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Efficient Digestion and Structural Characteristics of Cell Walls of Coffee Beans

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Screening of effective food-processing cellulase for digestion of cell walls of coffee beans was carried out, and the cellulase from *Trichoderma* sp. was selected. The digestion of the cell walls of green and roasted coffee beans was carried out by sequential procedures of alkali boiling (0.1 M Na_2CO_3 buffer, pH 10, and 0.1 M NaOH), cellulase digestion, autoclaving with 0.1 M NaOH, and cellulase redigestion. The total digestion yields were >95 and >96%, respectively. The cell walls became thin, and the final residues of the cell walls were easily broken into small pieces. The neutral sugar analysis of the digestion or the extract and the residues and the microscopy observations with staining with toluidine blue *O*, Yariv reagent, and calcofluor for the residue in each step were investigated. Four structures, the galactomannan-cellulose (center part), the membrane of the arabinogalactan protein, the cellulose-rich galactomannan layer, and the arabinogalactan protein-rich layers (outer part), were found in the cell walls.

KEYWORDS: Coffee bean; cell wall; cellulase; enzymatic digestion

INTRODUCTION

Coffee is considered to be a major tasty drink. The worldwide production of coffee beans is >5 million tons (*1*). Coffee beans have insoluble and thick cell walls (>48% of the beans weight), which are mainly made of galactomannans, arabinogalactan, and cellulose (*2*−*10*). The cell wall has been studied, but its detailed composition is still unknown over the past 40 years (*2*, *8*). Analysis of the composition requires it to be solubilized or extracted, but the cell walls are hard to digest or solubilize. The residues after the coffee extraction, which are the cell walls, are also not able to be used (*8*). The residues and the wastes are difficult to treat (*12*, *13*), although mannan, galactomannan, and arabinogalactan of the cell walls are expected for use as a food material (*14*). Many researchers have been studying the cell walls. Recently, Oosterveld et al. reported in detail the chemical extraction of the green and roasted coffee beans, the compositions, and the changes in galactomannan, arabinogalactan, and cellulose or xyloglucan (*4*, *5*). Fischer et al. also reported the chemical extraction of the mannan and its structures (*3*). Redgwell et al. reported arabinogalactan or the arabinogalactan proteins using stepwise chemical and alkaline extraction, and the observation of some mannan layers of the cell walls using the mannan antibody (*6*, *8*, *9*). These results are important information about the cell walls of the coffee bean. However, the insolubility of the cell walls makes them difficult to analyze and use (*2*, *8*). Galactomannan is the main insoluble polymer of carbohydrates (*4*, *5*). Some arabinogalactans are tightly cross-linked with protein (*2*, *6*−*9*). Glucan is proposed to exist as a mannan-cellulose in the cell walls (*2*). These characteristic compositions and structures would prevent the digestion and extraction for a long time. We have been studying the efficient digestion of the soybean and its characteristics (*15*−*17*). Coffee is also a bean; thus, the principal structures of the cells are similar (*15*, *17*). We carried out an investigation to determine an effective treatment and enzyme screening for the efficient digestion of the green and roasted coffee beans and achieved efficient digestion of the cell walls (>95%). We also show an outline and the structural characteristics of the cell wall based on the results of the efficient enzymatic digestion, the sugar composition from the digestion, the extracts, and the residues or the microscopy observations of the residual cell walls using toluidine blue *O*, Yariv reagent, and calcofluor staining in each procedure. At least four structures, the galactomannan-cellulose (center part), the membrane of the arabinogalactan protein, the cellulose-rich galactomannan layer, and the arabinogalactan protein rich layers (outer part), were found in the cell wall.

MATERIALS AND METHODS

Green and Roasted Coffee Beans. Coffee beans (*Coffee arabica*), the roasted coffee beans, and their cracked and milled powders were gifts from UCC Ueshima Coffee Co., Ltd., Kobe, Japan. The defatted powdered samples were prepared with a 10-fold volume of hexane

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extraction at room temperature for 24 h. The sample was then washed with hexane and dried at room temperature.

Enzymes. Cellulase (for food-processing cellulase from *Trichoderma* sp., 5000 units/g) was a gift from Daiwa-Kasei Co., Ltd. (Osaka, Japan). Pectinase (Pectinex Ultra SP, from *Aspergillus aculeatus*; 26000 units/mL) and Cellclast 1.5L FG from *A. aculeatus* were gifts from Novozyme Japan. Cellulase XL531 from *Aspergillus niger* was a gift from Nagase chemtex, Japan. Cellulocine GM5, AC40, and HC100, cellulases from *Aspergillus* sp. and TP25 and T2 from *Trichoderma viride* were gifts from HBI, Inc., Japan. All other reagents were of reagent grade.

Screening of Enzymes. The roasted residues was autoclaved with 0.1 M NaOH at 121 °C for 60 min. The treated residues were washed with water and 0.1 M acetate buffer (pH 5.0) and placed in a 96-well microplate; a 1 or 5% enzyme solution (0.1 M acetate buffer, pH 5.0) was mixed at 40 °C overnight, and the collapse or any visual change was observed.

Digestion of the Cell Walls of Sliced Green Coffee Beans. The green coffee beans were autoclaved with water at 121 °C for 10 min. The cooled beans were sliced, and the sliced sections were autoclaved with water or 0.1 N NaOH at 121 °C for 10 min. The sections were incubated in an eppen-tube with 1% the selected cellulase from *Trichoderma* sp. at 40 °C overnight.

Time Course of the Cellulase Digestion of the Cracked Green Beans and the Roasted Coffee Residues. The cracked coffee beans or the residues were autoclaved with a 50-fold volume of 0.1 M NaOH (121 °C for 60 min), filtered, washed, and dried at 40 °C. The dried samples (2 g) and 0.1 M acetate buffer (pH 5.0, 16 mL) were added to a glass bottle (30 mL). The glass bottle was then incubated with stirring (1200 rpm) at 40 °C for 24 h with 1% cellulase. The reaction mixture was centrifuged (3000 rpm, 10 min), and the supernatant was analyzed.

Sequential Alkali Treatment and the Cellulase from *Trichoderma* sp. Digestion of the Milled and Defatted Powders of the Green and Roasted Coffee Beans. The sequential alkali-heated treatments and the cellulase digestions were as follows: for the first extraction, 2 g of the defatted powder of the green coffee beans or the roasted coffee beans and a 50-fold volume of 0.1 M Na_2CO_3 buffer (pH 10) were mixed, and the suspension was boiled for 20 min. The suspension was centrifuged at 3000 rpm for 10 min, and the residue was collected. The residue was mixed with a 50-fold volume of 0.1 M NaOH, and the suspension was boiled for 20 min; the residue was centrifuged and washed with water. For the first cellulase digestion, the collected residue was digested by 1% of the cellulase of *Trichoderma* sp. (0.1 M acetate buffer, pH 5.0) at 40 °C overnight with stirring. The residue was collected by centrifugation at 3000 rpm for 10 min. For the second alkali extraction, the residue was autoclaved with a 20-fold volume of 0.1 M NaOH (121 °C for 10 min). For the second same cellulase digestion, the residue and the 1% of the cellulase of *Trichoderma* sp. (0.1 M acetate buffer, pH 5.0) were mixed and incubated at 40 °C overnight with stirring again. The residual samples in each step were collected by centrifugation (3000 rpm for 10 min), washed with water, freeze-dried, and weighed. The differential weight in the each step was estimated as the extracts or the digestion weight. The supernatants of each step were used for the analysis of sugar and protein. Each residue was used for sugar analysis and microscopy observation.

Estimation of Sugar and Protein. The amount of uronic acid was measured according to the 3-phenyl phenol method (*18*). The total sugar was estimated by using the phenol−sulfuric acid method (*19*). The reducing sugar was estimated according to the Nelson-Somogyi method (*20*). Protein was estimated by using the Bradford method (*21*). Each amount was calculated using a colorimetric standard curve with D-galacturonic acid, D-glucose, and serum albumin as the standards.

Neutral Sugar Analysis. Analysis of the neutral sugars was done using the alditol−acetate method (*14*). The composition of the neutral sugar was analyzed by a GC system with a capillary column of DB-225 (J&W Co., 0.25 mm × 30 m) connected to a Yanaco G-2800 (Yanaco Co., Ltd., Kyoto, Japan). The standard solution was adjusted with a 1% solution of each of the seven kinds of following sugars: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose.

Staining. Toluidine blue *O* (1% in 0.1 M phosphate buffer, pH 7) (*22*), Yariv regent (0.1 M in 0.1 M phosphate buffer containing 0.3 M NaCl, pH 7) (*23*), and Calcofluor white were used for the staining reagents of the coffee residue (*24*). The β-glucosyl−Yariv reagent was obtained from Wako Pure Chemistry Co., Ltd., Osaka, Japan. Calcofluor white, BactiDrop, was obtained from Remel, Lenexa, KS.

Light and Fluorescent Microscopic Observation. The microscopic observations and photos were done using an Olympus BH-21 (Olympus Optical Co., Ltd., Tokyo, Japan) light microscope and a digital DP-II microscope photographic device. Fluorescent microscopic observations and photos were done using an Nikon Eclipse E-600 (Nikon Co., Ltd., Tokyo, Japan) fluorescent microscope and a digital Hamamastu ORCA-ER C4742-95 camera (Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan).

RESULTS

Screening of Cellulase. Nine kinds of cellulases as food-processing enzymes (1%, 0.1 M acetate buffer, pH 5.0) were mixed with the alkali-treated coffee residue (0.1 M NaOH, 121 °C for 60 min) at 40 °C overnight. Only the cellulase of *Trichoderma* sp. caused the collapse of residues. The cells were dispersed, and the cell walls were partially digested.

Cellulase from *Trichoderma* sp. Digestion of the Sliced Section of Green Coffee Beans. The selected cellulase (1%, from *Trichoderma* sp.) digestion was carried out for the sliced green coffee beans treated by autoclaving with water (121 °C for 10 min). The microscopy observations are shown in **Figure 1**. The autoclaving treatment effectively stopped the formation of the brown color, which inhibited the cellulase digestion. The body complexes of the cells were removed by slicing and autoclaving with water, and the cell walls were easily observed. The decomposition of the cell walls was recognized as the autoclaving treatment and the cellulase effectively digested the cell walls of the coffee beans. The cells kept their shape without a single cell formation after the autoclaving. The residual cell walls were not completely digested even by 5% cellulase. In the cell walls without the body complex or residual coffee brew, the autoclaving with water (121 °C for 10 min) was sufficiently effective for the digestion of the outer cell walls.

Cellulase from *Trichoderma* sp. Digestion of the Boiled or Autoclaved Sliced Section of the Green Coffee Beans with 0.1 M NaOH. The alkali boiling (0.1 M NaOH, 100 °C for 10 min) and the cellulase digestion produced a digestion similar to that shown in **Figure 1**, but the alkali autoclaving (0.1 M NaOH, 121 °C for 10 min) and the cellulase digestion could produce a finger-pressure breakup in the sliced cell walls of the green coffee beans (**Figure 2**). It was found that alkali autoclaving was efficient for the cellulase digestion and the breakup of the cell walls.

Time Course of the Cellulase from *Trichoderma* sp. Digestion of the Cracked Green Coffee Beans and the Roasted Coffee Residues. Figure 3 shows the time course of the selected cellulase from *Trichoderma* sp. digestion for the alkali-treated cracked green coffee beans and roasted coffee residues. The roasted one was well digested with a yield of 38.5 ± 1.84%, and the green coffee yielded 16.3 ± 0.53% within 24 h. The digestion of the roasted coffee beans was higher than that of the green coffee beans. The green coffee beans contained the body complex and the oil in the cells as ∼50% w/w, and each ratio of the digestion could be estimated to be the reasonable values. The cells and the body complex were dispersed in the reaction mixture, and the cell walls were partially digested and became thinner. The cell walls were collapsed and partially digested, but the body complex were not digested. The sugar degree of polymerization of the digestion

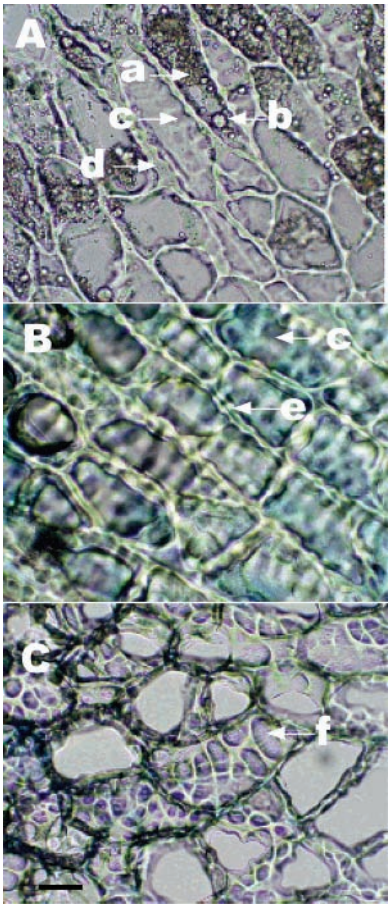

Figure 1. Microscopy of the cellulase digestion of the sliced green coffee. The green coffee bean was autoclaved (121 °C for 10 min) and sliced. The sliced section was autoclaved again with water (121 °C for 10 min). The section was dipped in the 1% cellulase solution (0.1 M acetate buffer, pH 5.0) at 40 °C overnight. The change of the section was observed under light microscopy: (A) slice of green coffee [body complex (a), oil drop (b), hole of the cell wall (c), and hump of the cell wall (d) were detected]; (**B**) slice without enzyme solution [body complexes of cells were removed by slicing and autoclaving; emphasis of shading was done by toluidine blue *O*; cell walls were firmly attached to each other (e), and single cell formation was not produced by autoclaving]; (**C**) slice autoclaved (121 °C, 10 min) and digested by cellulase (f) [only the outer part of the cell wall was digested]. Black bar represents 5 μm.

were estimated as 2.2 and 2.5, respectively. Small amounts of uronic acid were detected. Mannose, galactose, glucose, and arabinose were continuously released for the digestion. The neutral sugars from the final digestion of the green coffee beans and the roasted coffee residues were mannose (33.7 and 48.4 mol %), galactose (25.4 and 24.6 mol %), glucose (26.6 and 18.3 mol %), arabinose (12.3 and 8.6 mol %), and rhamnose (2.0 and 0 mol %).

Sequential Alkali Treatment and Cellulase Digestion of the Milled and Defatted Powder of the Green Coffee Beans. The results are summarized in **Table 1**. The first alkali treatment produced a strong brownish green extraction, and the treatment effectively dissolved and removed the body complex of each cell. Half the weight was then solubilized (53.7%) in the result. The subsequent cellulase digested the outer layer of the cell walls. Mannose, galactose, glucose, and arabinose were found in the supernatant of the digestion. The residues were rich in galactose and mannose; however, glucose was not detected from the short hydrolyzation (2 h) with 2 M trifluoroacetic acid, but the long hydrolyzation (24 h) provided the glucose. The second

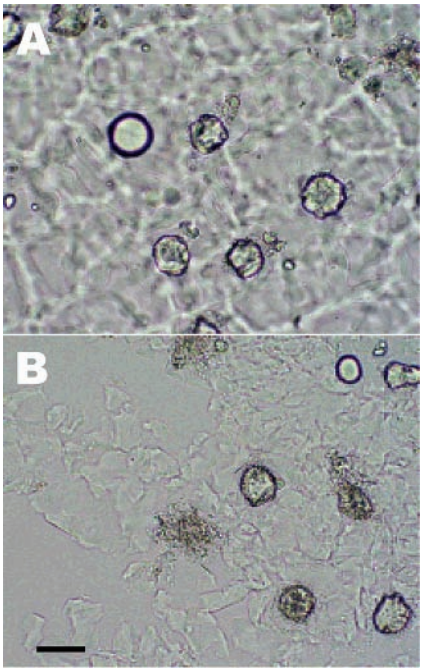

Figure 2. Breaking up of the cell walls of the sliced green coffee beans. The green coffee bean was autoclaved (121 °C for 10 min) and sliced. Sliced green coffee beans were autoclaved at 121 °C for 10 min with 0.1 M NaOH and washed with 0.1 M acetate buffer (pH 5.0). The sliced sample was treated with 5% of the *Trichoderma* sp. cellulase at 40 °C overnight. The cell walls were changed to transparent ones by keeping some layers (**A**), and the cell walls were easily broken by only finger pressure (**B**). The spheres are alkali-denatured residual body complexes in the cells and oil drops (**A**); some pressed and broken body complexes were also found (**B**). Black bar represents 5 μm.

autoclaving with alkali was essential for the effective second cellulase digestion, but the boiling with alkali could not produce an effective second cellulase digestion. The second alkali treatment provided arabinose and galactose in the extraction, and the ratio was found to be 1:3.8. The ratio of the residues was 1:2.5. Boiling with the same alkali concentration also produced arabinose and galactose in the same ratio. The secondary cellulase digestion caused fractionation of the residual cell walls with only finger pressure, and the secondary cellulase digestion caused the release of mannose, galactose, and glucose. The final residue contained mannose, galactose, and glucose. The ratio of arabinose and galactose was 1:3.7. The glucose and mannose were found in the ratio 1:17.4. The total extraction and digestion were achieved with the yield of 95.0%.

Sequential Alkali Treatment and Cellulase Digestion of the Milled and Defatted Powder of the Roasted Coffee Beans. Table 2 shows the results of the same sequential procedures used for **Table 1** carried out for the milled and defatted roasted coffee beans. The conditions of the extraction and the digestion were the same as those for the green coffee. The weight losses in each procedure were similar. The extraction and digestion were achieved in a yield of 96.1%. Comparison of the results of the roasted coffee residues and green coffee beans (**Table 1)** showed that the general results were similar. The extractions by autoclaving with 0.1 M NaOH were rich in mannose, but arabinose was not detected. The first cellulase digestion was rich in glucose. Arabinose was not found in the second alkali extraction, but galactose was found. The arabinose and galactose ratio of the residue after the secondary alkali treatment was 1:2.6. The ratio of the neutral sugar in the final residue was similar to that in the green coffee beans. The ratio

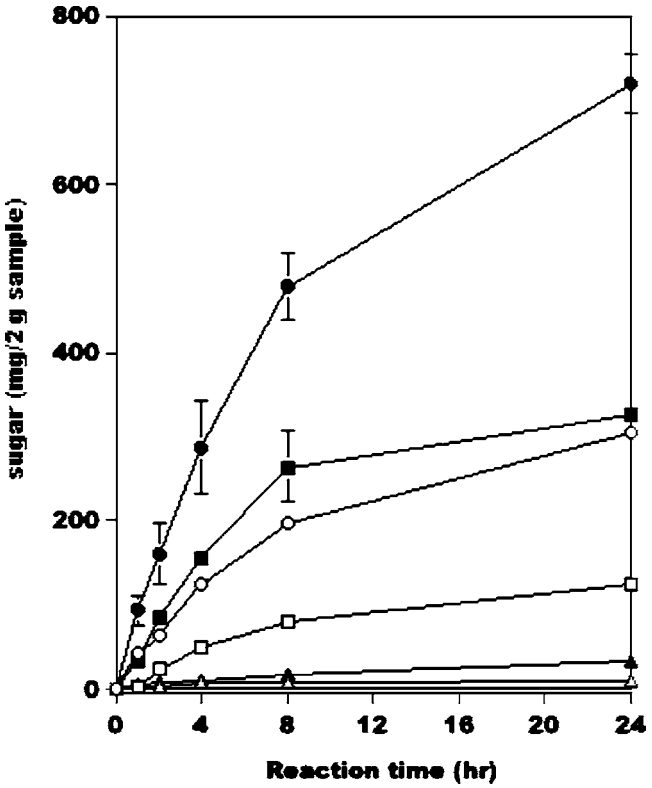

Figure 3. Time course of the digestion of the cracked green coffee beans and roasted coffee residues by the cellulase from *Trichoderma* sp. The cracked green coffee beans and the roasted coffee residues were autoclaved (121 °C, 60 min) with a 50-fold volume of 0.1 M NaOH, and the beans were washed, filtered, and dried. The dried samples (2 g) and 0.1 M acetate buffer (pH 5.0) (16 mL) were added to a glass bottle (30 mL). The glass bottle was incubated with stirring (1200 rpm) at 40 °C for 24 h with 1% cellulase. The supernatant of the reaction mixture was analyzed. Symbols: (○) total sugars of the reaction mixture of the green coffee beans; (□) reducing sugars of the reaction mixture of the green coffee beans; (△) uronic acids of the reaction mixture of the green coffee beans; (●) total sugars of the reaction mixture of the roasted coffee residues; (■) reducing sugars of the reaction mixture of the roasted coffee residues; (▲) uronic acids of the reaction mixture of the roasted coffee residues.

of arabinose and galactose in the final residue was also 1:2.6. The glucose and mannose were found in the ratio 1:11.6. Xylose was found in the final residues.

Change in the Cell Walls of Green Coffee Beans by Sequential Treatment of Alkali Extraction and Cellulase Digestion. Figure 4 shows the microscopy photos of the residues of the sequential treatments described above by staining of toluidine blue *O* (column **A**), Yariv reagent (column **B**), and calcofluor reagent (column **C**). The cell walls were completely broken by milling, and the body complexes in the cells were removed to solubilize. The cell walls only were clearly detected, and the characteristic holes of the cell walls were also detected. The cell walls gradually became thin by digestion of the cellulase. The characteristic hump junctions of the cell walls were well stained with all of the reagents. The alkali treatment decreased the staining parts of the cell walls (**Figure 4A-2**,**B-2**). The cell wall covered with a membrane was found after the first cellulase digestion, and the membrane was well stained by Yariv reagent (**Figure 4B-3**). The membrane became thin by the extraction of the second alkali treatment (**Figure 4B-4**). The residual cell walls were further digested by the second cellulase digestion (**Figure 4A-5**,**B-5**,**C-5**). In the final residue, the cell walls and the membrane became very thin (**Figure 5**). The cell walls were very easily broken into small pieces by finger pressing (**Figure 4A-6**,**B-6**,**C-6**). The final residue (**Figure 4C-5**) and the broken pieces of the cell walls (**Figure 4C-6**) were stained with calcofluor. The broken pieces of the cell walls were again well stained by Yariv reagent (**Figure 6**).

DISCUSSION

The efficient digestion of the cell walls of the green and roasted coffee beans was achieved, and the characteristics of the cell walls were investigated. The cell walls are insoluble and thick, and the digestion has been very difficult for a long time (*2*, *8*, *9*). The compositions mainly contain galactomannan, arabinogalactan, and cellulose, but their detailed characteristics and distributions are not yet clarified (*2*−*10*). Their efficient digestion is only slightly known. Especially, the characteristics or the localization of the strong binding of arabinogalactan (protein) and the (galacto) mannan-cellulose are important, but not totally known (*2*−*9*). We carried out the efficient digestion of the cell walls of the coffee beans by evaluating the results of the efficient digestion and extraction of the soybean seeds (*15*−*17*). The essential cell structures, such as the cell wall, body complex, and oil drop, are similar to each other, but the single cells could not be formed by autoclaving, and the same procedures could not be adapted. However, the cellulase screening and microscopic observation led us to solve this problem. In the cracked coffee beans and the coffee residues, the cells were not completely broken, and the body complexes were not completely solubilized by the autoclaving with alkali. They remained in the cellulase digestion, and the digestion yields were low. The milling of the coffee beans and the alkali solubilization of the body complex in the cells were effective for the efficient digestion and observation of the cell walls. Most of the cells were broken. The contents of the cells, which are body complexes, were easily removed with cell breaking and the alkali treatment. In this experiment, removal of the body complexes was done not by the alkaline protease but by the alkali extraction (*17*). The alkali treatment effectively removed the body complexes or residual coffee brew (*17*). The cell walls only were clearly observed. Autoclaving with alkali produced a better cellulase digestion than boiling in the milled powders and the sliced sections, and the breakup of the cell walls was observed (**Figures 2B** and **4)**.

Mannase would be expected for the digestion of coffee beans (*25*), but the food-processing cellulase from *Trichoderma* sp. was very effective, whereas the other cellulases containing mannase activity were not effective. The selected cellulase is the same food-processing enzyme found in the efficient digestion of the previously reported soybean seeds and soybean milk residues (*15*−*17*). The cellulase showed a strong glycosidase activity that acted on the raw soybean cells (*16*). The sugar degree of polymerization of the digestion of the coffee beans was also short, and the average was 2.2−2.5 (**Figure 4**). The cellulase could well act on the xylan or cellulose powder. Konjac mannan could be digested, but the galactomannan from locust beans of *Coretonia siliqua* seeds and gum guar were very weakly digested by the cellulase. The cellulase containing a strong mannase activity such as GM5 from *Aspergillus oryzae* could digest the galactomannan and the konjac mannan, but was not effective for the digestion of the coffee cell walls (data not shown). The real key activities of the enzyme for the digestion of the coffee beans were not clear. The main sugar compositions could not always lead to the expected enzymes; thus, the real screening of the enzymes should be carried out (*16*, *17*).

Table 1. Neutral Sugar Composition and Residual Percent of the Alkali Extraction and Cellulase Digestion of the Defatted Powder of Green Coffee Bean

	neutral sugar[a] (mol %)						composition[b] (mg/g)			
procedure	rham	ara	xyl	man	gal	glu	total sugar	total uronic acid	total protein	residue[c] (%)
first alkali extraction										
supernatant	6.0	13.9	nd	26.1	7.6	46.4	210.5 ± 25.1	15.1 ± 1.7	102.2 ± 12.4	
residue	nd	20.3	nd	51.7	28.0	nd				46.3 ± 1.24
first cellulase digestion										
supernatant	nd	10.2	nd	46.1	24.4	19.3	282.0 ± 55.8	16.5 ± 1.1	2.5 ± 0.6	
residue	nd	5.5	nd	70.4	24.1	nd				20.5 ± 0.21
second alkali extraction										
supernatant	nd	20.9	nd	nd	79.1	nd	83.5 ± 11.4	0.8 ± 0.1	2.8 ± 0.4	
residue	nd	10.5	nd	62.9	26.6	nd				13.5 ± 0.2
second cellulase digestion										
supernatant	nd	nd	nd	57.4	24.4	18.2	47.6 ± 9.5	1.6 ± 0.2	1.5 ± 0.1	
residue	nd	5.3	nd	71.1	19.5	4.1				5.0 ± 0.12

[a] The extraction (2–3 mg as sugar) and the residues (5 mg) were hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 2 h, and the mole percent was estimated. [b] Total sugar, uronic acid, and protein were analyzed using the phenol–sulfuric acid, 3-phenyl phenol, and Bradford methods. Values are expressed as av ± SD ($n = 4$). [c] Values were the residual weights of the washed and freeze-dried samples. Values are expressed as av ± SD ($n = 4$).

Table 2. Neutral Sugar Composition and Residual Percent of the Alkali Extraction and Cellulase Digestion of the Defatted Powder of Roasted Coffee Bean

	neutral sugar[a] (mol %)						composition[b] (mg/g)			
procedure	rham	ara	xyl	man	gal	glu	total sugar	total uronic acid	total protein	residue[c] (%)
first alkali extraction										
supernatant	nd	15.4	nd	25.2	45.0	14.4	193.7 ± 14.8	9.2 ± 1.7	95.6 ± 8.3	
residue	nd	7.9	2.3	63.1	23.9	2.8				45.6 ± 2.78
first cellulase digestion										
supernatant	nd	6.3	nd	55.0	7.9	30.8	320.3 ± 106.3	11.5 ± 1.0	4.1 ± 0.9	
residue	nd	6.0	2.4	64.6	21.6	5.4				22.2 ± 0.35
second alkali extraction										
supernatant	nd	nd	nd	27.8	72.2	nd	109.0 ± 21.7	0.6 ± 0.1	7.3 ± 1.2	
residue	nd	6.9	nd	70.3	18.8	3.9				10.5 ± 0.33
second cellulase digestion										
supernatant	nd	10.1	nd	43.3	13.5	33.1	20.8 ± 4.8	0.5 ± 0.1	1.4 ± 0.3	
residue	nd	8.9	7.5	55.3	23.6	4.7				3.9 ± 0.06

[a] The extraction (2–3 mg as sugar) and the residues (5 mg) were hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 2 h, and the mole percent was estimated. [b] Total sugar, uronic acid, and protein were analyzed using the phenol–sulfuric acid, 3-phenyl phenol, and Bradford methods. Values are expressed as av ± SD ($n = 4$). [c] Values were the residual weights of the washed and freeze-dried samples. Values are expressed as av ± SD ($n = 4$).

The arabinogalactan protein or arabinogalactan covalently cross-linking proteins was proposed in the coffee beans (*2*, *6*−*9*). The arabinogalactans of the coffee beans are hard to extract (*2*, *6*−*9*). Redgwell et al. recently reported the chemical and alkali extractions from the fine milled coffee beans, and the extracted arabinogalactans were positive to Yariv reagent (*8*, *9*). All of the extractions contained the arabinogalactan protein. All of the residues of the cell walls in our examination were also well stained with Yariv reagent, and these stainings agreed with the results of several stepwise extractions of arabinogalactan (proteins) by Redgwell et al. (*8*, *9*). Although the extraction of the strong adhesive arabinogalactan was reported to require a strong alkali such as 5 M NaOH or 8 M KOH (*2*, *8*, *9*), dilute alkali (0.1 M) was enough in our sequential extraction and digestion. However, the strongly binding arabinogalactan proteins still existed in the residual cell walls (**Figure 6**); therefore, further localization and its characteristics should be studied in the future. The outer cellulose-rich layer was first removed in our digestion by cellulase, and then the naked arabinogalactan protein (**Figure 4B-3**) could be easily extracted by the second alkali treatment (**Figure 4B-4**). The second alkali extraction was clearly rich in arabinose and galactose with the ratio of 1:3.8, whereas mannose was not detected in the extraction. The 1:2.5 ratio of arabinose and galactose was detected in the residue, and the ratio of the final residue was found to be 1:3.7 (**Table 1**). The ratios agreed well with the ones reported by Redgwell et al. (*8*, *9*). The residual cell walls after the first cellulase digestion was well stained, and the membrane was detected by Yariv reagent (**Figure 4B-3**). The inner membrane or the layer could be mainly composed of arabinogalactan proteins (**Figure 4B-3**). The fractionated pieces of the final residues were also again well stained by Yariv reagent (**Figure 6**). These results showed that the arabinogalactan proteins also existed in the central deep regions of the cell walls. The first and second cellulase digestions were well done after the heated alkali treatment. The arabinogalactan proteins may prevent cellulase digestion. The central parts of the cell walls were strongly attached to each other and shared with the cell walls of the next cells (**Figure 1B**). We think that this unique structure and the compositions could be a reason for no single cell formation. After autoclaving (121 °C, 10 min) with the dilute alkali extraction was completed, the second digestion by the same cellulase was done again. As a result, the residual cell walls became very thin, and then a drastic breakup by finger pressing was observed (**Figures 4** and **6**). This breaking of the cell walls was also found in the sliced green coffee (**Figure 2**). The second autoclaving with alkali would then be effective for the extraction and swelling of the final residues. The boiling with alkali was not sufficiently effective as described above. A similar effect of autoclaving was found for the primary cell walls of the

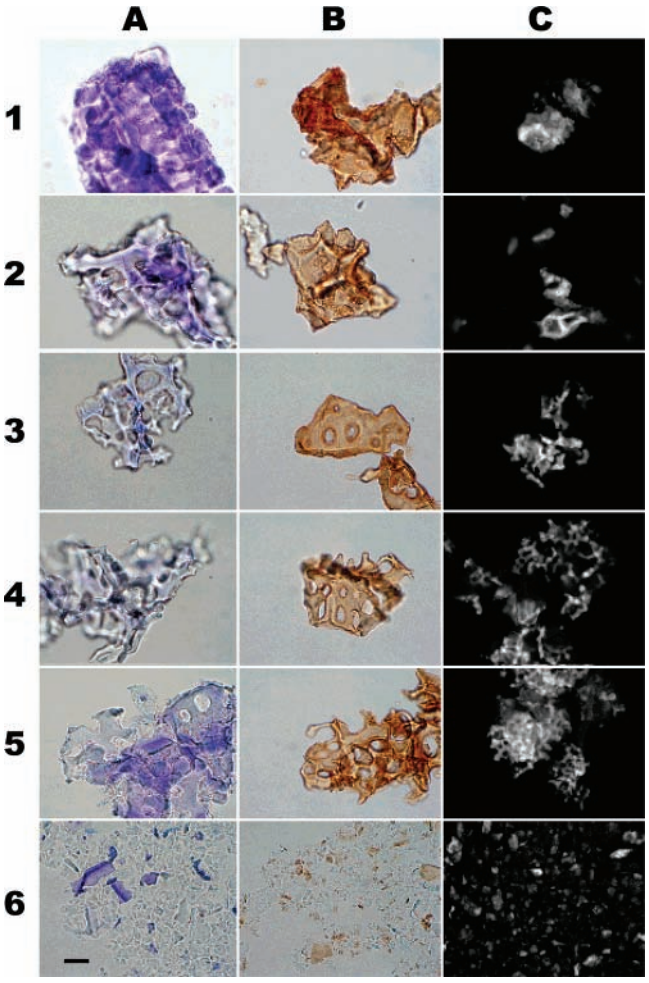

Figure 4. Microscopy of the residues of the sequential extraction and digestion. Sequential digestion of cell walls of the green coffee beans was observed by microscopy. Staining was done using toluidine blue *O* (column **A**), Yariv reagent (column **B**), and calcofluor white (column **C**). Lines: **1**, defatted powder of the green coffee beans; **2**, first alkali treatment; **3**, first cellulase digestion; **4**, second alkali treatment (alkali autoclaving); **5**, second cellulase digestion; **6**, finger-pressed final residue. Black bar represents 5 μm.

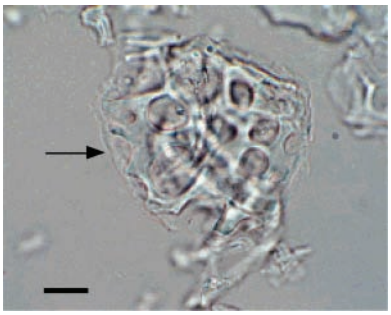

Figure 5. Microscopy of membrane covering cell walls of the green coffee bean. A thin and transparent membrane covering the cell wall was found. The arrow indicates that the membrane became thin and transparent after two alkali and cellulase digestions. The cell walls also became thin. The cell walls were also easily broken by finger pressure. Black bar represents 5 μm.

soybean digestion. The cellulase could not digest the raw soybean seed because the adhesives in the middle lamella stopped the cellulase digestion, but the naked single cells formed by autoclaving with water were easily digested (*15*−*17*). The final residues of the coffee beans seemed to be the outer part

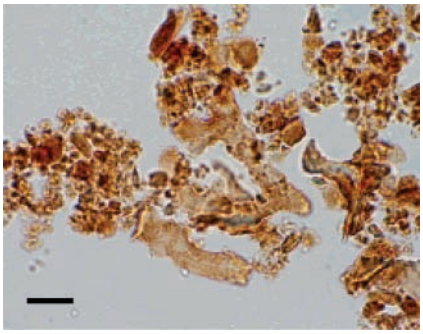

Figure 6. Microscopy of the broken final residues restained with Yariv reagent. The final residues broken by finger pressure were again stained with Yariv reagent and observed under microscopy. The fractionized cell walls were not stained with the Yariv reagent (**Figure 4B-6**), but were clearly stained with Yariv reagent after a second treatment. Black bar represents 5 μm.

or layer of the residual cell walls (**Figures 2** and **4A-6**). The final residue of the cell walls of the coffee beans was not easily hydrolyzed with 2 M trifluoroacetic acid or 1 M NaOH; therefore, a further analysis should be completed. Arabinose decreased during the roasting (*2*). It was noted that the second alkali extraction did not give arabinose in the roasted coffee beans, but the residue had arabinose and galactose in the ratio of 1:2.6. The arabinose of the arabinogalactan (protein)-rich membrane (**Figure 4B-3**) could be predominantly lost during the roasting (**Table 2**) (*2*).

Most of the glucans in the coffee beans are proposed to be cellulose; its existence was proposed as the mannan-cellulose in the coffee beans (*2*). The glucose was rich in the first cellulase digestion after the alkali treatment. The outer layer of the cell walls was easily digested by the selected cellulase. Staining by the iodide−H_2SO_4 was clearly positive, and the cell walls were swollen with a blue color (*22*) (data not shown). The residues of the cell walls were not stained by the iodide−H_2SO_4 method, and the glucose was not detected after the short hydrolysis (2 h) by the trifluoroacetic acid, but was detected after the long hydrolysis (24 h). Glucose was also detected in the second cellulase digestion. The glucose was also detected in the final residue; the ratio of glucose to mannan was 1:17.4. This result could indicate the existence of glucogalactomannan, but the calcofluor staining was clear in the residues of all treatments and the final residues (**Figure 4C**). The cellulose of the residual cell walls remained after the final cellulase digestion. These results would be due to the structural localization of the cellulose. The glucose could be from the mannan-cellulose. Layers or unique structures may exist. The cell walls of the coffee are composed of multiple mannan layers by the staining with the mannan antibody as done by Redgwell et al. (*8*). Our results agreed with their results. The results showed that there were at least four kinds of layers in the cell walls. The layers of the cell walls were composed of galactomannan-cellulose in the central deep part, which was very tightly bound to the next cell wall, the covering arabinogalactan protein layer or membrane, the cellulose-rich galactomannan as the outer layer, and the outer layer was again covered with arabinogalactan-protein (*8*).

Plants are organically structured carbohydrates, proteins, and so on (*26*). The complete structural and compositional understanding or their uses is difficult. Information about their chemical composition is essential for digestion or use, and the microscopic observations and the screening of the enzymes are assessment important tools. The efficient digestion of the cell

walls of the coffee beans would be useful for the high extraction of a coffee brew or use of the residue of the roasted coffee (*8*, *12*, *13*). Exploring the food functions (*14*) of the digested galactomannan of the coffee beans would be possible using our results.

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