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Effects of Cellulase from *Aspergillus niger* and Solvent Pretreatments on the Extractability of Organic Green Tea Waste

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As green tea is being consumed in larger amounts, more green tea waste is being produced. Following extraction, several bioactive compounds may exist in the waste including polyphenols and amino acids. It was found that an *Aspergillus niger* cellulase treatment of green tea waste increased the extractability of various nutritional and functional components after pretreatments with various extraction solvents such as cold water (CW), hot water (HW), sulfuric acid (SA), hydrochloric acid (HA), and methanol (Me). After the residue was treated with cellulase from *Aspergillus niger*, the amounts of polyphenols, total catechins, and reducing sugars in the HW extract were increased by 64.6, 941.2, and 350.9%, respectively. In particular, levels of epigallocatechin, epicatechin, and gallic acid were significantly enhanced compared to those in the nontreated control. However, protein extraction was not significantly affected, and cellulase treatment was not more efficient for caffeine extraction compared to phenolic extraction. Among the four extraction solvents, HW and SA showed relatively higher extractabilities as compared to the other groups (CW, HA, and Me). These results indicate that cellulase from *A. niger* can increase the extractability of green tea waste when combined with certain solvent pretreatments. Consequently, the residual functional compounds and essential nutrients from cellulase-treated green tea waste have the potential to be applied in the production of new functional foods.

KEYWORDS: Catechin; cellulase; extraction; green tea; solvent pretreatment

INTRODUCTION

Green tea is one of the most widely consumed beverages in many Asian countries. Numerous studies on the various beneficial effects of green tea, such as its antimutagenic (*1*−*3*), anticarcinogenic (*4*, *5*), and antioxidant activities (*6*−*8*) and cardio protective effect (*9*) have been reported. The beneficial effects of green tea are highly attributed to polyphenolic compounds, especially catechins, which make up 30% of the dry weight of green tea leaves (*10*). In green tea, several catechin derivatives exist, including (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3-gallate (EGCG), (+)-catechin, and (+)-gallocatechin (GC) (*10*, *11*). Among the five types of catechins, EGCG is the most abundant polyphenol in green tea extract (*10*), and the majority of functional properties of green tea are associated with the epi-catechins rather than the catechins (*12*). In addition to catechins, green tea leaves contain many other active components such as tannins, theanine, polysaccharides, and other flavonoids.

For the desirable utilization of active food components, such compounds must be efficiently released from the foods consumed and digested, and become available in the body. Therefore, the extractability and bioavailability of active functional food components are the most important factors that should be considered in evaluating the functionality of foods. Green tea extraction has commonly been conducted by brewing it in hot water, but not all of its valuable compounds are effectively extracted, leaving large portions in the waste. Moreover, epimerization and degradation of catechins can occur during the extraction process (*13*). Many strategies have been suggested for the effective extraction of foods, such as Soxhlet, heat reflux, boiling, and distilling methods (*14*). Ultrasonic (*15*), microwave (*16*), supercritical fluid (*17*), and high pressure extractions (*18*) are currently being introduced. Along with these methods, extraction conditions such as the extraction solvent, temperature, extraction time, and pH can be important factors for effective extraction. Enzyme treatment has also been attempted in order to improve extraction yields from natural resources. In previous studies, carotenoids were effectively extracted from marigold flowers using a variety of enzymes including pectinase, cellulase, hemicellulase, and proteolytic enzymes (*19*). The extractability of phenolic compounds from apple peel was also enhanced by enzyme-mediated extraction (*20*). Furthermore, various enzymes were used to improve extractions from garlic, soybeans, rosemary, and safflower (*21*−*24*). Since all of these enzymatic treatments could enhance cell wall degradation, the cellulolytic enzyme was tested to develop methods for the efficient utilization of green tea.

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It is well established that *Aspergillus niger* produces a number of cellulolytic enzymes with relatively high yield. Moreover, the enzymes are food grade and commercialized (*25*). In this aspect, we studied the effects of cellulase produced from *A. niger* on the extractability of organic green tea waste after various solvent pretreatments.

MATERIALS AND METHODS

Materials. Five kilograms of the green tea leaves were provided by Dae Han Tea Produce Co. (Seoul, Korea). Cellulase produced by *A. niger* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Catechin isomers including EGCG, EGC, ECG, and EC were purchased from Sigma-Aldrich, Inc. All other chemicals were of analytical reagent-grade.

Pretreament with Solvents. Stem-removed organic green tea leaves were ground to a powder. For extraction, samples of 50 g were repeatedly agitated in 1 L of cold distilled water (CW: 25 °C), hot water (HW: 90 °C), 2% sulfuric acid (SA), 2% hydrochloric acid (HA), and 95% methanol (Me) for 20 min at 250 rpm. After being extracted with the acids, the green tea extracts were neutralized to pH 7.0 with 1 N NaOH. After pretreatment, the solid residue separated from the aqueous phase was collected and dried overnight at 60 °C. The aqueous portions of the treated samples were filtrated with Whatman No. 42 filter paper (Whatman Int. Ltd., Maidstone, U.K.), evaporated at 40 °C using LABOROTA 4000 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and freeze-dried using Programmable Freeze-Dryer (Ilshin Lab Co., Gyeonggi-do, Korea).

Measurements of Cellulase Activity and Catechins. Five grams of dried green tea waste pretreated with various solvents was mixed with a compensating amount of cellulase (20 U) from *A. niger* in 200 mL of sodium acetate buffer with 0.02% sodium azide (pH 5.0), followed by incubation of the mixture at 37 °C by shaking at 60 rpm. One unit (U) of cellulase activity was defined as the amount of enzyme that released 1 μmol of glucose in 1 h, and 1 U of cellulase from *A. niger* was 1.36 mg. After being incubated for 24 h, the enzyme reaction was terminated by boiling for 2 min. The cellulase-treated samples were filtered, evaporated, and then freeze-dried for the analysis of protein, reducing sugar, polyphenol, and catechin contents. Cellulase activity was determined as follows: 50 mg of green tea powder in 10 mL of 50 mM sodium acetate buffer (pH 5) was incubated with 10 mg of enzyme at 37 °C for 2 h. Glucose content was determined by the DNS assay using 1 mL of supernatant after centrifugation at 8,000*g* for 5 min (*26*). A reaction mixture with no enzyme was prepared as a blank. To confirm the cellulase activity on glycosylated catechins in green tea, 1 mg/mL of EGCG was incubated with 0.2 U of cellulase for 24 h at 37 °C, followed by boiling for 2 min to terminate the enzymatic reaction. The catechins and gallic acid were analyzed using an HPLC system (Waters 515, Waters, Milford, MA. USA) equipped with two solvent-delivery systems, an auto sampler (Waters 717), and a photodiode array detector (Waters 2996). The column used for the analysis was an I.D CAPCELL PAK C18 (25 cm $\times$4.6 mm, 5 μm), a reverse-phase column (Shiseido Fine Chemicals, Tokyo, Japan). The mobile phase A was 0.1% acetic acid, phase B was acetonitrile, and gradient elution was performed by varying A and B at 1.0 mL/min of total flow. All of the solutions used were filtrated using 0.45 μm membrane filters and degassed. Ten microliters of each sample was injected and monitored at 280 nm to detect catechins, caffeine, and gallic acid. The identification and quantification of the compounds were carried out by comparing retention times and peak areas from calibration curves of EGCG, EGC, ECG, EC, caffeine, and gallic acid.

Determination of Protein, Polyphenols, and Reducing Sugars. Total protein content was determined by the Bradford assay using bovine serum albumin as a standard (*27*). One milliliter of Quick start Bradford Dye reagent (Bio-Rad, Hercules, CA, USA) was mixed with 20 μL of each sample solution dissolved in distilled water. After incubation for 10 min, the absorbance of the mixture was measured at 595 nm. The polyphenol and reducing sugar contents were measured by Folin−Ciocalteu's and DNS assays, respectively (*28*). The amount of each ingredient was calculated considering the extraction yield from the raw material and waste.

Statistical Analysis. All data were obtained from triplicate experiments and expressed as the mean ± standard deviation. Statistical differences of the experimental results were determined by analysis of variance (ANOVA) in the SAS statistical software package (SAS Inst. Inc., Cary, NC). Duncan's multiple-range test was used to determine differences between means, and $p < 0.05$ was considered to be statistically significant.

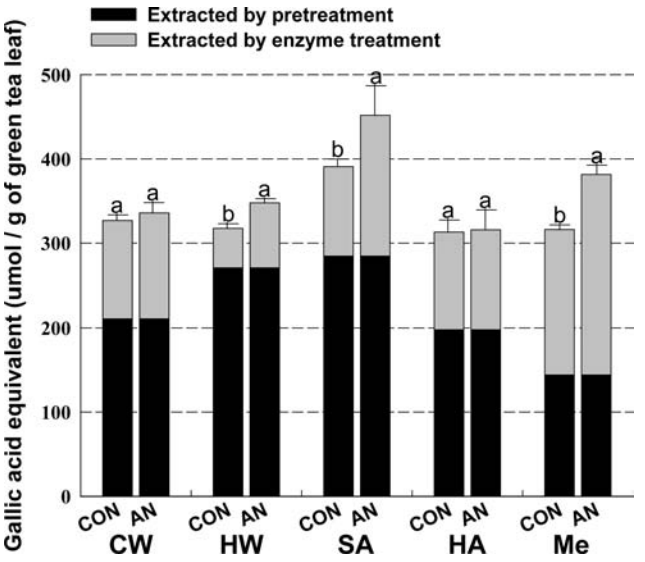

Figure 1. Total phenolics extracted by pretreatment and enzyme treatment of organic green tea leaf. CON, control with no enzyme; AN, *Aspergillus niger* cellulase. Data are presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$.

RESULTS AND DISCUSSION

Measurements of Total Polyphenols and Protein. According to the results of the analysis of total phenolic compounds, the most effective solvent pretreatment for releasing compounds from the green tea leaves was sulfuric acid. The pretreatments using cold water, hot water, sulfuric acid, hydrochloric acid, and methanol yielded 210.76, 270.57, 284.62, 197.88, and 144.08 μmol of GAE/1 g of organic green tea leaf, respectively. Enzyme treatment was found to increase polyphenol content from the green tea waste. After the cellulase treatment, sulfuric acid pretreatment yielded the highest phenolic extractability, and hydrochloric acid showed the lowest extractability among the groups tested. Moreover, after sulfuric acid pretreatment, the extractability of total phenolics from the green tea leaves was enhanced (132%), but there was no significant increase in extraction by cellulase treatment when the green tea samples were pretreated with cold water and hydrochloric acid (**Figure 1**). It was assumed that concentrated H^+ might increase the extractability by accelerating green tea cell wall degradation (*29*). The higher concentration of H^+ in sulfuric acid may cause the higher phenolic level extracted from green tea leaves than that resulted from pretreatment with hydrochloric acid. Furthermore, it is known that the release of phenolics is highly correlated to the reducing sugar level. Kapasakalidis et al. (*30*) and Kim et al. (*20*) showed that there was a correlation between total phenolic content and reducing sugar level when cell wall degrading enzymes were treated. Cell wall degrading enzyme treated black currant pomace showed elevated levels of total phenolics and reducing sugars (*30*).

In general, protein content is not expected to increase by cellulase treatment. In our study, protein levels were not enhanced compared to the control group after cellulase treatment. It is suggested that the cellulase used in this study had no effect on protein release from the green tea tissue. Among the pretreatment

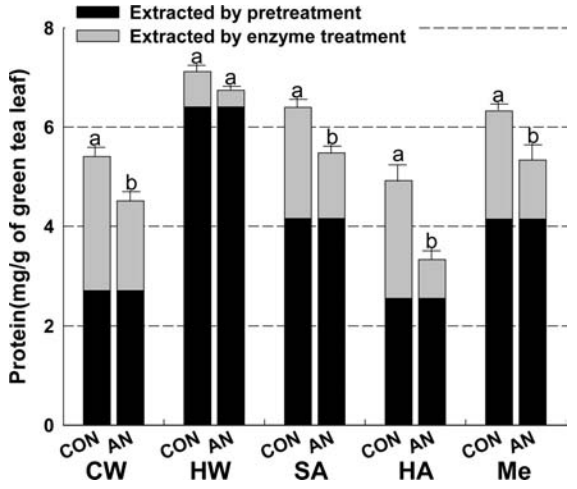

Figure 2. Effects of pretreatments and enzyme treatment on protein extraction from organic green tea. Data are presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$.

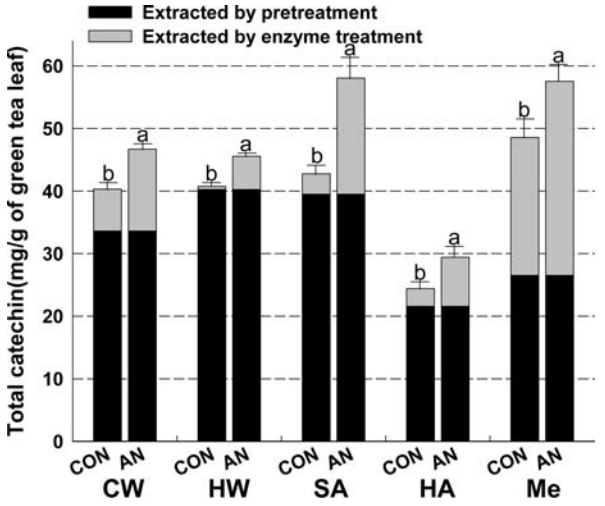

Figure 3. Effects of pretreatments and enzyme treatment on the total catechin contents of organic green tea. Data are presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$.

groups, hot water was the most efficient for increasing protein yield (**Figure 2**).

Effects of Pretreatments and Enzyme Treatment on the Catechin Composition of Green Tea Extract. The total catechin content extracted from 1 g of organic green tea leaves was 58 mg, approximately four times higher than the caffeine content when the waste was treated with cellulase after sulfuric acid pretreatment (**Figures 3** and **4**). In this study, green tea waste was pretreated with various solvents and degraded by enzymatic reaction to enhance catechin levels. It has been reported that the caffeine content of green tea is approximately 2.66% DW and that the total content of catechin compounds is 7.65% by distilled water (http://www.greentea.com). Through our optimized extraction method using a pretreatment (sulfuric acid) and cellulase treatment, 76% of the total catechins was extracted versus only 50% of the caffeine. When the green tea leaves were pretreated with methanol, a relatively lower amount of total catechins was extracted (26.5 mg/1 g). By cellulase treatment of the methanol-pretreated waste, an additional 16.9% of catechins was extracted

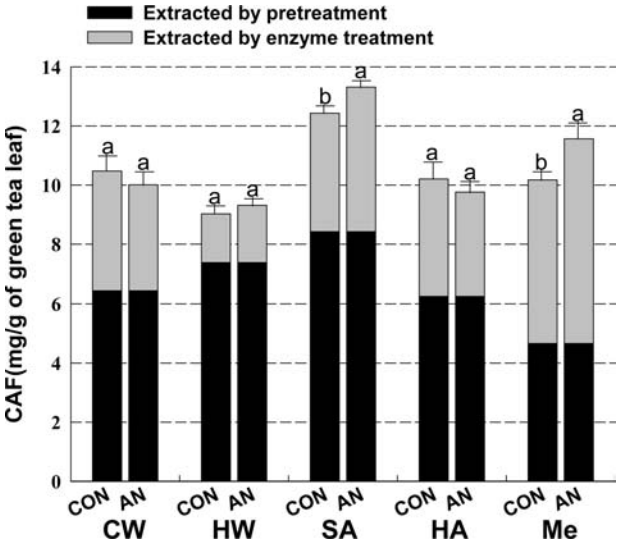

Figure 4. Effects of pretreatments and enzyme treatment on the caffeine content of organic green tea. Data were presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$.

from the waste compared to that from the nonpretreated control. Methanol pretreatment is assumed to facilitate the enzymatic extractability of catechins by cellulase treatment. It was shown that caffeine was not effectively released by cellulase in the cold water, hot water, and hydrochloric acid pretreatment groups. Cellulase treatment was found to be effective for caffeine extraction of the sulfuric acid and methanol extracted green teas; however, the extraction of caffeine was not as effective as that of catechins and polyphenols.

An HPLC system was applied to analyze the catechin composition of the green tea extract after pretreatments and enzyme treatment. **Table 1** shows the catechin profiles of each extract after enzyme treatment. Cellulase treatment did not increase galloylated catechins (EGCG and ECG) unlike the polyphenol and total catechin contents. These results imply that the elevated level of total catechins by cellulase treatment was not attributable to an increase in galloylated catechins. Cellulase treatment is supposed to facilitate the breakdown of galloylated catechins to catechins and gallic acid. Indeed, increased levels of the ungalloylated catechins, EGC and EC, were observed after cellulase treatment. This was confirmed by results in which gallic acid levels were significantly increased in the cellulase treated groups, and GCG and CG, the epimerized forms of EGCG and ECG, were not detected. **Figure 5** shows that cellulase broke the glycosidic bonds of EGCG leading to gallic acid and EGC. When EGCG was treated with cellulase, the concentrations of gallic acid and EGC increased approximately 9 and 13 times, respectively, compared to those in the control group. Simultaneously, total EGCG concentration was significantly reduced (data not shown). The increased level of catechins is attributed to the cellulose hydrolysis of glycosidic bonds of gallolated catechins.

Cellulase Activity on Cell Wall Degradation. After pretreatment, followed by cellulase treatment of the green tea waste, the DNS assay was performed to measure the amount of reducing sugar extracted. Since cellulase hydrolyzes cellulose in plant cell walls to produce glucose or shorter saccharides, the level of reducing sugar produced after enzyme treatment can be indicative of cellulase activity. Reducing sugar content as measured by the DNS assay was significantly higher by the treatment of cellulase from *A. niger* (**Figure 6**). Moreover, total phenolic and catechin contents were significantly increased by cellulase treatment.

Table 1. Catechin Profiles in Extract from Enzyme Treatment[a]

pretreatment	enzyme	EGCG (mg/g)	EGC (mg/g)	ECG (mg/g)	EC (mg/g)	GA (mg/g)
CW	control	2.633 ± 0.96 a	1.770 ± 0.48 b	1.322 ± 0.16 a	0.944 ± 0.1 b	0.194 ± 0.01 b
	treated	3.844 ± 0.61 a	6.666 ± 1.19 a	0.930 ± 0.06 b	1.639 ± 0.11 a	2.071 ± 0.13 a
HW	control	ND[b]	ND	0.182 ± 0.04 a	0.327 ± 0.07 b	0.021 ± 0.01 b
	treated	0.262 ± 0.1 a	3.440 ± 1.42 a	0.170 ± 0.01 a	1.438 ± 0.14 a	1.400 ± 0.34 a
SA	control	1.097 ± 0.77 b	ND	1.295 ± 0.06 a	0.867 ± 0.02 b	0.152 ± 0.03 b
	treated	4.288 ± 0.53 a	10.644 ± 1.5 a	0.957 ± 0.09 b	2.657 ± 0.15 a	3.054 ± 0.13 a
HA	control	0.574 ± 0.49 b	ND	1.396 ± 0.15 a	3.948 ± 0.34 a	0.266 ± 0.05 b
	treated	1.172 ± 0.25 a	4.207 ± 0.95 a	0.516 ± 0.09 b	3.509 ± 0.21 b	3.163 ± 0.29 a
Me	control	7.353 ± 0.76 a	11.375 ± 0.97 b	1.542 ± 0.06 a	1.766 ± 0.09 b	0.327 ± 0.02 b
	treated	5.018 ± 0.54 b	21.38 ± 1.7 a	1.149 ± 0.16 b	3.458 ± 0.14 a	2.105 ± 0.19 a

[a] CW, cold water; HW, hot water; SA, sulfuric acid; HA, hydrochloric acid; Me, methanol. Control, with no enzyme; treated, with cellulase from *Aspergillus niger*. All values were presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$. [b] ND: not detected.

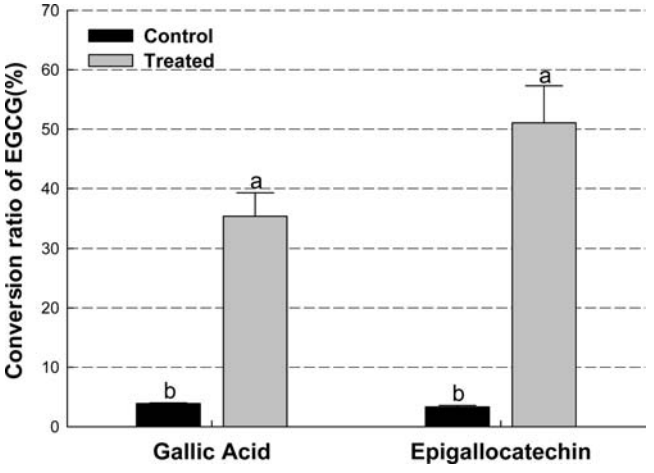

Figure 5. Effects of cellulase from *Aspergillus niger* on major catechins in green tea. Control, not treated with cellulase; treated, treated with cellulase. Data were presented as the mean ± SD, $n = 3$. Data with different letters were statistically different at $p < 0.05$.

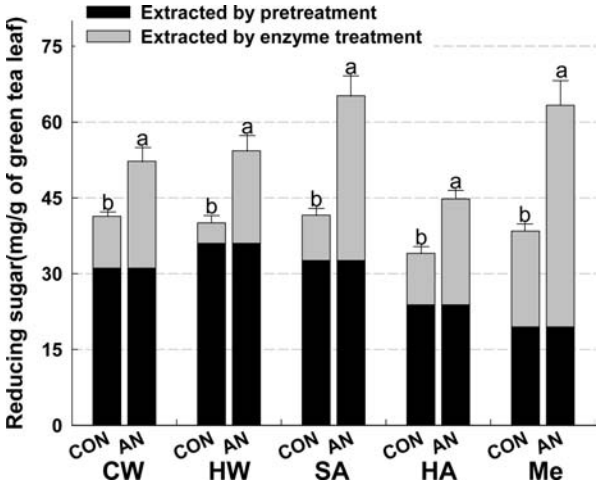

Figure 6. Variations in reducing sugar content by pretreatment and enzyme treatment of organic green tea leaf. Data were presented as the mean ± SD, $n = 3$. Data with different letters in the same pretreatment were statistically different at $p < 0.05$.

A. niger cellulase has *endo*-glucanase (EC 3.2.1.4) activity. The complete hydrolysis of cellulose into glucose requires the synergistic action of several cellulases, including *endo*-glucanase, cellobiohydrolases, and β-glucosidase activities (*31*). In this study, the cellulose in green tea was hydrolyzed only by an *endo*-glucanase. Since a lower amount of reducing ends is expected to be produced by *endo*-glucanase alone than a cocktail of different cellulases, relatively fewer reducing sugars were detected in the cellulase treated groups despite higher extractions of polyphenols and catechins. In this study, it was shown that cellulase from *A. niger* has useful effects to release higher levels of polyphenols from green tea waste compared to untreated waste. Thus, it might be desirable to optimize combinations of hydrolytic enzymes, yielding the most efficient extraction of green tea waste.

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