carrier gas, helium; injection temperature, 230°C; oven temperature, 40°C at t = 0 to 2 min, then to 230°C at 40°C min^{-1}; ionization, electron-impact mode (70 eV).

The microscale protocol was carried out by employing disposable 1.5-ml microcentrifuge tubes, a vortex, and a mini centrifuge for the extraction process as follows. Five milligrams of the extract-free sample was placed into a 1-ml glass tube with a screw cap [Mighty vial (No. 03),

Table 1. A comparison of the conventional and microscale thioacidolysis protocols.

	Conventional protocol (Matsui et al. 1994)	Microscale protocol (present study)	Robinson's protocol (Robinson and Mansfield 2009)
Reaction			
Vessel	23-ml glass tube with the screw cap	1-ml glass tube with the screw cap	5-ml glass tube with the screw cap
Amount of sample	10 mg	5 mg	10 mg
Device and condition	Oil bath (100°C, 4 h)	Heat block (100°C, 4 h)	Heat block (100°C, 4 h)
Internal standard	Docosane (120 μg)	Docosane (60 μg)	Docosane (120 μg)
Work up	Extraction with diethyl ether three times using a separatory funnel	Extraction with diethyl ether three times using 1.5-ml microcentrifuge tube (or 1-ml glass tube), vortex and mini-centrifuge	Extraction with the mixture of H_2O–CH_2Cl_2 (2 : 1, v/v) once using vortex
	Solvent (about 30 ml) evaporation with a rotary evaporator	Solvent (about 0.1 ml) evaporation with a centrifugal concentrator	Solvent (about 1.5 ml) evaporation with a centrifugal concentrator
Throughput/8 h	4–6 samples	At least 40 samples	50 samples

catalog number 0102-01, Maruemu Co., Osaka, Japan]. The thioacidolysis solution (0.9 ml) and 6 μl of docosane (10 mg ml^{-1} in methanol) were added to the tube, and the reaction mixture in the tube was heated in a heat block (Dry Thermo Unit DTU-Neo, catalog number 0063286-000; Aluminum block B-1120A, catalog number 0063293-000; Taitec Co., Saitama, Japan) at 100°C for 4 h. Then, the tube was cooled on ice. Two hundred microliters of the reaction mixture was transferred to a 1.5-ml polypropylene microcentrifuge tube with low DNA adsorptivity (1.5-ml DNA LoBind tube, catalog number 022431021, Eppendorf AG, Hamburg, Germany). The reaction was stopped by adding 100 μl of 1 N $NaHCO_3$. Then the solution was adjusted to pH 3–4 by adding 1 N HCl. To the solution 250 μl of diethyl ether was added, and the tube was vortexed and centrifuged in a mini centrifuge. Then, the extraction was repeated twice. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. One hundred microliters of the solution was dried under high vacuum, and the residue was analyzed by GC-MS as described above.

In separate experiments, work-up of the microscale protocol was carried out exactly as described above but with the following tubes: polypropylene microcentrifuge tube with low protein adsorptivities (1.5-ml Protein LoBind tube, catalog number 022431081, Eppendorf AG, Hamburg, Germany), polypropylene microcentrifuge tube (1.5-ml Microtube, catalog number 131-415C, WATSON Co., Ltd., Tokyo, Japan), and glass tube (1-ml microtube No. 1, catalog number 0407-02, Maruemu Co., Osaka, Japan). In addition, the polypropylene microcentrifuge tubes (WATSON Co., Ltd.) and the glass tubes were also used after siliconization as follows: beakers containing the tubes and Sigmacote (catalog number SL2-25, Sigma-Aldrich Corp., St. Louis, MO, USA), respectively, were placed in a desiccator. The beakers were kept *in vacuo* in the desiccator for 2 h at r.t. Then, the tubes were taken out of the beaker and kept in a plastic bag until use. For comparison, the extract-free samples were also analyzed exactly according to the protocol of Robinson and Mansfield (2009), and the reaction protucts were analyzed by GC-MS as described above.

The GC-MS analysis of the products obtained from the leaf sheaths of *O. sativa* by the conventional and our microscale protocols showed the presence of *erythro* and *threo* diastereomers of *p*-hydroxyphenyl- (t_R=16.97 and 17.22 min; *m/z* 388 $[M]^+$, 265, 239, 205; Figure 2A, 2Ba), guaiacyl- (t_R=20.15 and 20.54 min; *m/z* 418 $[M]^+$, 403, 269; Figure 2A, 2Bb), and syringyl- (t_R=24.83 and 25.55 min; *m/z* 448 $[M]^+$, 299, 269, 265; Figure 2A, 2Bc) trithioethylpropane compounds (Figure 1). They were identified by comparison of the mass spectra with the previous reports (Nakatsubo et al. 2008; Rolando et al. 1992). Both conventional and microscale protocols gave

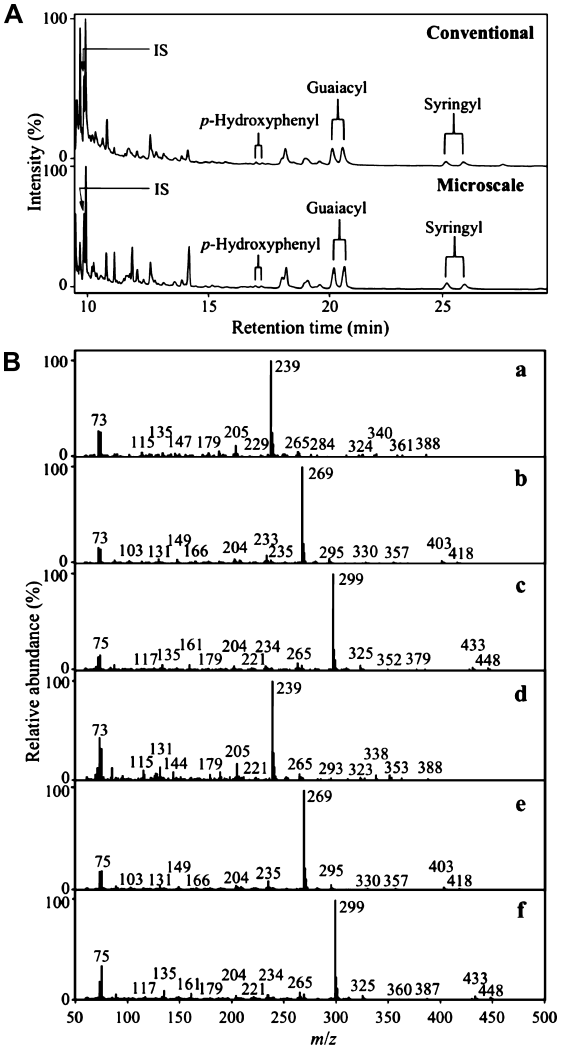


Figure 2. GC-MS chromatograms and mass spectra of thioacidolysis products from *Oryza sativa* leaf sheath. (A) Total ion chromatograms of TMS ethers of thioacidolysis products. Two peaks of guaiacyl, syringyl, and *p*-hydroxyphenyltrithioethylpropane compounds are due to *erythro* and *threo* diastereomers. Conventional, conventional protocol; Microscale, microscale protocol. (B) Mass spectra of TMS ethers of thioacidolysis products. a–c, conventional protocol; d–f, microscale protocol. a and d, *p*-hydroxyphenyltrithioethylpropane; b and e, guaiacyltrithioethylpropane; c and f, syringyltrithioethylpropane.

the same products (Figure 2A, 2Bd–2Bf), indicating that the microscale thioacidolysis proceeds successfully as the reaction with the conventional protocol.

Because each sample must be extracted one-by-one using a separatory funnel, the conventional protocol requires long time for the work-up. On average, only four to six samples are handled within a standard workday (8 h). On the other hand, our microscale protocol can simultaneously deal with many samples. At least 40 samples can be handled in a single cycle within a standard workday. As a result, the throughput is approximately ten-fold greater compared with the conventional protocol, which is similar to the previous report (Robinson and Mansfield 2009).

Table 2. Yields of guaiacyl- and syringyltrithioethylpropane compounds obtained using various tubes for work-up.

Tubes	G/IS	S/IS
Conventional protocol (Matsui et al. 1994)		
Glass	0.39±0.06[a]	0.30±0.05[a]
	1.76±0.14[b]	3.12±0.42[b]
Microscale protocol (present study)		
Polypropylene (DNA LoBind tube)	0.56±0.02[a]	0.44±0.03[a]
	2.12±0.09[b]	3.68±0.17[b]
Polypropylene (Protein LoBind tube)	0.51±0.02[a]	0.36±0.00[a]
Polypropypene	0.51±0.01[a]	0.36±0.01[a]
Siliconized polypropylene	0.71±0.02[a]	0.54±0.01[a]
Glass	0.49±0.01[a]	0.36±0.00[a]
Siliconized glass	0.67±0.04[a]	0.50±0.05[a]
Robinson's protocol (Robinson and Mansfield 2009)		
Glass	0.36±0.02[a]	0.23±0.01[a]
	1.35±0.07[b]	2.32±0.17[b]

G (or S)/IS: the peak area ratio of total of *erythro* and *threo* guaiacyl- (or syringyl)trithioethylpropane compounds to internal standard (docosane). Plant samples are [a] *Oryza sativa* and [b] *Acacia mangium*. $n=3$.

Next, we examined the effects of work-up tube materials on the yields of guaiacyl- and syringyltrithioethylpropane compounds in our microscale protocol based on the peak area ratios of guaiacyl and syringyl compounds (guaiacyl, M^+, m/z 418; syringyl, M^+, m/z 448) to internal standard (docosane, M^+, m/z 310), referred to as G/IS and S/IS, respectively (Table 2). The yields of the products from *O. sativa* leaf sheaths varied among the work-up tubes. However, the variation was smaller than the yield difference between the microscale and the conventional experiments. Thus, the microscale experiments with each work-up tube gave higher yields (G/IS and S/IS values in the ranges of 0.49 to 0.71 and 0.36 to 0.54, respectively) than conventional protocol (G/IS, 0.39 and S/IS, 0.30) for *O. sativa* leaf sheaths (Table 2). Statistical analysis by Student's *t*-test showed that the differences are significant ($p<0.05$, $n=3$). A similar result was obtained for a wood sample. The yields with the microscale protocol with DNA LoBind tube using *A. mangium* (G/IS, 2.12±0.09; S/IS, 3.68±0.17) were higher than those of conventional protocol (G/IS, 1.76±0.14; S/IS, 3.12±0.42) (Student's *t*-test, $p<0.05$, $n=3$; Table 2).

On the other hand, G/IS and S/IS values obtained by Robinson's protocol were 0.36±0.02 and 0.23±0.01 in *O. sativa*, and 1.35±0.07 and 2.32±0.17 in *A. mangium*, respectively (Table 2). Thus, the yields with our microscale protocol were higher yield than those of Robinson's protocol (Student's *t*-test, $p<0.05$, $n=3$). The reaction mixture in a 5-ml glass vial was extracted once with a mixture of H_2O and methylene chloride (2:1, v/v) using vortex in Robinson's, while, in our protocol, the reaction mixture was extracted three times with diethyl ether by vortexing, and organic and aqueous layers were clearly separated in a mini centrifuge. The repeated extraction with clear biphasic separation in our protocol might result in higher product yields. In addition, siliconization of work-up tubes increased the product yields (Student's *t*-test $P<0.05$, $n=3$; Table 2). Our protocol with the high yield facilitates thioacidolysis of small scale samples.

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