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Influence of Proteins on the Lignin Decomposition Behavior of Japanese Cedar (*Cryptomeria japonica*) Wood by Supercritical Methanol Treatment

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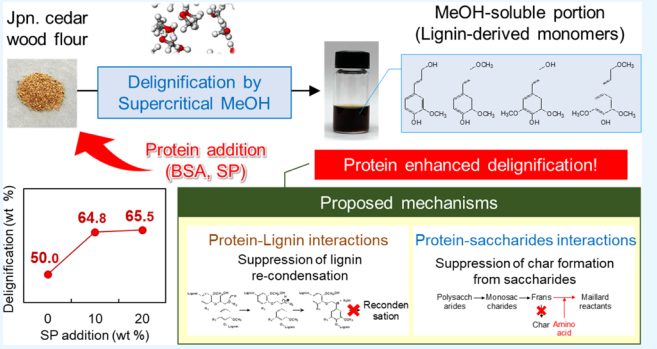
ABSTRACT: The effect of adding protein on the decomposition behavior of lignin in Japanese cedar under supercritical methanol conditions (270 °C/27 MPa) was studied. The Klason method was used to detect the lignin content in the insoluble residue following to a 30 min treatment. Adding either an animal (bovine serum albumin) or plant (soy) protein enhanced delignification from 50 to 65% of the lignin-based wt %. This result was attributed to enhanced lignin depolymerization owing to inhibited lignin recondensation and/or the suppressed formation of polysaccharide-derived char via reactions between the protein and polysaccharides. Although the solubilization of lignin was promoted and the yield of lignin-derived low-molecular-weight compounds increased, the selectivity of major monomers such as coniferyl alcohol (CA) and γ-methylated CA decreased. The addition of proteins has a substantial impact on the decomposition behavior of cell wall components under supercritical methanol conditions. This information provides insights into the use of protein-rich lignocelluloses.



INTRODUCTION

Lignin, one of the main cell wall components of woody biomass, is the second most abundant natural polymer after cellulose.[1] With the depletion of fossil fuels and growing global interest in using renewable resources, lignin from biomass is a potential source of energy and value-added chemicals that can replace fossil resources.

Several strategies enable further use of lignin in the degradation process of woody biomass, with super- and subcritical fluid technologies prominent because of their potential in the efficient dissolution and decomposition of lignin and polysaccharides without adding acid or base catalysts, owing to their unique properties. Ionic products and the dielectric constant of super- and subcritical fluids can be controlled by changing the temperature and pressure, which play significant roles in the decomposition of lignocelluloses.[2] For example, the dielectric constant of supercritical water varies from 3 to 20, whereas that of standard water is ∼80.[3] The low dielectric constant of super- and subcritical fluids facilitates solubilization of hydrophobic substances, thereby promoting lignin decomposition.[4,5] By subcritical water treatment (230−270 °C, 10 MPa), approximately 60% of lignin in Japanese cedar (*Cryptomeria japonica*) was reported to be decomposed and dissolved as soluble products in a semi-flow reactor.[6]

Since the super- and subcritical fluid technology is potential on lignocellulose decomposition, various solvents had been tested in this field, with alcohols representing competitive candidates. Yamazaki et al. treated Japanese beech with several supercritical alcohols (270 and 350 °C, with pressure dependent on the solvents used) and determined that supercritical methanol achieved the highest delignification.[7] For lignin-derived products in the methanol-soluble portion, the main monomeric products were lignin precursor compounds, such as coniferyl alcohol and sinapyl alcohol and their γ-methyl ether compounds (produced via methylation). Thus, supercritical methanol treatment is a promising strategy for further utilization of lignin because of the high yields of monomers.

In addition to the role of solvents, the obtained products and their yields are also defined by the biomass source itself because components interact with each other during treatment. Among the biomass resources available, herbaceous plants and algae have a high protein content ranging from 100 to 500 g/

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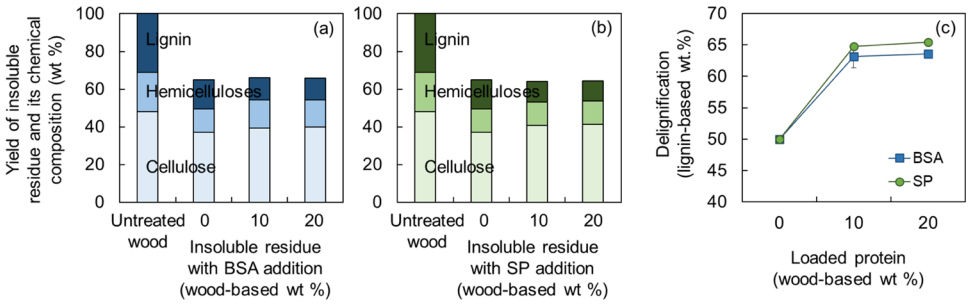

Figure 1. Yield of the insoluble residue and its chemical composition from Japanese cedar wood treated with supercritical methanol (270 °C/27 MPa/30 min) and different loading amounts of (a) BSA and (b) SP and (c) their delignification ratio.

kg.[8] Proteins with many functional groups are expected to affect thermochemical treatment under high-temperature and high-pressure conditions. For example, for bio-char production by hydrothermal carbonization, a high protein content can improve the hydro char yield accompanied by the accumulation of nitrogen in the solid fuel.[9] This is caused by the Maillard and Mannich reactions between protein and cell wall components. However, for bio-oil production that occurs under conditions of high reaction severity, as studied with model compounds, the Maillard reaction can inhibit the carbonization of sugar-derived furans and N-containing compounds such as amines, N-heterocycles, and nitriles that are retrieved in the aqueous portion.[10] As for the decomposition of protein-containing biomass by the supercritical methanol, Zeb et al. treated macroalgae at 300 °C and achieved a higher bio-oil yield (66 wt %) than that obtained by using supercritical water.[11]

In cases of supercritical methanol treatment of woody biomass, the topochemistry method revealed that locally distributed inorganic substances, albeit in trace amounts, affect decomposition behavior. However, insoluble residue from demineralized beech wood still contained some secondary products, which is probably caused by the distribution of proteins in parenchyma cells.[12] The effects of proteins on lignin decomposition behavior during supercritical methanol treatment have not been fully investigated. Therefore, in this study, we examined the effects of proteins on lignin decomposition behavior by adding either a plant or animal protein (i.e., soy protein or bovine serum albumin) to Japanese cedar (*Cryptomeria japonica*) wood flour and subjected these samples to supercritical methanol treatment (270 °C/27 MPa/30 min).

MATERIALS AND METHODS

Samples and Chemicals. Japanese cedar (*C. japonica*) sapwood wood flour (purchased from Nakawood Co., Japan and was sieved to be 0.15−0.5 mm size) was extracted with acetone for 4 h using a Soxhlet apparatus and then dried at 105 °C for 24 h to prepare the wood sample. For the model study, Japanese cedar chlorite holocellulose and milled wood lignin (MWL) were prepared according to existing methods.[13,14]

All chemicals used in this study were purchased from Nacalai Tesque Inc., Kyoto, and of reagent grade without purification. The proteins (bovine serum albumin (BSA) and soybean protein (SP)) were also purchased from Nacalai Tesque Inc., and the purities are >96 and >92%, respectively.

Supercritical Methanol Treatment and Fractionation. 150 mg of extractive-free cedar wood (or 100 mg of holocellulose or 50 mg of MWL) was used with 0, 10, or 20% protein (bovine serum albumin (BSA) or soybean protein (SP), wood-based wt %) added for the supercritical methanol (270 °C, 27 MPa; T_C 239 °C, P_C 8.09 MPa) treatment. The treatment was conducted by enclosing the sample in a 5 mL batch-type reactor with 4.9 mL of methanol and immersing the reactor in a salt bath at 270 °C. The schematic and experimental information about the batch-type treatment system have been shown in previous studies.[15,16] After a 30 min holding process, the reaction was stopped by cooling in a water bath, and the obtained product was fractionated by filtration into a methanol-soluble part and an insoluble residue. All experiments were duplicated.

Characterization of Each Fraction. The lignin content in the insoluble residue was determined by the Klason method to calculate the delignification ratio.[17] Subsequently, cellulose and hemicellulose contents were estimated from constituent monosaccharides in the hydrolysates obtained from the lignin determination. The hydrolysates were analyzed by high-performance anion-exchange chromatography (HPAEC, Prominence, Shimadzu Corp., Kyoto, Japan) with an electrochemical detector (DECADE Elite, Antec Scientific, Zoeterwoude, Netherlands) and the following conditions: column, CarboPac PA-1 (4 × 250 mm, Thermo Fisher Scientific, Waltham, MA, USA); mobile phase, 30 mM aqueous NaOH; flow rate, 1.0 mL min−1; column temperature, 35 °C.

Structural analysis of the methanol-soluble fraction was achieved by gas chromatography−mass spectrometry (GC−MS) using a Shimadzu-2010 Plus gas chromatograph (Shimadzu Corporation) coupled with a Shimadzu QP 2010 Ultra mass spectrometer (Shimadzu Corporation). Instrumental conditions: column, Agilent CPSil 8CB (length: 30 m; diameter: 0.25 mm); injector temperature, 250 °C; split ratio, 10:1; column temperature, 60 °C (2 min), 4 °C min^{-1} to 150 °C, 150 °C (1 min), 10 °C min^{-1} to 300 °C, 300 °C (6 min); carrier gas, helium; flow rate, 2.75 mL min^{-1}. The MS scan parameters included a scan range of 35−600 *m/z* and a scanning interval of 0.3 s.

Gel permeation chromatography (GPC) was performed with the Shimadzu LC-20A to elucidate the molecular weight distributions of the soluble parts under the following conditions: column, Shodex KF-801 connected with KF-802, KF-802.5, and KF-803 in series; flow rate, 0.6 mL min^{-1}; eluent, tetrahydrofuran (THF); detector, UV (λ = 280 nm); temperature, 50 °C.

High-performance liquid chromatography (HPLC) analysis was carried out with a Shimadzu LC-20A (Shimadzu Corporation) to quantify the main monomeric products in the soluble fraction under the following conditions: column, Cadenza CD-C18; flow rate, 1.0 mL min^{-1}; eluent, methanol/

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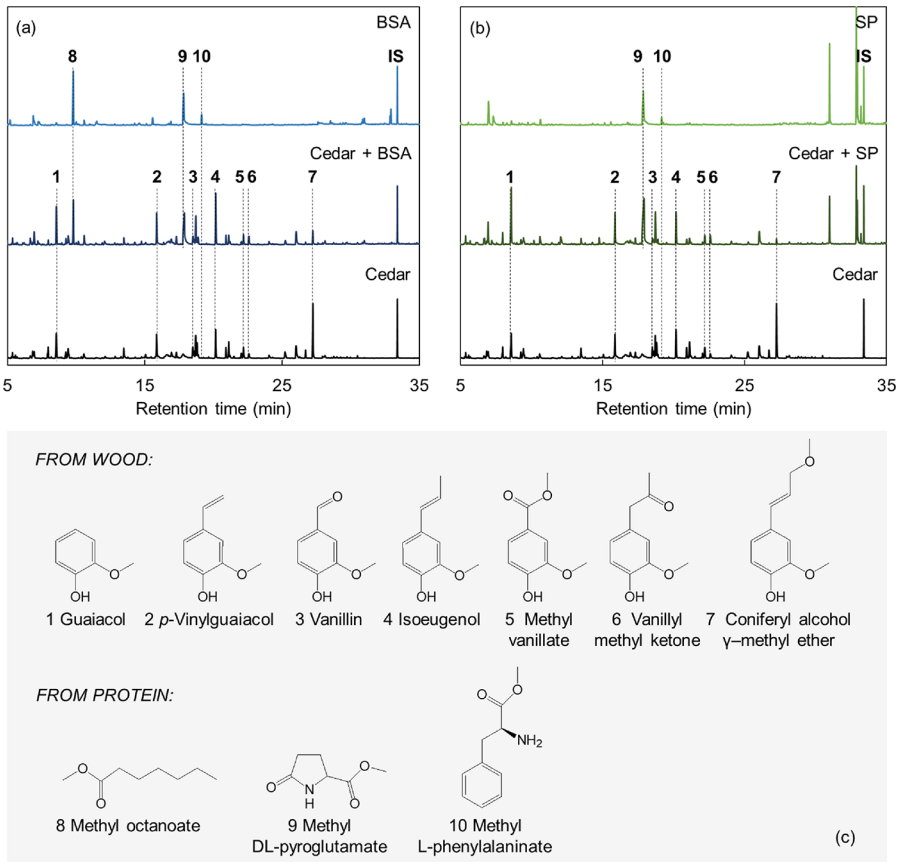

Figure 2. GC−MS chromatograms of the methanol-soluble portion obtained from (a) cedar, cedar + 10% BSA and only BSA; (b) cedar, cedar + 10% SP, and only SP, as treated by supercritical methanol (270 °C/27 MPa/30 min); and (c) identified monomeric compounds. IS, internal standard.

water 20/80 to 100/0 (50 min); detector, UV (λ = 280 nm); oven temperature, 40 °C.

Elemental analysis of insoluble residues was conducted at the Laboratory for Organic Elemental Microanalysis, Faculty of Pharmaceutical Science, Kyoto University, Japan. The proton concentration of the methanol-soluble fraction was determined using a pH meter (Horiba D-52, HORIBA, Ltd., Kyoto, Japan) after a 10-fold dilution with distilled water.

RESULTS AND DISCUSSION

Characterization of the Insoluble Residue. Figure 1 shows the chemical composition of the insoluble residue of cedar wood flour after treatment with supercritical methanol (270 °C/27 MPa/30 min) when a sample contained BSA (Figure 1a) or SP (Figure 1b). For comparison, the two proteins were treated independently with supercritical methanol, and no solid residue was retrieved in either protein sample. Thus, the residue from cedar wood in the presence of proteins was derived solely from the cell wall component. The addition of BSA to the supercritical methanol treatment of Japanese cedar did not change the insoluble residue yield, but the lignin content in the residue decreased, as shown in Figure 1a. Even the addition of 10% BSA reduced about 13% residual lignin. In contrast, the yields of residual saccharides were slightly improved. A similar result was observed when adding SP to the Japanese cedar sample (Figure 1b). A comparison of the delignification ratio, which was calculated based on the initial lignin amount, revealed that adding either BSA or SP enhanced delignification significantly from 50 to 65% (Figure 1c). In addition, elemental analysis of insoluble residues with protein added contained nitrogen (Figure S1). The presence of nitrogen indicates that although residues were thoroughly washed with methanol, protein-derived structures were incorporated into the insoluble residues.

Characterization of the Methanol-Soluble Portion. The methanol-soluble portion was characterized using GC−MS, HPLC, and GPC analysis. GC−MS analysis provided structural information and variation in the yields of the lignin-derived monomers (Figure 2). Treating Japanese cedar with supercritical methanol without the addition of protein yielded lignin-derived monomers from typical pyrolysis reactions, such as guaiacol (1) and isoeugenol (4), and methylated monomers such as methyl vanillate (5) and coniferyl alcohol γ-methyl ether (7, CA-γ) (Figure 2a, bottom chromatogram). Coniferyl alcohol (CA, a prevalent monomeric product from lignin, the precursor of 7) was not detected by GC−MS, probably because it is unstable and repolymerized during GC analysis; however, the presence of CA was verified by HPLC, as described later (Table 1). For BSA-added samples (Figure 2a, middle chromatogram), the same lignin-derived monomers as the normal supercritical methanol treatment were detected. Thus, the addition of BSA did not lead to the formation of monomers with different side-chain structures. However, adding BSA appeared to change the yields. For example, the peak area representing CA-γ (7) decreased significantly (the quantification result is shown in Table 1), whereas the yields of guaiacol (1), *p*-vinylguaiacol (2), isoeugenol (4), and vanillyl methyl ketone (6) increased to varying degrees. In addition to

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Table 1. Yields of Typical Lignin- and Saccharide-Derived Monomeric Compounds in the Methanol-Soluble Portion from Cedar and Soy Protein (SP)-Added Cedar Treated with Supercritical Methanol (Wood-Based wt %)

	yields (wood-based wt %)	
compounds	cedar	cedar + SP
coniferyl alcohol (CA)	0.08	0.05
CA γ-methyl ether	0.20	0.03
5-hydroxymethylfurfural	0.03	0.02
furfural	0.16	0.05

lignin-derived monomers, protein-derived monomers such as methyl DL-pyroglutamate (9) and methyl L-phenylalaninate (10), which were detected in the treatment of only BSA (Figure 2a, top chromatogram), were obtained. Similar trends were observed when SP was added to the sample (Figure 2b).

For quantitative discussion, the yields of major monomeric products from lignin and polysaccharides were quantified by HPLC (Table 1). The yields of CA and CA-γ (7), which are the major lignin-derived monomers, were reduced significantly in the methanol-soluble portion when SP was added. Moreover, the yields of furans such as 5-hydroxymethylfurfural (5-HMF) and furfural, which are essential undesirable degradation products of saccharides, also decreased when proteins were added.

GPC analysis revealed the molecular weight distribution of the methanol-soluble portion (Figure 3). In the presence of BSA, the molecular weight (MW) distribution of the lignin-derived products in the methanol-soluble portion shifted slightly toward lower MWs. Additionally, the relative contents of low-molecular-weight compounds such as monomers and dimers increased. The reduction in the molecular weight of lignin facilitates delignification. The reduction in the molecular weight of lignin in the soluble portion was also observed in the presence of SP. In summary, there were no obvious differences between the addition of BSA and SP. Elemental composition analysis of C, H, O, N, and S for BSA and SP revealed negligible differences (Table S1). Although the composition of S differed between BSA (0.5%) and SP (1.6%), this difference did not cause a significant difference in decomposition behaviors. Thus, further discussion will be based on the results using SP.

Mechanisms Responsible for Enhanced Delignification by Protein Addition. Mechanisms responsible for improving delignification by adding protein will be discussed by evaluating lignin−protein interactions and polysaccharide−protein interactions. Recondensation may have been suppressed because the molecular weight of the soluble portion decreased (Figure 3). For example, super- and subcritical solvent treatments are typically carried out under acidic conditions, and lignin recondensation reactions occur under such acidic conditions.[18] In particular, carbocations are an important intermediate of lignin recondensation, and some proteins act as carbocation scavengers to inhibit recondensation reactions.[19] In another possible mechanism, nitrogen-containing cyclic compounds (e.g., methyl DL-pyroglutamate (9), which was detected in GC−MS (Figure 2)) can act as radical scavengers[20] and may inhibit the radical reaction of recondensation. Inhibition of recondensation by either carbocations or radical scavengers may have promoted the depolymerization of lignin (i.e., delignification). In either case, a similar reduction in the molecular weight of lignin and

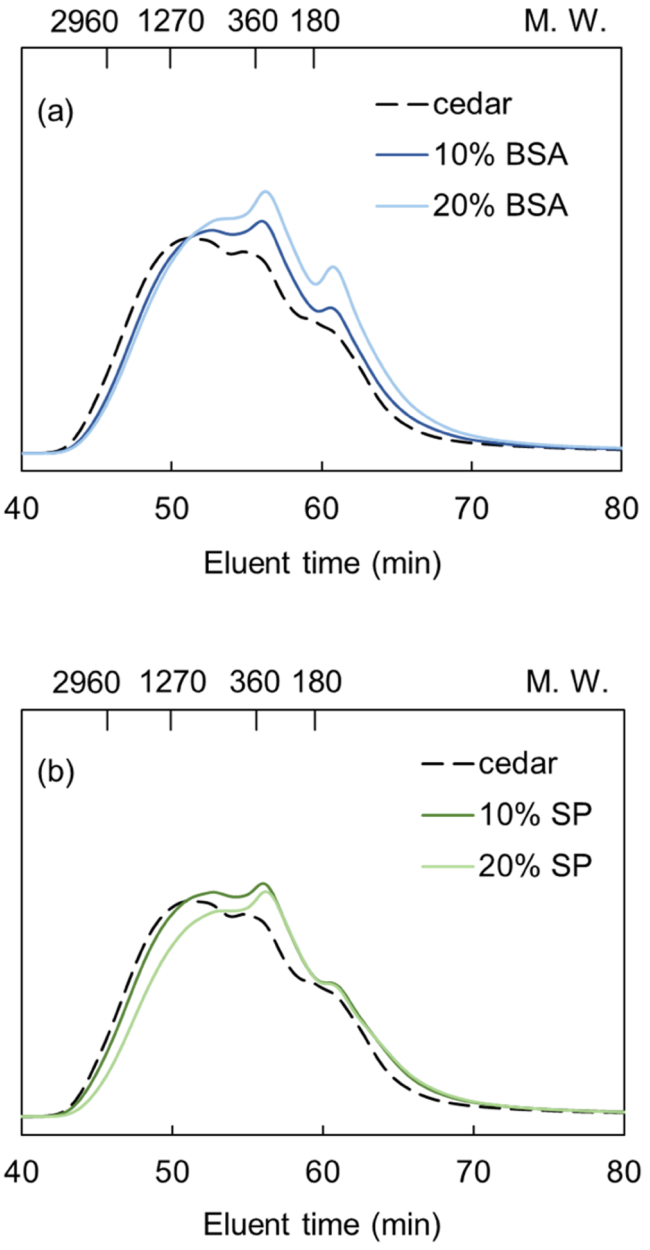

Figure 3. GPC chromatograms of the methanol soluble portion from (a) cedar, cedar + 10% BSA and cedar + 20% BSA, and (b) cedar, cedar + 10% SP, and cedar +20% SP.

incorporation of protein into lignin would have occurred in the lignin model compound in the absence of polysaccharides. Therefore, we investigated the effect of adding SP to MWL in supercritical methanol treatment. Most of the MWL, which is lignin once extracted with a solvent, was solubilized in supercritical methanol, and the amount of residue present was very low for samples with and without protein (Figure S2b). According to the elemental composition analysis of the residue, the nitrogen content in the residue from MWL with protein added was high (Figure S3). Given that free protein was removed by thoroughly washing the MWL with ample methanol, the protein structures would have been incorporated into the lignin molecule. Residue from holocellulose treated with the addition of SP did not contain nitrogen (Figure S3), suggesting the selective incorporation of protein into lignin. Furthermore, according to the GPC analysis of the methanol-

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soluble portion from MWL, it was apparent that the molecular weight of dissolved lignin was reduced by adding SP (Figure 4). This observation indicates that recondensation of lignin was suppressed by the reaction between lignin and protein via either carbocations or radical scavengers.

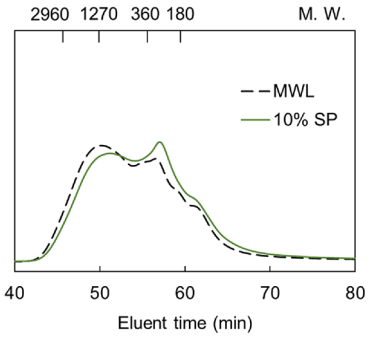

Figure 4. GPC chromatograms of the methanol-soluble portion from MWL and MWL + 10% soy protein (SP).

The interaction between polysaccharides and proteins also should be considered. In a previous study examining the delignification of Japanese beech under supercritical methanol treatment and based on a topochemical viewpoint, the char-like substances were likely overestimated as a Klason lignin.[12] This possibility was examined by estimating the chemical composition (cellulose, hemicellulose, and Klason lignin) and elemental composition (Table 2). As a result, residue without adding protein showed a lower O/C ratio (0.363) when compared with the average O/C ratio of protein-added residues (0.393) and lignin model compounds, indicating that Klason lignin in the residue without adding protein seems to contain low oxygen substances (i.e., char-like structure).

Table 2. Estimated O/C Ratios of the Klason Lignin for Various Samples

sample	estimated O/C ratio
residue from SCM	
without protein	0.363
ave. of protein-added samples	0.393 ± 0.021
model compounds	
MWL	0.373
coniferyl alcohol	0.400

Furans such as furfural and 5-HMF are carbonization intermediates in hydrothermal treatment, resulting in their polymerization to hydrochar.[21] Fan et al. reported that the Maillard reaction between amino groups and carbonyl groups of reducing sugars suppresses the formation of furans under protein-containing conditions, resulting in the suppression of char formation.[10] Similarly, in the supercritical methanol treatment, the formation of furans in the methanol-soluble portion of the protein-added system was noticeably suppressed (Table 1). Also, the recovery of methyl L-phenylalaninate (10), which containing amino groups, decreased significantly when the protein was treated with wood flour, indicating that it was consumed (Figure 2). Therefore, char formation appeared to be suppressed. Using a model compound (holocellulose, in Table S2) also confirmed that the yield of furans was reduced when adding SP. In addition, as explained above, radical scavengers contributed to the suppression of polymerization, resulting in the inhibition of char formation. Thus, because char was recovered as lignin by the Klason method, the suppression of char formation may have contributed to the higher apparent delignification ratio when estimated by the Klason method.

Based on the discussion above, plausible mechanisms for the high delignification ratio observed in protein-added supercritical methanol treatment are presented in Figure 5. Although further experiments are required to confirm the mechanism, it was apparent that both lignin−protein and polysaccharide−protein interactions affected the high delignification behavior.

Discussion on the Lower Selectivity of Lignin-Derived Monomers when Adding Protein. Although the addition of protein enhanced delignification, the yields of the major lignin-derived monomers (CA and CA-γ) decreased (Table 2), indicating a reduction in the selectivity of lignin-derived monomers. Such a decrease in CA and CA-γ was also observed for MWL without polysaccharides (Table S2). Moreover, the higher content of 6, which is a type of Hibbert ketone typically generated from acidolysis of woody biomass,[22]

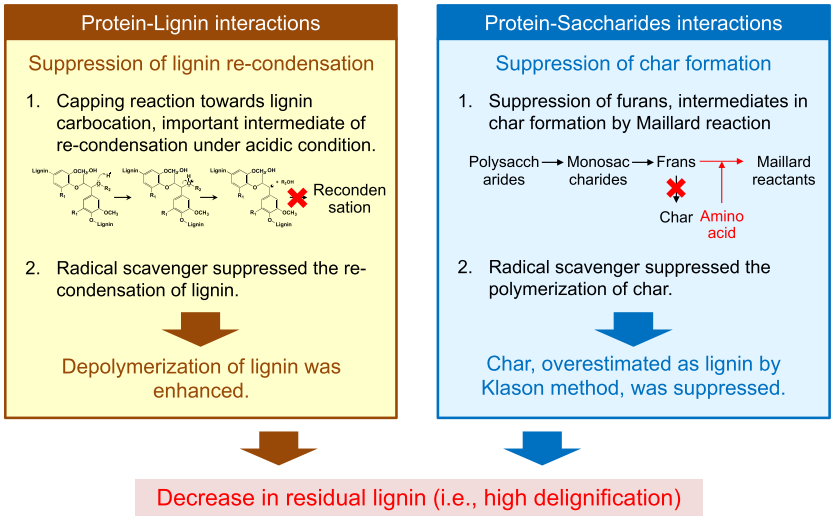

Figure 5. Plausible mechanisms of high delignification from protein-added supercritical methanol treatment.

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was observed in the methanol-soluble portion of the protein-added system. The side-chain structure of lignin-derived monomers was suggested to be affected and probably catalyzed by additional acidic groups from the protein. However, pH testing revealed that the soluble portion without adding protein (pH = 5.8) was more acidic than the protein-added systems (ave. pH = 6.6). The carboxylic acid groups in proteins likely lost their acidity because of esterification by methanol, whereas the amino groups were probably retained. Thus, the soluble portion with added protein showed less acidity. Although an explanation for the increase in the typical product of acidosis remains unresolved, such a change in reaction media by adding protein probably affected the selectivity of the reaction in the lignin side-chain structure.

Furthermore, because the reaction media in the protein-added system was less acidic, among the two mechanisms of lignin recondensation shown in Figure 5, the radical scavenger rather than the carbocation scavenger would have a more substantial impact on the suppression of lignin recondensation.

CONCLUSIONS

The influence of adding protein on the decomposition behavior of lignin in Japanese cedar under supercritical methanol conditions was discussed in this study. The addition of proteins enhanced delignification, represented by the reduced Klason lignin content in the residue. This observation can be attributed to the inhibition of lignin recondensation and/or polysaccharides-derived char formation. Although the total yield of low-molecular-weight products in the methanol-soluble portion increased, the selectivity of monomers decreased with protein addition. The additional protein was revealed to have a strong impact on the decomposition behavior of lignin under supercritical methanol conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03716.

Nitrogen content in insoluble residue from cedar wood treated with supercritical methanol in the absence and presence of BSA or SP; yield of insoluble residue and its chemical composition (C: cellulose; H: hemicellulose; L: lignin) from holocellulose and milled wood lignin (MWL) treated with supercritical methanol and different loading amounts of SP; nitrogen content in insoluble residue from cedar wood, MWL, and holocellulose treated with supercritical methanol and different loading amounts of SP; elemental composition of BSA and SP used in this study; and yields of typical lignin- and saccharide-derived monomeric compounds in the methanol-soluble portion from holocellulose and milled wood lignin (MWL) treated with supercritical methanol with/without 10% SP (wood-based wt %) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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