Study on a New Preparation of D-Glucose Rich Fractions from Various Lignocelluloses through a Two-step Extraction with Sulfuric Acid

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Abstract: Preparing D-glucose with comparatively high yield and purity from many kinds of lignocelluloses through a basically unified method is an important technology because almost all of the yeasts show limitative use of carbon sources in ethanol fermentation. Although cellulase is of great interest in the production of D-glucose, the enzyme degradation demands a pre-treatment for loosening the hard structure of lignocelluloses. A typical acid hydrolysis of lignocelluloses digesting directly to monosaccharides is also used; however, the hydrolysate contains not only a mixture of pentose and hexose but also some chemicals inhibiting the growth of yeast. Therefore, representative samples of hardwood (Japanese oak), softwood (Japanese cedar) and annual plants (sugar cane bagasse) were examined in order to look for a convenient method of collecting oligosaccharides from these biomasses employing the concept that high-molecular-weight molecules with hard structure are gradually loosened and then degraded into low-molecular-weight molecules with strong alkaline or strong acid under mild conditions. Potassium hydroxide was suitable for the annual plant but insufficient for wood in the segregation of hemicellulose and cellulose. Concentrations of sulfuric acid for extracting selectively the majority of hemicelluloses from annual plants and wood were 42.5 and 44% (v/v), respectively. Lignocellulose (100 mg) was treated with the concentrated sulfuric acid (10 mL) at 20°C for 5 h under 120 rpm shaking condition. A treatment with 55% (v/v) sulfuric acid of the residual material under the same conditions was appropriate for preparation of oligosaccharides composed chiefly of D-glucose.

Key words: D-glucose, lignocellulose, sulfuric acid, wood, annual plant

From misgivings about global warming, lignocelluloses have been sought for use as renewable sources of energy. Lignocellulosic materials are chiefly composed of cellulose, hemicellulose and lignin.¹⁾ For their conversion into fuel ethanol, a method degrading the very hard structure of lignocelluloses is an important strategy and most of the prevailing research is characterized by the process of saccharification.²⁻⁴⁾ In the case of low concentrations of acid (0.5–15%), biomass was treated at high pressure (10–15 atm) and high temperature (100–250°C) and also at low temperature (35–45°C) in the case of a high concentration of acid (70–80%).⁵⁻¹⁰⁾ Lignocelluloses of softwood, hardwood and annual plants differ in components and conformations from each other;¹¹⁾ however, it is generally thought that ratios of hemicellulose, cellulose and lignin are approximately 3 : 4 : 3 for wood and 3 : 4 : 2 for annual plants.^{12,13)} The remarkable difference among hardwood, softwood and annual plants is observed in the sugar components of hemicelluloses.

From recent environment problems, efficient utilizations of building wastes, agricultural residues such as straw of rice, bagasse of sugar cane and thinnings of trees like Japanese cedar and cypress have been noticed increas-

ingly. From the point of view concerning the diversity of plant biomass, a uniform method for preparing D-glucose rich fractions from various lignocelluloses might be a useful technology because a large part of yeasts are able to ferment only a few saccharides like D-glucose and sucrose to alcohol. In this study, model lignocelluloses of hardwood, softwood and annual plant residue were examined under various conditions with inorganic matter in order to find a new method of obtaining D-glucose with high purity from the biomass remnants.

MATERIALS AND METHODS

Lignocellulose materials. Japanese oak (*Quercus serrata*) as a typical hardwood, Japanese cedar (*Cryptomeria japonica*) as a typical softwood and sugar cane bagasse (SCB) as a typical annual plant residue obtained from Oji Forest and Product Co., Ltd., Tokyo were used. Their powdery samples with around 8% moisture content and approximately 500 µm chip size were examined. In the case of SCB, the sample (100 g) suspended in 500 mL of 75% (v/v) ethanol was shaken at 120 rpm at 30°C overnight in order to eliminate sucrose remaining in the bagasse. The sucrose-free SCB (SCB-s) was filtered and then lyophilized. The amount of sucrose in SCB was approximately 20% (w/w).

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Alkaline treatment of lignocellulose in various concentrations. In a Teflon tube (50 mL), 100 mg of lignocellulose was suspended in 10 mL of potassium hydroxide solution of various concentrations from 10–30% (w/v) and shaken at 120 rpm at 30°C for 72 h. The tube was chilled in an ice bath to stop the reaction and then centrifuged at 6000 rpm at 0°C for 30 min. One mL of the each supernatant was neutralized with 10% (v/v) sulfuric acid and then the amounts of total sugar (TS) estimated by phenol sulfuric acid method¹⁴⁾ and the reducing strength of sugar (RS) by the modified Park-Johnson method.¹⁵⁾ The values of average degree of polymerization (DP) were estimated by dividing the value of TS by that of RS.

Acid treatment of lignocellulose in various concentrations. In a Teflon tube (50 mL), 100 mg of lignocellulose was suspended in 10 mL of sulfuric acid solution of various concentrations from 10–60% (v/v) and shaken at 120 rpm for 5 h. A series of tests was conducted both at 20 and 30°C. The tube was chilled in an ice bath to stop the reaction. The samples treated with acid concentrations higher than 45% (v/v) were mixed with 10 mL of water because of the gelatinous liquid. All samples were centrifuged at 6000 rpm at 0°C for 30 min. One mL of the each supernatant was neutralized with 2 N sodium hydroxide and the amounts of TS and RS were estimated and DP values were calculated.

Analysis. The neutral sugars of D-glucose, D-xylose, D-mannose, L-arabinose and D-galactose were analyzed by GC/Mass chromatography (GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan). Derivatives of alditol acetates were prepared as described by Blakeney *et al.*¹⁶⁾ Chromatographic separation was performed with a 30 m × 0.25 mm SPTM 2380 fused SILICA capillary column operating with 1 mL/min helium as the carrier gas. The temperature was kept at 210°C for 3 min and heated up to 240°C within 2 min, at an increasing rate of 15°C/min and that continued for 7 min to complete the total analysis time of 12 min.

RESULTS AND DISCUSSION

Characteristics of saccharides obtained by alkaline treatment.

As the ratio of hemicellulose and cellulose in lignocelluloses is generally 3 : 4, the amount of hemicellulose and that of cellulose could be estimated roughly as 30 and 40 mg, respectively, in 100 mg of sample. As shown in Fig. 1A, the TS amounts of SCB-s (annual plant residue) showed around 35–38% (w/w) in all potassium hydroxide solutions tested and it could be thought that almost all hemicellulose was extracted. The average DP showed values of around 8–11 (Fig. 1B). On the other hand, the TS amounts for Japanese oak (hardwood) and Japanese cedar (softwood) were 17–20% (w/w) and 16–18% (w/w) in all potassium hydroxide solutions tested, respectively (Fig. 1A). It could be suggested that even strong alkaline treatment could not extract hemicelluloses sufficiently in the case of wood. Even longer treatment for one week at 30°C and stronger treatment at the high temperature of 70°C did not remarkably increase the TS amount (data not shown). The average DP showed comparatively large values of around 14–16 (Fig. 1B). The results suggested that

Fig. 1. Total sugar amount % (w/w) (A) and average DP value (B) of saccharides extracted from lignocelluloses treated with various concentrations of potassium hydroxide.

Lignocelluloses 100 mg each of Japanese oak (hatched), Japanese cedar (white) and SCB-s (black) were treated with 10–30% (w/v) potassium hydroxide (10 mL) at 30°C for 72 h under 120 rpm shaking condition (Data are average of two separate experiments).

the treatment of strong alkaline might be segregate hemicellulose and cellulose for annual plant residues but was inadequate for woods.

Characteristics of saccharides obtained by acid treatment.

As shown in Figs. 2A and 3A, the TS amounts showed unique rising patterns with the first increase at 30.0–42.5% (v/v) sulfuric acid concentrations and the second increase at 45.0–55.0% (v/v) sulfuric acid concentrations and it could be thought that there were two kinds of extracted materials. Up to around 42.5% (v/v) sulfuric acid, it could be suggested that lots of chemical linkages of hemicellulose were broken preferentially and those of cellulose were substantially kept. At the second sharp increase of TS amount, crystalline lattices of cellulose were disrupted and converted into a completely amorphous state forming a gelatinous substance.¹⁷⁾ It could be thought that cellulose was extremely susceptible to hydrolysis of sulfuric acid at concentrations higher than 60.0% (v/v).

Regarding the treatment at 20°C, the TS amount of SCB-s was 38% (w/w) on the treatment of 42.5% (v/v) sulfuric acid; it could be suggest that almost all hemicellulose was extracted from SCB-s. On the other hand, the

Fig. 2. Total sugar amount % (w/w) (A) and average DP value (B) of saccharides extracted from lignocelluloses treated with various concentrations of sulfuric acid at 20°C.

Lignocelluloses 100 mg each of Japanese oak (hatched), Japanese cedar (white) and SCB-s (black) were treated with 10–60% (w/v) sulfuric acid (10 mL) at 20°C for 5 h under 120 rpm shaking condition (Data are average of two separate experiments).

TS amount for woods was around 22–25% (w/w) on the treatment of 42.5% (v/v) sulfuric acid and that of 45.0% (v/v) sulfuric acid was around 37.5–39.0% (w/w) (Fig. 2A). For the extraction of saccharides derived from hemicelluloses of woody biomass, it could be suggest that 44.0% (v/v) sulfuric acid was suitable to achieve the expected TS amount of 30% (w/w) according to the general ratio of sugar composition. These suggestions could be confirmed from the data of their sugar components described later (see Table 2).

TS amounts for 55% (v/v) sulfuric acid at 20°C showed the highest values of 71, 70 and 76% (w/w) for Japanese oak, Japanese cedar and SCB-s, respectively (Fig. 2A). It could be recognized that these values were very similar to total amounts of saccharides derived from both hemicellulose and cellulose.¹⁸⁾ The average DP values of saccharides obtained under the condition at 20°C were 1.5–2.7 for SCB-s and 1.7–3.8 for woods (Fig. 2B), and those of saccharides obtained under the condition at 30°C were 1.0–1.8 for SCB-s and 1.1–2.9 for woods (Fig. 3B). TS amounts on the treatment of 55% (v/v) sulfuric acid at 30°C fell slightly to 66, 68 and 73% (w/w) for Japanese oak, Japanese cedar and SCB-s, respectively (Fig. 3A), suggesting over-decomposition of hemicellulosic sugars. The treatment of 50% (v/v) sulfuric acid at 30°C did not show so high TS amounts as 55% (v/v) sul-

Fig. 3. Total sugar amount % (w/w) (A) and average DP value (B) of saccharides extracted from lignocelluloses treated with various concentrations of sulfuric acid.

Lignocelluloses 100 mg each of Japanese oak (hatched), Japanese cedar (white) and SCB-s (black) were treated with 10–60% (w/v) sulfuric acid (10 mL) at 30°C for 5 h under 120 rpm shaking condition (Data are average of two separate experiments).

furic acid at 20°C. So, it could be thought that the 55% (v/v) sulfuric acid is an appropriate concentration for cellulose extraction under the treatment at 20°C for 5 h.

Tentative test of the two-step extraction.

As the first step, SCB-s (100 mg) was suspended in 42.5% (v/v) sulfuric acid (10 mL), and 100 mg each of hard and soft woods was also done in 44% (v/v) sulfuric acid (10 mL). The reaction mixtures were subjected to 120 rpm shaking condition at 20°C for 5 h and then centrifuged at 6000 rpm at 0°C for 30 min. TS amounts of the supernatants of Japanese oak, Japanese cedar and SCB-s were 28.0, 29.0 and 37.0% (w/w), respectively and their average DP values were 2.7, 3.1 and 2.0, respectively (Table 1).

As the second step, the precipitates were re-suspended in 55% (v/v) sulfuric acid (10 mL) and subjected to 120 rpm shaking condition at 20°C for 5 h. Reaction mixtures were mixed with water (10 mL) under chilled condition and then centrifuged at 6000 rpm at 0°C for 30 min. TS amounts of the resulted supernatants were 46.2, 44.6 and 40.0% (w/w), for Japanese oak, Japanese cedar and SCB-s respectively and their average DP values were 3.2, 3.5 and 2.7, respectively (Table 1). Sugar components of each extraction were analyzed for further verification.

Table 1. Total sugar (TS) amounts and average DP values of oligosaccharides derived from hemicellulose and cellulose obtained by the two-step extraction with sulfuric acid and suitable condition for preparation of their monosaccharides.

Temperature (°C)	Saccharification of hemicellulose oligosaccharide obtained by the first-step extraction												Saccharification of cellulose oligosaccharide obtained by the second-step extraction											
	Japanese oak				Japanese cedar				SCB-s				Japanese oak				Japanese cedar				SCB-s			
	0 h	2 h	4 h	6 h	0 h	2 h	4 h	6 h	0 h	2 h	4 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h
TS % (w/w)	28.0	27.8	27.6	27.5	29.0	28.6	28.5	28.5	37.0	36.4	34.7	33.7	46.2	46.0	45.5	44.6	44.5	44.0	40.0	39.4	38.3			
50 Average DP value	2.7	2.1	1.8	1.7	3.1	2.7	2.3	2.1	2.0	1.3	1.2	1.1	3.2	1.8	1.6	3.5	1.7	1.4	2.7	1.5	1.2			
TS % (w/w)	28.0	27.3	26.7	26.2	29.0	28.2	27.6	27.1	37.0	34.7	34.2	33.0	46.2	45.8	45.2	44.6	44.0	43.6	40.0	37.6	36.8			
60 Average DP value	2.7	1.3	1.2	1.1	3.1	1.4	1.2	1.1	2.0	1.2	1.1	1.0	3.2	1.3	1.2	3.5	1.2	1.2	2.7	1.2	1.1			
TS % (w/w)	28.0	24.7	23.6	23.2	29.0	21.5	20.7	19.4	37.0	34.0	32.4	31.3	46.2	44.9	44.1	44.6	43.4	41.5	40.0	36.0	34.2			
70 Average DP value	2.7	1.2	1.2	1.0	3.1	1.3	1.2	1.0	2.0	1.1	1.0	1.0	3.2	1.2	1.0	3.5	1.1	1.0	2.7	1.1	1.0			

Table 2. Properties of hemicellulosic and cellulosic oligosaccharides obtained by the two-step extraction with sulfuric acid from lignocelluloses of Japanese oak, Japanese cedar and SCB-s.

Neutral sugar (%)	Hemicellulosic oligosaccharide (first-step extraction)			Cellulosic oligosaccharide (second-step extraction)		
	Japanese oak	Japanese cedar	SCB-s	Japanese oak	Japanese cedar	SCB-s
L-Arabinose	2.2	8.4	6.3	ND	ND	0.3
D-Xylose	67.4	37.3	84.6	2.5	3.5	4.3
D-Mannose	0.5	29.0	2.0	ND	3.7	ND
D-Galactose	8.3	5.5	ND	ND	ND	ND
D-Glucose	21.6	19.8	7.1	97.5	92.8	95.4

ND, Not detected.

Determination of appropriate saccharification condition of the oligosaccharide.

The obtained supernatant (5 mL) of the first-step extraction (oligosaccharides of hemicellulose) was mixed with 2.5 mL of water gradually to drop the acid concentration for the following saccharification. The obtained supernatants (10 mL) of the second-step extraction (oligosaccharides of cellulose) were also used for the saccharification. The solutions were saccharified under various conditions combining reaction temperatures from 50°C to 70°C and reaction times from 2 h to 6 h by shaking at 120 rpm in a water bath. Appropriate saccharification conditions were chosen considering synthetically both the average DP values and TS amount (Table 1). They were decided to be at 60°C for 2 h for oligosaccharides of hemicellulose and at 60°C for 6 h for those of cellulose.

Sugar components of oligosaccharides obtained by the two-step extraction.

From data for the first step extraction in Table 2, it was found that oligosaccharides of Japanese oak (hardwood) and SCB-s (annual plant) were mainly composed of D-xylose and that of Japanese cedar (softwood) composed of D-xylose, D-mannose and D-glucose. The principal component of hemicellulose in hardwoods and annual plants is glucuronoxylan and that of softwoods is glucomannan.¹⁹⁾ A higher amount of arabinose was observed both in Japanese cedar and SCB-s compared to the Japanese oak. Hemicelluloses of softwood and annual plants differ from that of hardwood in the occurrence of arabinofuranose

units linked α -(1→3) to the xylan backbone.¹⁹⁾ SCB-s showed minor amounts of D-galactose and D-mannose.²⁰⁾ These results showed that the oligosaccharides of first-step extraction were derived mainly from their hemicellulose portions. In fact, minor amount of glucose from amorphous cellulose may be incorporate with this fraction.

From data for the second step extraction shown in Table 2, oligosaccharides of Japanese oak, Japanese cedar and SCB-s were mainly composed of D-glucose (>90%), supporting the assumption that they were oligosaccharides derived mainly from cellulose portions. These results suggested that the two-step extraction of biomass could be used to obtain two kinds of oligosaccharides derived mainly from hemicellulose and cellulose.

There are two famous technologies for degradation of lignocellulose with sulfuric acid. One method with dilute sulfuric acid is based on a treatment using high temperature and high pressure and degrades mainly hemicelluloses. Some hemicellulosic sugars are fermented to bio ethanol and the residual cellulose is further digested with enzymes for subsequent ethanol fermentation.²¹⁾ Another method with concentrated sulfuric acid is based on a treatment using 70–80% sulfuric acid at low temperature (35–45°C) and degrades both hemicellulose and cellulose.²²⁾ So, a mixture of pentoses and hexoses is obtained. As hardly any yeasts can use D-xylose for ethanol fermentation, it is more desirable to take measures against D-glucose. The method in this paper is close into part both methods described above. The concentrations of sulfuric

acid, treatment condition and temperature are similar to those of the concentrated sulfuric-acid method and the separation of component sugars from hemicellulose and cellulose is similar to the dilute sulfuric-acid method. It could be estimated that almost all lignin might be left in the residue from the residual color and yields of sugars. From these comparisons, this method has one merit in the separated preparation of component sugars relating hemicellulose and cellulose and another merit of using low temperature and atmospheric pressure.

As provision for the problem of global warming, ethanol fermentation from agricultural residues such as straw of rice and bagasse of sugar cane, thinnings of Japanese cedar and cypress have attracted interest. From the point of view of efficient utilization of remnants of various biomasses, a uniform method for preparing D-glucose might be a useful technology. So, it could be proposed that 43.5% (v/v) sulfuric acid for the first extraction and 55.0% (v/v) sulfuric acid for the second extraction is appropriate for the treatment of all biomass hardwood, softwood and annual plants. The residual acid after the acid treatment and saccharification is removed by ion exchange chromatography to obtain sugars comprising mainly D-glucose for ethanol fermentation as described in our next paper.²³⁾

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リグノセルロースの硫酸 2 段階抽出から純度の高いグルコース画分を得る新しい調製法の研究

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酵母がエタノール発酵できる糖は限られているため、基本的に統一された方法により、多くのリグノセルロースから純度が高く収率もよいグルコースを調製する技術は重要である。セルラーゼはグルコース生産において注目されているが、酵素分解には硬質なりグノセルロースを柔らかくする前処理が必要である。酸加水分解で単糖にまで分解する方法もあるが、リグノセルロースは5単糖と6単糖の混合物であることのほかに、酵母生育阻害物質も含んでいる。そこで、広葉樹(コナラ)、針葉樹(スギ)、一年草(バガス)をモデル試料とし、マイルドな条件下で強アルカリや強酸を使用し、硬質な高分子を緩やかにほぐして低分子化するという考えに基づき、これらのバイオマスからオリゴ糖を調製する方法を見つけることを目的として研究を行った。その結果、ヘミセルロースとセルロースを分けるためには、アルカリ処理は一年草には適用できたが木材には不十分であった。それに対し硫酸処理は、一年草には42.5%硫酸が、木材には44%硫酸がヘミセルロース系オリゴ糖を抽出するのに優れていた。さらにこの処理の残渣を55%硫酸で処理するとセルロース系オリゴ糖が抽出された。抽出されたそれぞれのオリゴ糖の糖組成分析から、前段処理で得られたオリゴ糖はヘミセルロース関連の単糖であること、単糖の種類も広葉樹、針葉樹、一年草の特長が出ていること、後段処理からは90%以上の純度があるグルコースが得られたことからセルロース系オリゴ糖であることが確認できた。