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Comparison of inhibition effects of various isolated lignins on enzymatic hydrolysis of cellulose

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Abstract−Various isolated soluble and insoluble lignins isolated from ammonia recycle percolation (ARP), dilute sulfuric acid, and hot-water pretreatments and two commercially available lignins (alkali and organo-solv lignin) were tested to evaluate their inhibitory effects on the enzymatic hydrolysis of cellulose. The purpose of this study was to compare the effects by adding various isolated lignins in the enzyme hydrolysis. The results showed that enzyme reaction was generally inhibited by addition of lignins. The order of lignins from most to least inhibitory effects were alkali, ARP-soluble, water-soluble, acid-soluble, ARP-insoluble, and organo-solv lignins. With 0.344 g ARP-soluble or alkali lignin/g-glucan lignin loadings, 72-h digestibilities were decreased by 16% and 20%, respectively. It was also observed that the digestibilities were decreased, as the lignin loadings increased. The hindering effect by ARP-insoluble or organo-solv lignin was not significant.

Key words: Enzyme Inhibition, Pretreatment, Alkali Lignin, Water Soluble Lignin, Acid Soluble Lignin, Organo-solv Lignin

INTRODUCTION

Upon pretreatment, biomass undergoes considerable changes in physical properties and chemical composition. There are many factors that affect the enzymatic digestibility of the lignocellulosic biomass because it has heterogeneous characteristics and is very resistant for enzymatic breakdown. It has been reported that the lignin content is one of the major hindering factors of enzyme hydrolysis of lignocellulosic biomass [1-6]. Lignin forms a solid seal around cellulose micro-fibrils and exhibits limited covalent association with hemicellulose preventing enzyme from binding to cellulose [1,7,8]. Many previous studies also indicated that lignin-enzyme interactions make significant contributions to the decrease in rate observed during hydrolysis of lignocellulose substrates [9]. There have been numerous efforts for enhancing the enzymatic hydrolysis rate thereby increasing the yield of lignocellulosic biomass, including representative treatment methods such as hot-water treatments (autohydrolysis) [10-13], acid treatments [14-18] and alkali treatments [19-25]. We have previously reported that the removal of lignin using aqueous ammonia substantially improved microbial activity and enzyme efficiency [24-30]. With regards to the effects of various pretreatments on lignins, it was reported that the alkali treatment cleaves ester bond of lignin-carbohydrate complexes (LCCs) and breaks down the original lignin with a large molecular weight and three-dimensional network structure into one with a small molecular weight and linear structure [31]. Removal of carboxylic acids in lignin was reported to improve the enzymatic hydrolysis yield [32]. Other previous studies also indicated that removal of methyl groups on lignin enhanced the enzyme hydrolysis rate and yield of lignocellulosic biomass [33,34]. Kumara et al. [35] reported that, among many pretreatment methods (ARP, DSA, and HW), ARP most significantly reduces both methyl group and carboxylic acids from the corn stover lignin and HW and DSA pretreatments also reduce them.

The aim of this study was to quantitatively compare effects of the lignins derived from various pretreatment methods. Making a fair and direct comparison between different types of lignins can be difficult. Furthermore, the characteristic of isolated lignin is presumably somewhat different from the original characteristic. In spite of these challenges, studying the isolated effects of lignins is worth attempting, because there have been few experiments for a quantitative test. In this investigation, attempts were made to determine the isolated effects of lignin on the enzymatic digestibility using pure cellulose (α-cellulose) and pretreated corn stover as substrates.

Three different pretreatment methods using various chemical reagents (ammonia recycle percolation (ARP), hot water, and dilute sulfuric acid) were used in order to generate lignins using corn stover. The inhibitory effects of various lignins were compared by enzymatic hydrolysis of α-cellulose.

MATERIAL AND METHODS

1. Materials

Air-dried ground corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was screened to a nominal size of 9-35 mesh. The initial composition of corn stover as determined by NREL was: 36.1 wt% glucan, 21.4 wt% xylan, 3.5 wt% arabinan, 1.8 wt% mannan, 2.5 wt% galactan, 17.2 wt% lignin, 7.1 wt% ash, 3.2 wt% acetyl group, 4.0 wt% protein, and 3.6 wt% uronic acid.

The α-cellulose was purchased from Sigma-Aldrich (St Louis, MO, Cat. # C-8200, Lot No. 11K0246). Composition of α-cellulose was determined to be 95.0 wt% glucan, 3.0 wt% xylan, 1.6 wt% mannan, and >0.1 wt% ash.

Cellulase enzyme, Spezyme CP (Genencor International, Lot # 301-00348-257), was provided from Genencor. An average activ-

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ity of the enzyme (Spezyme CP), as determined by NREL was: 31.2 filter paper unit (FPU)/ml. The β-glucosidase enzyme, Novozyme 188 (Novo Inc., lot no. 11K1088), was purchased from Sigma-Aldrich (St. Louis, MO). Activity of β-glucosidase was 750 CBU/ml.

The AL (Sigma-Aldrich Cat # 37,095-9) and OSL (Sigma-Aldrich Cat # 37,101-7) were purchased from Sigma-Aldrich. The average molecular weight of OSL, MW is 3,140.

2. Experimental Setup and Operation

The reactor system for the ARP, DSA, and HW pretreatments consists of a flow-through column reactor with preheating coil, an HPLC (high performance liquid chromatography) pump (Series II pump, Chrom Tech, Inc., MN), a temperature-programmable GC (gas chromatography) oven (Hewlett Packard 5890, HP Inc., Ontario, Canada), solution reservoirs and a sample cylinder tank. The reactor (70 cm^3 of internal volume) was constructed from a 16.5 cm length of 2.3 cm I.D. SS-316 tubing. Two 1,000 ml SS 304 cylinders were used as receiver tanks for collecting the liquid products.

Ten grams of (dry) corn stover was packed into the flow-through reactor. The oven was preheated for 15 minutes, and 300-350 psi of N_2 backpressure was applied to the reactor system to maintain the system pressure above the saturated pressure of chemicals and water. Pretreatment reagents were pumped by HPLC pump to the reactor. At the completion of the run, the solid in the reactor was flushed with water to remove the residual sugar in the treated biomass. The wet solids obtained from the reactor were separated into two portions: One was dried for measurement of weight loss and subjected to composition analysis, while the others were subjected to the enzymatic digestibility test.

3. Pretreatment Conditions for Lignin Separations

3-1. DSA Pretreatment

Corn stover was treated with 0.07 wt% of H_2SO_4, 210 °C, 2.5 ml/min of flow rate, and 30 min of reaction time using flow-through column reactor.

3-2. Hot-water Pretreatment

Corn stover was treated with hot-water, 210 °C, 2.5 ml/min of flow rate, and 30 min of reaction time using flow-through column reactor.

3-3. ARP pretreatment

Corn stover was treated with 15 wt% of ammonia, 170 °C, 5.0 ml/min of flow rate, and 12 min of reaction time using flow-through column reactor.

4. Lignin Preparations

After pretreatment, the solids and liquid hydrolysates were separated by fluted filter paper (Fisher Cat# 09-790-14F) and solids were then washed with de-ionized (DI) water using vacuum filter until the wash water had a neutral pH. The liquids were directly used for soluble lignin tests after the secondary hydrolysis (see below) and the solids were subjected to lignin separation step.

4-1. Soluble Lignin

To concentrate the hydrolysates, the effluents produced from the pretreatments were put into the rotary evaporator (Buchi Corp., New Castle, DE) to evaporate water and ammonia. After evaporation, the liquid was subjected to a secondary acid hydrolysis to hydrolyze the sugar oligomers to monomers, and then the sugars in the liquid were determined by HPLC. The conditions of secondary hydrolysis were 4 wt% sulfuric acid, 121 °C, and 1 hour. This liquor contains soluble lignin as well as soluble sugars, mainly xylan. Glucose contents in all effluents were determined in the negligible level. Xylose concentration was varied upon pretreatment methods, but xylose contents in hydrolysate were very low. Although xylose concentration is low and negligible, hydrolysates were subjected to the secondary hydrolysis because it was reported that xylooligomers were more inhibitory to cellulase than xylose for an equivalent amount of xylose [36]; therefore, we assumed the influences of xylose on cellulase activity were negligible in this study. We also assumed that soluble lignins in the hydrolysates were equal to the amount of lignin removed from the solids.

4-2. Insoluble Lignin

Treated corn stover was subjected to two step acid hydrolysis (1st stage - 72 wt% H_2SO_4, 30 °C, 1 h; 2nd stage - 4 wt% H_2SO_4, 121 °C, 1 h). Filter and collect the solid residues. We assumed that the sugars in the solid were zero and the remaining solids were mostly lignins and some ash, because sugars were entirely solubilized and washed out through the acid hydrolysis conditions.

5. Enzymatic Digestibility Test

The enzymatic digestibility of glucan in corn stover and α-cellulose was determined in triplicate according to the NREL Chemical Analysis and Testing Standard Procedure [37]. The conditions of the enzymatic digestibility tests were pH 4.8 (0.05 M sodium citrate buffer) on a shaker bath agitated at 150 rpm at 50 °C. Enzyme loading of 15 FPU of Spezyme CP/g-glucan supplemented with 30 CBU of Novozyme 188/g-glucan was used. The initial glucan concentration was 1% (w/v) based in 100 ml of total liquid and solid. The 250 ml screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (New Brunswick Scientific, Edison, NJ). Various isolated lignins were loaded with three different levels of dosages, which are 0.086, 0.172, and 0.344 g/100 ml-working volume.

Samples were taken periodically at appropriate sampling times (6, 12, 24, 48, 72, and 96 h) and analyzed for glucose, xylose, and cellobiose content using HPLC.

Enzyme digestibility was determined at glucan loading of 15 FPU/g of glucan with b-glucosidase supplemented at 30 CBU/g of glucan. Substrate and enzyme blanks were run in parallel as controls.

$$\text{Enzyme digestibility } [\%] = \frac{\text{Total released glucose} \times 0.9}{\text{Initial glucan lading}} \times 100$$

0.9 is the conversion factors of glucose to equivalent glucan.

6. Analytical Methods

Solid analysis of sugar and lignin was performed following the procedures of NREL Chemical Analysis and Testing Standard Procedures [37]. Each sample was analyzed in triplicate. The moisture contents of solid samples were measured by infrared moisture balance (Denver Instrument, IR-30). The sugars in the liquid samples were determined after secondary acid hydrolysis to convert oligomeric sugars to monomeric sugars. The conditions of the secondary hydrolysis were 4 wt% sulfuric acid and 121 °C for one hour. For the lignin analysis, the hydrolysis solution was vacuum filtered, and the filtered hydrolyzed solid sample was dried and weighed. The dried samples were then combusted in a furnace at 575±25 °C for 16 h to determine the ash content [37]. The difference of the two weights was taken as the acid insoluble lignin. The absorbance of the hydrolysis liquor in the aliquot obtained from the vacuum filter sample at 320 nm on a UV-Visible spectrophotometer measured the acid soluble lignin.

Sugars in the hydrolysates were determined by HPLC using a Bio-Rad Aminex HPX-87P column coupled with a refractive index detector.

RESULTS AND DISCUSSIONS

1. Effects of Various Lignins on α-Cellulose Hydrolysis

The enzyme digestibility tests of the α-cellulose for various lignin loadings were conducted using 15 FPU of cellulase/g-glucan and 30 CBU of β-glucosidase/g-glucan with initial solid loading of 1% glucan, and the results are summarized in Fig. 1-7. The control in the figure means that the enzymatic hydrolysis was performed with α-cellulose alone without any addition of isolated lignin.

1-1. ASL Effects on Enzyme Digestibility

Fig. 1 summarizes the enzymatic digestibility results of α-cellulose with addition of ASL. Compared to the result of control sample with no ASL, the test indicated that enzyme hydrolysis rates and yields were inhibited by adding isolated ASL at all lignin loadings. The 72-h digestibility of control was 85%, while the digestibilities of the higher lignin loadings were 81% with 0.172 g ASL loading and 77% with 0.344 g ASL loading. However, the 72-h digestibility at 0.086 g ASL loading was as high as that of control. Therefore, enzyme hydrolysis tests with varying ALS loadings showed

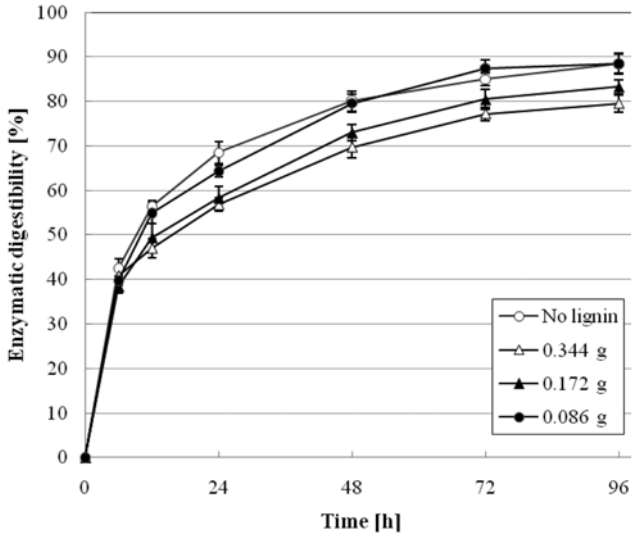

Fig. 1. Effect of ASL on enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; pretreatment conditions: 0.07 wt% of H_2SO_4, 210 °C, 2.5 ml/min of flow rate, and 30 min of reaction time using percolation reactor; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

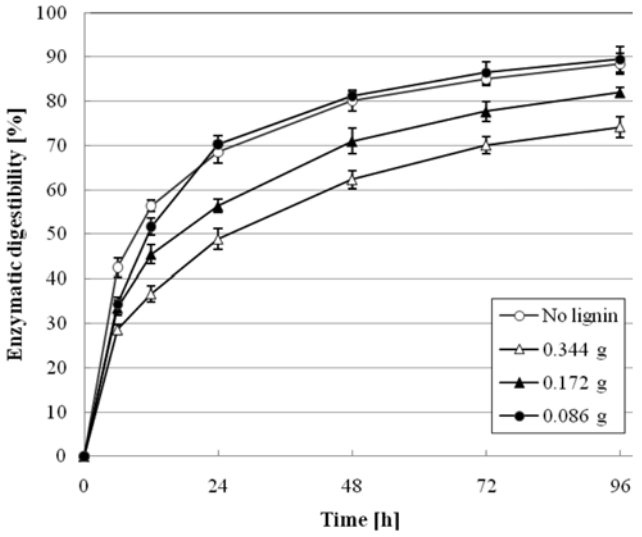

Fig. 2. Effect of WSL on enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; pretreatment conditions: water, 210 °C, 2.5 ml/min of flow rate, and 30 min of reaction time using percolation reactor; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

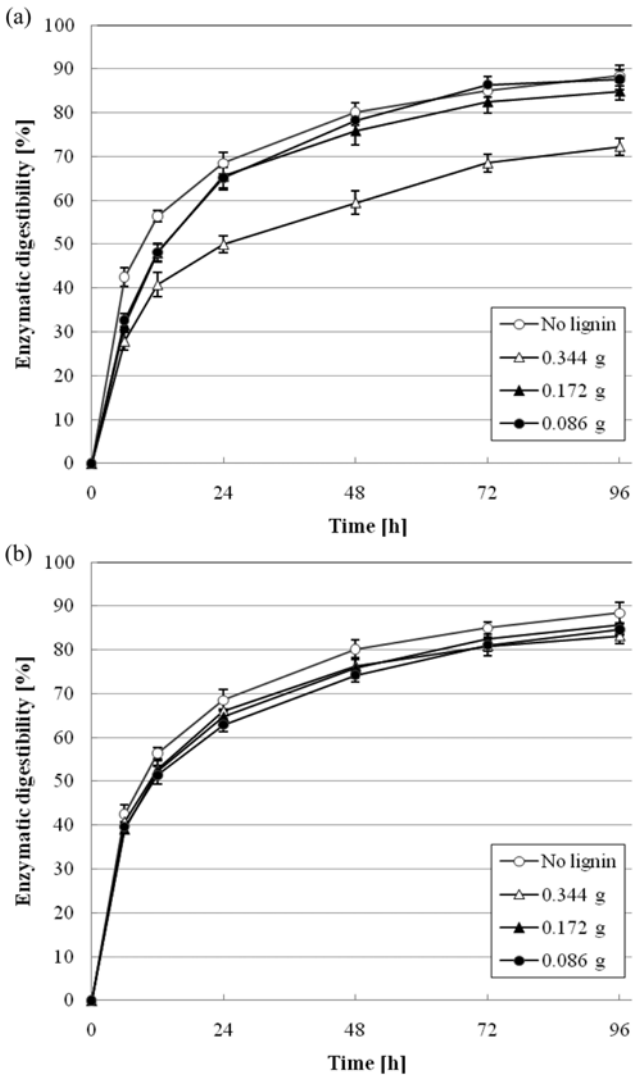

Fig. 3. Effects of ARP-SL and ARP-ISL on enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; pretreatment conditions: 15 wt% of ammonia, 170 °, 5.0 ml/min of flow rate, and 12 min of reaction time using percolation reactor; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

that enzyme digestibility was inhibited by ASL as the ASL loadings increased. However, the difference between highest and lowest digestibilities was only 8%.

1-2. WSL Effects on Enzyme Digestibility

Fig. 2 shows that the enzymatic hydrolysis of α-cellulose was inhibited as WSL loadings increased. Similar trend in ASL case (Fig. 1) was also observed in the case of digestibility test with addition of WSL. WSL decreased enzyme activity, and 72-h digestibilities with two high lignin loadings, 0.172 g-WSL/g-glucan and 0.344 g-WSL/g-glucan, were 78% and 70%, respectively. However, the inhibition effect of 0.086 g-WSL/g-glucan was minimal, which was about the same with that of α-cellulose. The gap between highest and lowest digestibilities was about 15%, which was higher than that of ASL case.

1-3. ARP-SL and ARP-ISL Effects on Enzyme Digestibility

Enzyme digestibility tests with ARP-SL and ARP-ISL were conducted and the results are presented in Fig. 3-1 and Fig. 3-2. Over the range of ARP-SL loadings from 0.086 g-ARP-SL/g-glucan to 0.172 g-ARP-SL/g-glucan, ARP-SL reduced the digestibilities less than 3% compared to that of α-cellulose (Fig. 3-1). However, with 0.344 g-ARP-SL/g-glucan loading, the initial hydrolysis rate was significantly decreased, resulting in 69% at 72 h of hydrolysis.

In the digestibility test with varying ARP-ISL loadings (Fig. 3-2), there was no significant difference, which resulted in similar digestibilities compared to that of control at all levels of lignin loadings. ARP-ISL was the residual lignin in solids after ARP pretreatment. It may be concluded that the insoluble lignin after ARP pretreatment does not significantly affect the enzymatic hydrolysis in these test conditions compared to the other lignins. On the other hand, the decreases of enzymatic hydrolysis rate were obviously observed in the test with addition of other soluble lignins, such as ASL, WSL, and ARP-SL (Fig. 1-3).

1-4. AL Effects on Enzyme Digestibility

Digestibility test of α-cellulose upon different Sigma AL loading is summarized in Fig. 4. It was observed that the AL, black fine powder form, was dissolved into the water within 12-24 hours. The enzymatic digestibility with 0.344 g-AL/g-glucan loading was 66%. As compared to the digestibility of control, the 72-h digestibility test with 0.344 g-AL/g-glucan lignin loading was decreased by 20%, while the addition of 0.086 g-AL/g-glucan case affected hydrolysis rate unrecognizably. In general, as the AL loadings increased, the digestibility of samples decreased. The result in this test using AL was very similar to the result in the test using ARP soluble lignin in Fig. 3-1. It is probable that these two alkaline soluble lignins had similar characteristics and inhibitory effects on enzymatic hydrolysis using cellulase enzyme.

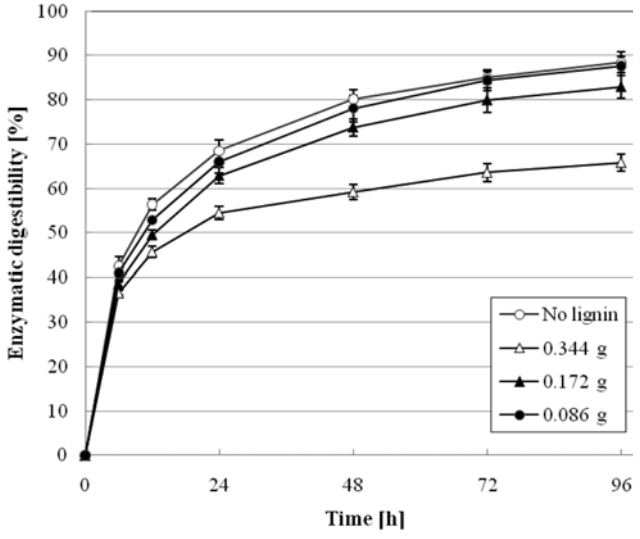

Fig. 4. Effect of AL on enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

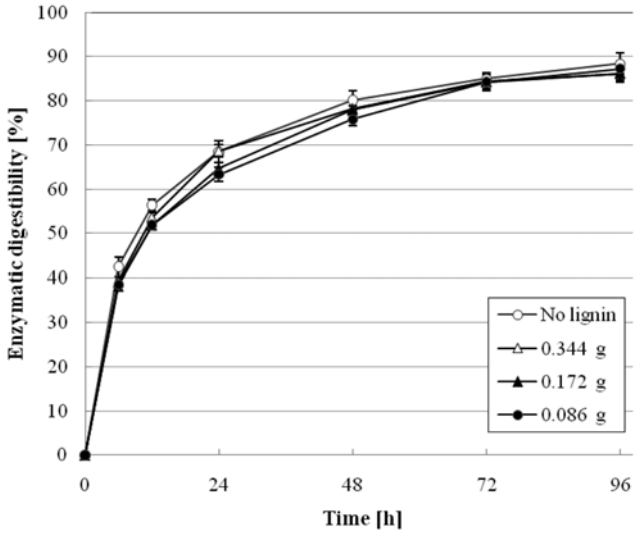

Fig. 5. Effect of OL on enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

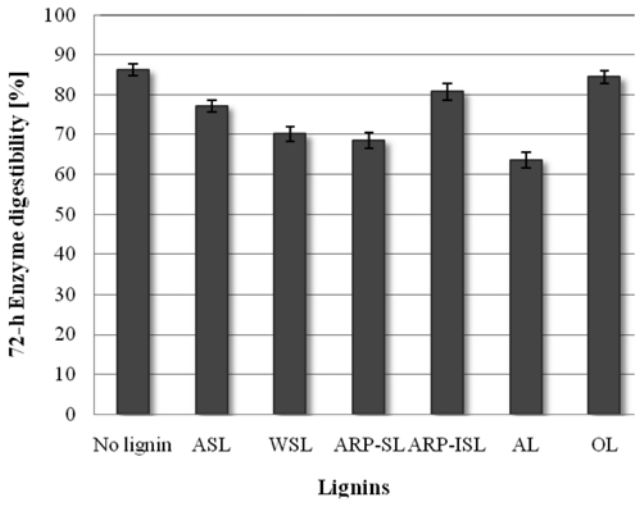

Fig. 6. Comparison of various lignin effects on 72-h enzymatic digestibility. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; all numbers in legend are gram of lignin loading per reactor; values are given as mean±SD (n=3).

1-5. OS Effects on Enzyme Digestibility

Like other previous tests, three different loadings of OSL were tested and the results presented in Fig. 5. It clearly indicated that the OSL did not have significantly negative effect on the enzymatic hydrolysis of cellulose, even at high OSL loadings.

2. Comparison Between the Various Lignins (in Relation to Pretreatments)

Fig. 6 summarizes 72-h enzymatic digestibilities of various lignin types with 0.344 g of lignin loading. OSL yielded a negligible effect on the enzymatic hydrolysis. In Fig. 6, AL, ARP-SL, WSL, ASL, ARP-IS, and OSL were the order of lignins upon its inhibitory effects, and their 72-h digestibilities were 64, 69, 70, 77, 81, and 84%, respectively. It showed that two alkaline soluble lignins, AL and ARP-SL, had the highest inhibitory effects among various lignins. However, the hydrolysis rate of AL case was slightly higher than that with WSL and ARP-SL before 24 h of hydrolysis (Fig. 2, Fig. 3-1, and Fig. 4). Fast initial rate in spite of AL addition can be explained as follows: AL was not dissolved well in the liquid; therefore, AL had no interaction with enzyme until AL powder became soluble in 24 h. It also seemed that the effects of WSL and ARP-SL on the cellulose hydrolysis were relatively significant, while the effect of ARP-ISL was small. It was inferred that ARP-ISL recovered after ARP treatment did not significantly affect the enzymatic hydrolysis; therefore, the high digestibilities of ARP-treated biomass samples in previous reports may be attributed to this reason that the remaining lignin part in solid may exist as inactive form [24-30]. ASL also suppressed the enzyme activity and it reduced 9% of 72-h digestibility, which was not significant as compared to 18-23% of ARP-SL and AL. It was supposed that the alkali- or hot water treatment induced lignin had higher relativities on enzymes.

3. Effects of Various Pretreatment on Corn Stover Compositions

Corn stover was pretreated using three different pretreatment methods including DSA, HW, and ARP and their pretreatment conditions are described in Materials and Methods section. Compositional changes and enzymatic digestibilities of solid and liquid samples are presented in Table 1. For enzymatic digestibility upon three different pretreatment methods, all pretreatment methods enhanced enzymatic digestibility remarkably as compared to 15.7-21.2% of untreated corn stover with 15-60 FPU/g-glucan enzyme loading. It should be noted that DSL pretreatment also improved enzymatic digestibility significantly at lower enzyme dosage even though digestibility test resulted in minimal inhibition effect by isolated ASL shown in Fig. 2. It was also seen that hot water treatment increased the enzymatic digestibility up to 89% with 15 FPU of cellulase/g-glucan loading (Table 1). Both DSL and HW pretreatments have been known as effective methods for hemicellulose removal from biomass.

It was reported that the hemicellulose inhibits the enzymatic reaction [6,38,39]. Along this line, it was assumed that xylan removal was responsible for the enhanced digestibility in the DSL and HW treated corn stover. If the lignin was a main factor hindering the enzymatic reaction, alkali treatment was more effective pretreatment method than acid treatment. Kim et al. also reported that the lignin-free corn stover improved the digestibility of corn stover than that of xylan-free corn stover [25].

4. Effect of ARP-SL on Enzymatic Hydrolysis of ARP-treated Corn Stover

Enzymatic hydrolysis test was conducted using actual pretreated

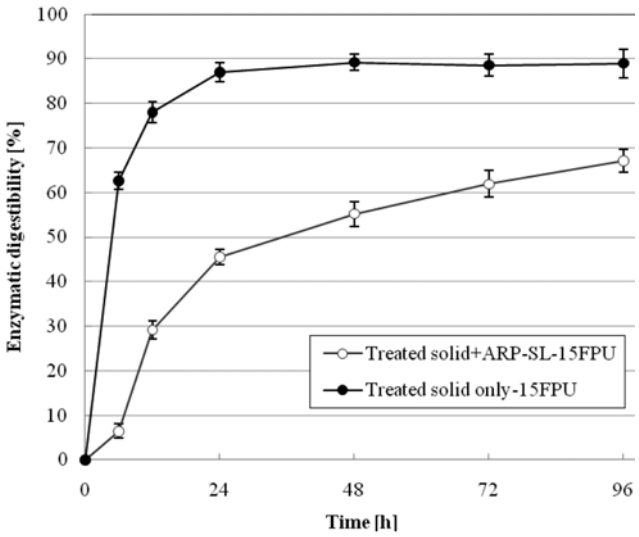

Fig. 7. Digestibility test of ARP-treated corn stover with ARP-SL. Enzymatic hydrolysis conditions: 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm; all numbers in the legend are gram of lignin loading per reactor; pretreatment conditions: 15 wt% of ammonia, 170 °C, 5.0 ml/min of flow rate, and 12 min of reaction time using percolation reactor; values are given as mean±SD (n=3).

Table 1. Effect of various pretreatments on compositions of solids and liquids, and enzymatic digestibility of treated-solids[a]

Pretreatment	Solid				Liquid		Total		Digestibility[d]	
	S.R.[b] [%]	Lignin[c] [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	60 FPU [%]	15 FPU [%]
Untreated	100.0	17.2	36.1	21.4	-	-	36.1	21.4	21.2	15.7
DSA	49.8	12.5	31.9	0.2	4.8	8.5	36.7	8.7	89.9	87.6
HW	56.9	13.6	35.4	1.0	1.2	11.1	36.5	12.1	93.6	88.9
ARP	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	95.3	90.1

[a]Carbohydrate and lignin contents are expressed as wt% of oven-dry untreated biomass; The data in the table show the mean value (n=2, SD< 0.3 for glucan and xylan, SD<0.5 for lignin (SD; standard deviation)

[b]S.R. stands for solid remaining after reaction

[c]Lignin=acid soluble lignin+acid insoluble lignin

[d]Enzymatic hydrolysis conditions: 60 or 15 FPU/g-glucan, 0.05 M citrate buffer, pH 4.8, 50 °C, 150 rpm

biomass (ARP-treated corn stover) and ARP-SL. The ARP-SL of 0.344 g was added to 1.0 gram glucan in a flask. The digestibility test results are summarized in Fig. 7. ARP-treated corn stover with no effluent was also put through the same test as control. The digestibility with the ARP effluent was substantially lower than that of ARP-treated corn stover with no effluent. The initial hydrolysis rate of enzymatic hydrolysis proceeded much slower than that of sample with effluent; the 6-h digestibility reached only 6.4% as opposed to 62.6% of sample without the effluent (Fig. 7). The 72-h digestibility (61.9%) was also lower than 88.5% of ARP-treated solid only case. Results in Fig. 7 confirmed that the enzyme reaction of pretreated biomass was also inhibited by the soluble lignin of the ARP effluent, which reduced mainly the hydrolysis rate.

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NOMENCLATURE

Abbreviations of various pretreatment methods and lignins are as follows:

ARP : ammonia recycle percolation
DSA : dilute sulfuric acid
HW : hot-water soluble
ARP-SL : soluble lignin separated by ARP pretreatment of corn stover
ARP-ISL : insoluble lignin separated by ARP pretreatment of corn stover
AL : alkali-lignin purchased from Sigma
ASL : corn stover derived soluble lignin separated by DSA pretreatment
WSL : corn stover derived soluble lignin separated by HW pretreatment
OSL : organo-solv lignin purchased from Sigma-Aldrich

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