NOTE

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Development of an acid soil conditioning agent from lignin by ozone treatment III: improvement of ability of lignin to form an aluminum complex and to reduce aluminum toxicity after ozone treatment

Abstract Lignin prepared by a modified Klason method (KSL) was modified with ozone followed by alkaline treatments. The original and modified KSLs were subjected to a plant growth test with radish (*Raphanus sativus* L. var. *radicula* Pers.) in the presence of $AlCl_3$ (37.5 μM) to assess their ability to reduce aluminum toxicity. The formation of an aluminum complex with these KSLs was also examined by potentiometry. Neither the original nor alkaline-treated KSLs had an effect on aluminum toxicity or the formation of a complex, except for the alkaline-treated KSL at a maximal dose of 250 mg/l, which reduced aluminum toxicity. However, all the KSLs modified with ozone and alkali were effective at forming a complex and reducing aluminum toxicity. The dose of saponified ozone-treated KSLs required to reduce aluminum toxicity became lower and their water solubility increased as the ozone treatment was prolonged. These findings clearly show that water solubility and the ability to form a complex with and reduce the toxicity of aluminum were improved by ozone treatment of KSL.

Key words Aluminum · Complex · Lignin · Aluminum toxicity · Ozone

Introduction

Rapid and highly efficient oxidation of lignin can be accomplished using ozone treatment.[1] Ozone can oxidize both phenolic- and nonphenolic-type structures in lignin.[2,3] Thus, the ability of lignin to reduce aluminum toxicity should be improved by ozone treatment due to the introduction of carboxyl groups, because the carboxyl group plays an important role in reducing aluminum toxicity.[4,5] Aluminum toxicity is the major factor limiting plant growth in acidic soil.[6–9] The authors reported that ozone treatment of kraft lignin improved its water solubility and its ability to reduce aluminum toxicity.[10] Water solubility and the ability to reduce aluminum toxicity were further improved by saponification of ozone-treated kraft lignin.[11] Ester bonds were reported to be formed by ozone treatment of lignin and lignin model compounds.[3,12–16] Improvements in water solubility and the ability to reduce aluminum toxicity of saponified ozone-treated kraft lignin were thought to be due to the conversion of ester bonds to carboxylate by saponification. Kraft lignin became water soluble and was effective at reducing aluminum toxicity following alkaline treatment,[11] although the original kraft lignin did not show any such effect.[10] The IR spectra of kraft lignin before and after alkaline treatment were very similar, except for conversion of the carboxyl group band in kraft lignin to that of carboxylate after alkaline treatment.[11] The improved ability of alkaline-treated kraft lignin to reduce aluminum toxicity may be explained by increased water solubility caused by conversion of the carboxyl group to its salt form and aluminum becoming accessible to the structures in kraft lignin that are able to reduce its toxicity. These results indicate that water solubility and introduction of carboxyl groups are important factors for improving the ability of lignin to reduce aluminum toxicity.

The effect on ozone treatment was relatively unclear in the case of kraft lignin, because kraft lignin became water soluble and was effective at reducing aluminum toxicity following alkaline treatment.[11] However, the ability to reduce aluminum toxicity was slightly less effective with alkaline-treated kraft lignin than with saponified ozone-treated kraft lignins.[11] Aluminum toxicity is reduced by the formation of a complex,[4,5] but it is unclear whether ozone treatment improves the ability of lignin to form a complex with aluminum. In the present study, lignin prepared by a modified Klason method (KSL) was used as the starting material for modifications with ozone. Klason lignin is expected to be a more suitable starting material than kraft lignin to investigate the effect of ozone treatment, because Klason lignin is water insoluble.[17]

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Experimental

Preparation of lignin by a modified Klason method

Japanese cedar (*Cryptomeria japonica* D. Don) wood meal (sapwood, 40 mesh passed) was extracted with ethanol–benzene (1:2, v/v) for 6 h using a Soxhlet extractor. The extracted wood meal (2.0 g, oven-dried weight) was treated with 10 ml of 72% sulfuric acid (w/w) at room temperature for 4 h and then with 3% sulfuric acid at 121°C for 0.5 h in an autoclave.[18] The residue was collected by filtration onto a glass filter, washed several times with water, suspended in water, freeze-dried, and dried in vacuo at 30°C overnight. The yield of residue, referred to as KSL, was 31.2 wt% with respect to wood meal.

Modification of KSL with ozone followed by alkali

The KSL was modified with ozone according to a method described previously.[10] The KSL (1 g) was suspended in 60 ml of a solvent consisting of acetic acid:water:methanol (16:3:1, v/v/v) and oxidized by bubbling with oxygen containing ozone (ca. 3.7%, w/w) at a rate of 0.5 l/min using an ED-OG-R3Lt ozone generator (Ecodesign, Saitama, Japan) for 0.5, 1.0, 2.0, or 4.0 h at 0°C with stirring. The reaction mixture was bubbled with oxygen for an additional 10 min and then evaporated to dryness. The KSLs modified with ozone for 4 h were suspended in water and freeze-dried. The residues were dried under reduced pressure at 30°C overnight. The yields of all ozone-treated KSLs were in the range 113.4%–121.9 wt%. The KSLs modified with ozone for 0.5, 1.0, 2.0, and 4.0 h are referred to as 0.5H-O_3, 1.0H-O_3, 2.0H-O_3, and 4.0H-O_3, respectively.

The KSL and ozone-treated KSLs (1 g) were each suspended in 0.1 M NaOH (250 ml) and maintained at room temperature with stirring overnight. The suspensions were then neutralized to around pH 7 with diluted HCl and subjected to dialysis against deionized water for 3 days using a dialysis membrane [molecular weight cutoff (MWCO): 3500, Spectra/Por 3, Spectrum, Compton, CA, USA]. The suspension remaining in the dialysis tube was evaporated, freeze-dried, and dried in vacuo at 30°C overnight. The products from KSL, 0.5H-O_3, 1.0H-O_3, 2.0H-O_3, and 4.0H-O_3 are referred to as KSLA, 0.5H-O_3A, 1.0H-O_3A, 2.0H-O_3A, and 4.0H-O_3A, respectively. The yields of KSLA, 0.5H-O_3A, 1.0H-O_3A, 2.0H-O_3A, and 4.0H-O_3A were 97.9, 90.9, 88.9, 77.2, and 60.7 wt%, respectively, based on the starting materials.

Water solubility test

Modified KSLs (20 mg) were suspended in deionized water (20 ml) and maintained at room temperature with stirring for 1 h. The insoluble part was removed by filtration and washed with deionized water several times. The filtrate and washings were combined and evaporated to dryness. The residue was further dried in vacuo at 30°C overnight and then the amount of the residue was determined. Water solubility was expressed as the weight ratio of the residue obtained to the starting material used.

Measurement of ozone consumption by KSL

The ozone consumption of the KSL was determined according to the method described in a previous article,[10] with some modifications. Ozone that did not react with the KSL and passed through the reactor during ozone treatment was trapped in a 15% (w/w) potassium iodide solution (300 ml), and the amount trapped was determined by iodometric titration. The 15% potassium iodide solution was renewed every 15 min during ozone treatment. A blank test was performed without KSL.

Determination of methoxyl group content

The methoxyl group content of modified lignins was determined according to the method of Goto et al.,[19] except that *n*-propyl iodide was used instead of ethyl iodide as an internal standard.

Infrared spectroscopy

Fourier-transform infrared (FT-IR) spectra of the original and modified KSLs were recorded on an FT/IR-410 spectrometer (JASCO, Tokyo, Japan) as a KBr disk containing 1% finely ground sample.

Formation of complexes

KSL, alkaline-treated KSL (KSLA), and saponified ozone-treated KSLs (18 mg) were each suspended in deionized water (50 ml), and the suspensions were adjusted to pH 4.8 with diluted HCl and/or NaOH. The formation of aluminum complexes was determined by successively adding 100 μl of 0.1 M $AlCl_3$ to a suspension containing KSL or modified KSLs at room temperature with stirring.[20] Changes in pH were measured after every addition of 0.1 M $AlCl_3$. Measurements were made in duplicate. A blank test was conducted without the modified lignins.

Plant growth test

The plant growth test was reported in detail previously.[10] Ten young sterilized seedlings of radish (*Raphanus sativus* L. var. *radicula* Pers.) were transplanted to a nylon mesh holder floating on 700 ml of hydroponic solution. The nutrient solution contained 1.2 mM $CaCl_2$ at pH 4.8. The plant growth test was performed in the presence and absence of $AlCl_3$ (37.5 μM) and modified lignins (10–250 mg/l) at 23.5°C with continuous air bubbling. The pH of the hydroponic solutions was adjusted to 4.8 with diluted HCl and/or NaOH every day during the plant growth test. Primary root lengths were measured with a ruler after a 3-day growth

period. Abnormal values were removed using Dixon's Q method ($n = 10$).[21] The results were evaluated statistically using the t test.[21]

Results and discussion

Modification of KSL with ozone followed by alkali

Lignin prepared by a modified Klason method (KSL) was used as the starting material for modification with ozone in this study. KSL was prepared by treating 2 g of wood meal with 72% sulfuric acid (10 ml) and then with 3% sulfuric acid (see Experimental). In a preliminary experiment, the yields of the Klason lignin prepared by the usual procedure (1 g of wood meal[22]) and that by using 2 g of wood meal were 32.5 wt% with respect to wood meal in both cases. The methoxyl group content of the KSL and Klason lignin prepared by the usual procedure was 4.5 mmol/g in both cases. These results indicate that acid hydrolysis was sufficient to prepare the KSL. The KSL obtained in this study was insoluble in water.

The KSL was modified with ozone according to a method described previously.[10] The ozone consumption of KSL, measured by iodometric titration, was 1.4, 2.9, 5.5, and 10.5 mmol/g for the 0.5-, 1.0-, 2.0-, and 4.0-h ozone treatments, respectively. Figure 1 shows the IR spectra of the KSL and ozone-treated KSLs. The band due to ester and/or carboxyl groups at approximately 1710–1730 cm^{-1} was very small for KSL; however, it clearly increased in intensity while the bands due to aromatic rings at 1600, 1510, and 1425 cm^{-1} decreased in intensity as the ozone treatment was prolonged. These findings indicate the formation of ester and/or carboxyl groups and the decomposition of aromatic rings in the KSL following ozone treatment.
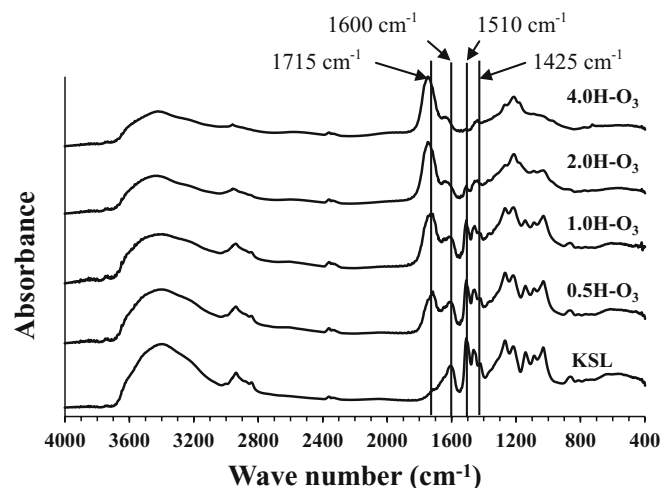
Ester bonds were formed by ozone treatment of lignin and lignin model compounds.[3,12–16] Kratzl et al.[12] indicated the presence of ester bonds in ozone-treated lignins by comparison of their methoxyl and carboxyl contents before and after saponification. Kaneko et al.[16] suggested the presence of ester bonds in ozone-treated thiolignin based on the fact that its molecular weight decreased on saponification. The ozone-treated KSLs were subjected to saponification to cleave ester bonds into carboxyl groups because the carboxyl group is known to play an important role in forming a complex with aluminum[4,23,24] and reducing its toxicity.[4,5] The ability to reduce aluminum toxicity was improved by saponification in the case of ozone-treated kraft lignins.[11] In this study, dialysis (MWCO: 3500) was employed as the post-saponification treatment instead of cation exchange[11] because ozone-treated KSLs did not completely solubilize in 0.1 M NaOH. The product yields obtained after saponification and dialysis decreased as the ozone treatment was prolonged, indicating that the molecular weight of KSL decreased with the ozone and alkaline treatments. The original KSL was also treated with alkali in the same manner, and the product, which was insoluble in water, was named KSLA. Figure 2 shows the water solubility of KSLA and saponified ozone-treated KSLs. Saponified ozone-treated KSLs had an insoluble portion, although this portion decreased as ozone treatment was prolonged (Fig. 2). It was reported that all kraft lignins modified with ozone and alkali were completely soluble in water.[11] Figure 3 shows the IR spectra of the KSLA and saponified ozone-treated KSLs. The intensity of the bands at approximately 1710–1730 cm^{-1} observed in ozone-treated KSLs were clearly smaller and that due to carboxylates at 1600 cm^{-1} was larger after saponification of ozone-treated KSLs (see Figs. 1 and 3), indicating the conversion of the ester and/or carboxyl groups into a sodium salt. However, the IR spectrum of KSLA was similar to that of KSL.
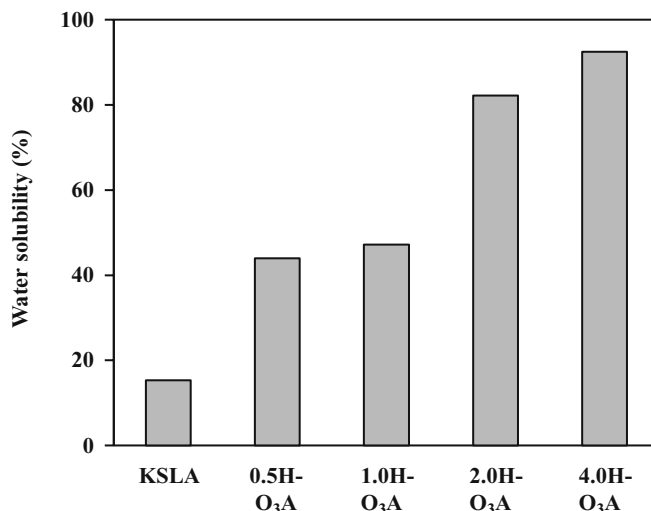

Fig. 1. Infrared spectra of lignin prepared by a modified Klason method (*KSL*) and ozone-treated KSLs. *0.5H-O₃*, *1.0H-O₃*, *2.0H-O₃*, and *4.0H-O₃*, indicate KSL modified with ozone for 0.5, 1.0, 2.0, and 4.0 h, respectively

Fig. 2. Water solubility of alkaline-treated KSL (*KSLA*) and saponified ozone-treated KSLs. *0.5H-O₃A*, *1.0H-O₃A*, *2.0H-O₃A*, and *4.0H-O₃A*, indicate the saponified (alkali-treated) versions of ozone-treated KSLs
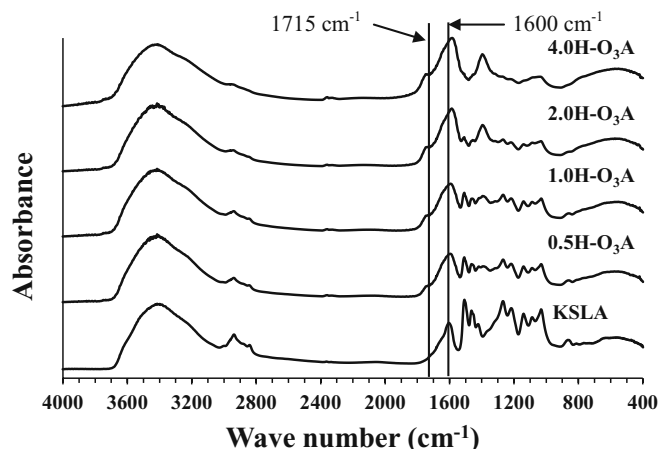
Fig. 3. Infrared spectra of alkaline-treated KSL (*KSLA*) and saponified ozone-treated KSLs

Fig. 4. Changes in pH of the solutions containing KSL, KSLA, and saponified ozone-treated KSLs on the addition of 0.1 M AlCl₃. *Blank* indicates a solution without modified lignins

Complexes of original and modified KSLs with aluminum

The toxicity of aluminum is known to be reduced by the formation of a complex.[4,5] Bartlett and Riego[25] reported that citric acid and ethylenediaminetetraacetic acid (EDTA) reduced aluminum toxicity in plant growth tests with maize. The Al–citrate (1:1) and Al–oxalate (1:3) complexes isolated from the cell sap of hydrangea and buckwheat, respectively, did not cause symptoms of toxicity in plant growth tests with corn.[26–29] Complexes of KSL, KSLA, and saponified ozone-treated KSLs with aluminum were examined by potentiometry. The formation of a complex between aluminum and an organic ligand leads to the release of protons from carboxyl and other acidic groups.[5] It was reported that the pH of solutions containing EDTA or other low-molecular-weight compounds that form complexes with aluminum decreased after Al was added.[20] The pH of a solution containing alkaline-treated kraft lignin or saponified ozone-treated kraft lignins also decreased more than that without modified kraft lignins (blank) after Al was added.[20] This finding showed that kraft lignin was effective at forming aluminum complexes both before and after ozone oxidation.

Figure 4 shows changes in the pH of suspensions containing KSL, KSLA, and saponified ozone-treated KSLs (0.5H-O₃A, 1.0H-O₃A, 2.0H-O₃A, and 4.0H-O₃A) after the Al solution was added. The effect of complexation with modified KSLs was examined at pH 4.8, the same pH used in the plant growth test described later. A decrease in pH was observed after 0.1 M AlCl₃ was added, even in the solution without lignin (blank), because of the acidity of the 0.1 M AlCl₃ solution itself. The pH profiles of the suspensions containing KSL and KSLA were almost the same as that of the blank after 0.1 M AlCl₃ was added. However, the pH of the suspensions containing saponified ozone-treated KSLs (0.5H-O₃A, 1.0H-O₃A, 2.0H-O₃A, and 4.0H-O₃A) clearly decreased more than that of the blank, showing that complexes formed between these modified KSLs and aluminum.
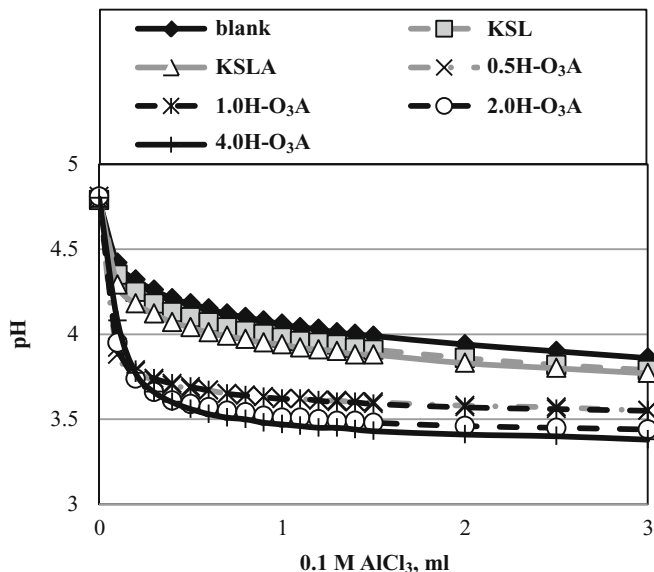
These results show that ozone treatment effectively improved the ability of KSL to form an aluminum complex.

Reduction of aluminum toxicity with KSL and modified KSLs

KSL, KSLA, and saponified ozone-treated KSLs were subjected to plant growth tests. The effect of these modified lignins on root elongation in radish is shown in Fig. 5. Aluminum toxicity was evaluated by investigating the inhibition of root growth, because toxicity first appears in the roots of plants.[6] As shown in this figure, KSL and KSLA had no effect on the recovery of root elongation, except for KSLA at a maximal dose of 250 mg/l. However, a reduction in aluminum toxicity was attained with all saponified ozone-treated KSLs, if a reduction in aluminum toxicity is defined as a recovery of root elongation to the level without aluminum [0(-Al)]. The doses of saponified ozone-treated KSLs required for reducing aluminum toxicity became lower as the ozone treatment was prolonged. These results show that ozone treatment is also effective for improving the ability of KSL to reduce aluminum toxicity. These tendencies were not observed clearly when kraft lignin was used as the starting material. Not only saponified ozone-treated kraft lignins but also alkaline-treated kraft lignin reduced aluminum toxicity, although the latter was slightly less effective.[11] As shown in Fig. 5, root elongations were accelerated above that of the blank when the KSLA and saponified ozone-treated KSLs were used. These phenomena have also been observed in the case of kraft lignins oxidized with alkaline oxygen or ozone.[10,11,20,30] It is not clear at present why root elongation was accelerated with these modified lignins. However, the ability of saponified ozone-treated KSLs to
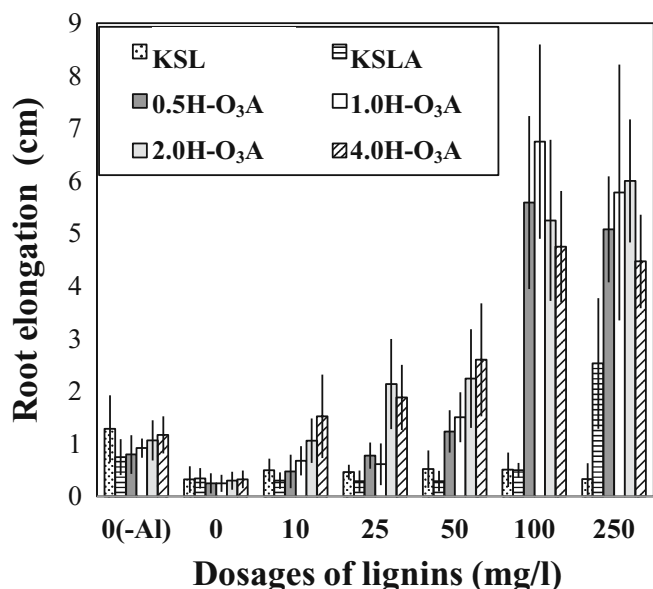
Fig. 5. Effect of KSL, KSLA, and saponified ozone-treated KSLs on root elongation in radish. The hydroponic solution contained 1.2 mM $CaCl_2$ and 37.5 μM $AlCl_3$ at pH 4.8. The growth period was 3 days ($n = 10$). *Error bars* represent the SDs. *0(-Al)* indicates a hydroponic solution without aluminum

reduce aluminum toxicity is thought to contribute, at least partly, to the recovery of root elongation in radish, because complexation is the key to the reduction of toxicity.

In this study, dialysis was used as the post-saponification treatment for ozone-treated KSLs. Some low-molecular-weight organic acids, which are capable of reducing aluminum toxicity and complexation, are formed when lignin is treated with ozone.[10,11,31] These compounds may be removed after saponification during dialysis. A more efficient saponified ozone-treated KSL for reducing aluminum toxicity would be obtained if the low-molecular-weight fraction could be retained after alkaline treatment.

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