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Phenolic Extraction from Apple Peel by Cellulases from *Thermobifida fusca*

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With the optimization of the pretreatment conditions for the crude *Thermobifida fusca* cellulase activity and phenolic release from apple peel, we focused on the activity of individual purified cellulase related to the antioxidant activity. The overall phenolic release was significantly increased in a synergistic manner with combined pretreatment, not with individual pretreatment such as boiling, acid, and pectinase treatment. Approximately 60 mg of reducing sugar equivalent were produced per g of apple peel by treatment with *T. fusca* crude extract, and up to 3 times more reducing sugars were released when the apple peel was boiled and then treated with acid and pectinase. There was good correlation between the release of phenolics and reducing sugar by cellulase treatment and also between the amount of total phenolics and antioxidant capacity by each enzyme treatment ($r^2 > 0.95$). Among the tested enzymes purified from *T. fusca* cell extract, cellulase activity on apple peel was the highest with cellulase 6A (Cel 6A; 43% digestion), and the highest antioxidant capacity was obtained by incubation with Cel 6B (16 mg vitamin C equiv/g). Synergism in the activity was found from the combined treatment with Cel 6A and 6B in both cellulase activity and antioxidant capacity after 20 h of incubation. Cel 9A (progressive endocellulase) exhibited greater cellulase activity and antioxidant capacity than Cel 9A cd which lacks in cellulose-binding module, indicating that the cellulose-binding domain might play important roles in cellulolysis of apple peel. This study could provide some insights into the action mechanism of various cellulases on the digestion of cellulose-containing byproducts and expand the opportunity for cellulase utilization in the extraction of functional ingredients from the plant-derived byproducts.

KEYWORDS: Antioxidant; apple peel; cellulase; phenolic; pretreatment; *Thermobifida fusca*

INTRODUCTION

Many agricultural residues and byproducts from the food industry contain a variety of phenolics which are associated with the prevention of degenerative diseases, and this may be ascribed to their action as antioxidants (*1*, *2*). Among them, peels and seeds of fruits are accumulated in large amounts during food processing (*3*, *4*). Since apple peel could provide health benefits (*5*), its proper utilization is desirable. Recently, the phenolic extraction from agricultural and industrial wastes has gathered great attention because they could be cheap and safe sources of strong antioxidants (*6*−*8*). The antioxidant capacity of many phenolics from natural sources surpasses that of vitamin C and vitamin E in aqueous- and lipid-phase oxidation models, respectively, so that phenolics have become widely accepted as health-promoting agents with potential to protect humans from a variety of degenerative diseases (*8*, *9*).

Bioactive phytochemicals are present as soluble, suspended, or colloidal forms in complexes with the cell wall components after the cell wall is ruptured (*10*). Many of these phytochemicals are degraded by harsh extraction conditions. Therefore, the extraction process should be designed to minimize the loss of bioactive phytochemicals and to obtain an acceptable yield. Because the cell-wall-hydrolyzing enzymes such as cellulase, hemicellulase, and pectinase hydrolyze the plant materials, they have often been proposed as tools for the extraction (*11*). The enzyme treatment will depolymerize plant cell wall polysaccharides resulting in changes in functional properties of the fruit material. This will improve the yield and composition of phenolics and dietary fiber from complexes within the cell

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wall matrix (CWM) (*12*−*15*). However, there have been limited studies on these enzymes to extract natural antioxidants from plant materials (*16*). Increased recovery of these valuable compounds from agricultural byproducts is possible by digestion with the cell-wall-hydrolyzing enzymes.

Thermobifida fusca is a nonpathogenic, thermophilic, and filamentous soil bacterium which produces a variety of cellulases including endo- and exocellulases. These enzymes are of particular interest due to their high thermostability, making them useful in industrial applications (*17*). The enzymes have been well-characterized and used to design suitable cellulase for biodegradation in our previous studies (*17*, *18*). The aim of the present study was to evaluate the effect of pretreatment methods and cellulases obtained from a bacterium, *T. fusca*, on the recovery of phenolics of apple peels and to study the effect of specific cellulases on the phenolic recovery and antioxidant capacity.

MATERIALS AND METHODS

Chemicals. Ammonium hydroxide, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as its diammonium salt, Folin and Ciocalteu's phenol reagent, gallic acid, bovine serum albumin (BSA), isopropyl-β-D-thiogalactoside (IPTG), pectinase, and *p*-hydroxybenzoic acid hydrazide (PAHBAH) were obtained from Sigma Chemical Co. (St. Louis, MO). The 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals U.S.A., Inc. (Richmond, VA). Ascorbic acid, sulfuric acid, and hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals used were analytical grade.

Preparation and Pretreatment of Apple Peel. Apple peels were obtained from apple cv. Gala in a local grocery in Ithaca, NY, and freeze-dried prior to enzymatic incubations. Freeze-dry apple peel pretreatments were performed in sodium acetate buffer (50 mM, pH 5.5) by heat-treatment (95 °C, 20 min), acid (2% sulfuric acid), or incubating with pectinase (1 unit/10 mL) at pH 4.0, 25 °C. Acid-treated samples were neutralized by 0.5 N NaOH before cellulase treatment.

Preparation of Concentrated Crude Cellulases. Crude enzyme extract was obtained from batch fermentation of *T. fusca* ER1, a protease negative mutant strain. After cultured to cells in 250 mL of Hagerdahl minimal medium containing 0.2% glucose for 18 h in a 50 °C shaker, enzyme extracts were desalted and concentrated 30 times (*19*). For individual enzyme production, *Escherichia coli* BL21 codon plus strain was used as a host for the production of enzymes coded by specific coding sequence in a large quantity. Each *E. coli* strain, which contains plasmids with corresponding coding sequence for Cel 6A, Cel 6B, Cel 9A, or Cel 9A cd (catalytic domain) was grown on M9 media. When the optical density at 600 nm reached 0.7, cells were induced with IPTG and were grown overnight. Enzymes were prepared as described previously (*17*, *18*), and each cellulase was used at 1 nmol/5 mg of apple peel/mL.

Cellulase Activity. Protein concentrations of the crude enzyme extracts were determined by the Bradford method using BSA as a standard (*20*). Enzymatic treatment of apple peel (1% w/v) was performed at 50 °C in sodium acetate buffer (50 mM, pH 5.5) with 0.02% sodium azide to prevent microbial growth. The enzyme reaction was terminated by boiling for 1 min. To measure the amount of reducing sugar produced, 1.5 mL of PAHBAH solution (0.5% w/v PAHBAH in 0.5 M HCl and 0.5 M NaOH (1:4)) was added to the samples (5−10 μL) which were placed in a boiling water bath for 4 min. After cooling the reaction mixture to room temperature, the optical densities were measured at 410 nm (*21*). Cellulolysis of apple peel by the concentrated *T. fusca* extracts was expressed as mg of reducing sugar released per μg of protein. The activity by individual cellulase was expressed as percentage (%) reducing sugar produced by 1 nmol of protein/mL from dry weight of apple peel, and phenolic release was measured based on the same protein concentration.

Determination of Total Phenolics. Total phenolics in the supernatant obtained after each cellulase treatment were determined by spectrophotometric analysis after reactions with Folin and Ciocalteu's phenol reagent (*22*). In brief, an aliquot (1 mL) of appropriately diluted extracts or standard solutions of gallic acid was added to a 25 mL volumetric flask containing 9 mL of distilled, deionized water (ddH_2O). A reagent blank using ddH_2O was prepared. One mL of Folin and Ciocalteu's phenol reagent was added to the mixture while shaking. After 5 min, 10 mL of 7% Na_2CO_3 solution was added with mixing. The solution was immediately diluted to the same volume (25 mL) with ddH_2O and mixed thoroughly. After 90 min at 23 °C, the absorbance was read against a blank at 750 nm. Total phenolics were expressed as mg of gallic acid equiv (GAE)/100 g of dry apple peel sample.

Table 1. Characteristics of Cellulases Present in Crude Extracts of *Thermobifida fusca*

name	catalytic domain family	MW (kD)	mode of action	binding domain structural family
E1	Cel 9B	101.2	endo	II
E2	Cel 6A	43.0	endo	II
E3	Cel 6B	59.6	exo	II
E4	Cel 9A	90.4	endo	II/III
E4 cd	N/A	51.4	endo	N/A
E5	Cel 5A	46.3	endo	II
E6	Cel 48A	104.0	exo	II

Vitamin C Equivalent Antioxidant Capacity (VCEAC) Assay Using ABTS Radicals. ABTS radicals were used to measure antioxidant activities of apple peels according to the method of Kim et al. (*23*). Briefly, 1.0 mM AAPH was added to 2.5 mM ABTS in phosphate-buffered saline (PBS; pH 7.4; 0.1 M K_2HPO_4/KH_2PO_4 buffer; 150 mM NaCl). The mixed solution was heated at 68 °C in a water bath. The resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.65 ± 0.020 at 734 nm with additional PBS. An amount of 20 μL of sample was added to 980 μL of the ABTS radical solution. The mixture was incubated in a 37 °C water bath under restricted light for 10 min. The reduction of absorbance at 734 nm was measured 10 min later. A control (20 μL of 50% methanol and 980 μL ABTS radical solution) was run with each series of samples. The radical stock solution was prepared daily, and the antioxidant capacity of apple peel was expressed on a dry weight basis as mg/100 g of vitamin C equiv.

Statistical Analysis. All data are presented as the mean ± SD for at least three replications. Statistical analyses were conducted using SigmaStat program (version 8.0; Jandel Corp., San Rafael, CA). Statistical analysis of experimental results was based on the analysis of variance. Significant difference was statistically considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

The plant cell wall consists of a rigid complex of biopolymers such as cellulose, pectin, and lignin, and these materials were chemically and physically treated before being incubated with cellulase to enhance the rate and the extent of release of antioxidants, such as phenolics. Thus, the optimizations of the pretreatment conditions are the first and important step. In this study, we investigated the effects of three individual and one combined pretreatments on cellulase activity and phenolic release using *T. fusca* crude cellulase, which includes a variety of exo- and endocellulases (**Table 1**). Cellulase activity was measured by reducing sugar, and approximately 60 mg of reducing sugar equivalent were produced per g of apple peel without pretreatment of apple peel (control). There was no increase in crude cellulase activity with incubation time for this control. We estimated three individual pretreatment effects on cellulase activity. As shown in **Figure 1**, pectinase pretreatment was shown to be the most efficient to enhance the cellulase activity resulting in up to a 3-fold increase after 42 h of incubation, whereas boiling or acid treatment alone represented little impact on the cellulase activity. Since each pretreatment

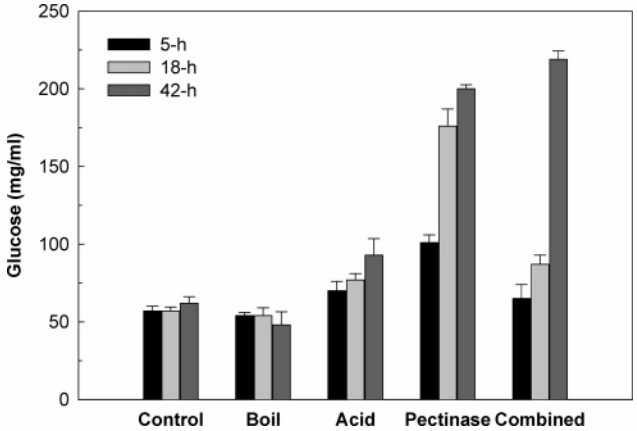

Figure 1. Effect of pretreatment on the cellulase activity of crude *T. fusca* extract. Samples were measured for enzyme activity at 5, 18, and 42 h during the incubation. Enzyme activity was evaluated based on the reducing sugar produced per μg of protein released from the dry weight of sample from three replicates. Control: no pretreatment. Combined: pectinase, boiling (90 °C 20 min), acid (2% sulfuric acid).

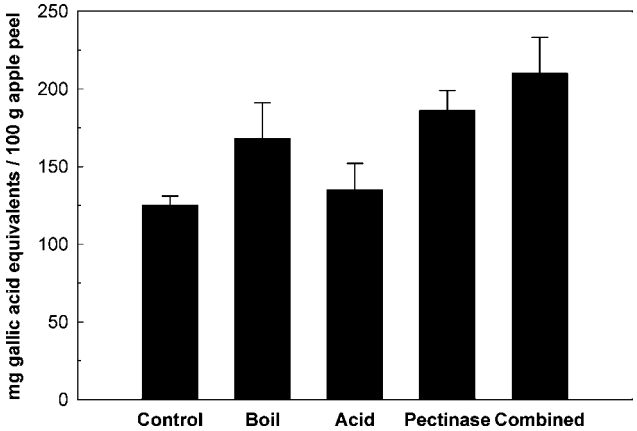

Figure 2. Effect of pretreatments on phenolic release by crude cellulases from *T. fusca.* Samples were measured at 42 h of incubation. Phenolic release was expressed as mg of gallic acid equiv per 100 g of dry sample from six replicates. Control: no pretreatment. Combined: pectinase, boiling (90 °C 20 min), acid (2% sulfuric acid).

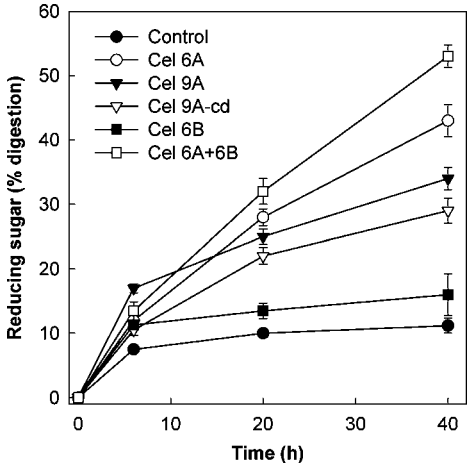

Figure 3. Enzyme activity of individual *T. fusca* cellulases. Samples were pretreated by boiling, acid, and pectinase before being incubated with individual cellulases for 40 h at 50 °C. Enzyme activity of individual cellulase was expressed as percentage (%) of reducing sugar produced by 1 nmol of protein/mL from the dry weight of samples from three replicates. Control: no cellulose treatment.

process makes the apple peel more susceptible to enzymatic digestion in a different way by removal of surrounding hemicelluloses and/or lignin along with modification of the cellulose microfibril structure, we combined three individual pretreatment conditions; pectinase treatment followed by boiling and acid treatment (at 95 °C for 20 min). When compared to the pectinase pretreatment, the crude cellulase activity, however, was not significantly increased by the combined pretreatments. This indicated that the pectinase treatment alone was enough to increase the reactivity of cellulase. Another interesting result was the time dependence of cellulase activity for pectinase and combined pretreatment conditions. Although the cellulase activity was not significantly increased after 5 h of incubation compared to the control, it increased up to 3-fold after 42 h of incubation. Since the accessibility of cellulase to cellulosic materials is a key step in the hydrolysis of insoluble plant cell walls, this time-dependent manner of cellulase activity was mainly due to the increased surface area for cellulase caused by hydrolysis of cell wall by pretreatments. Holtzapple et al. showed that steam explosion treatment (260 °C) was an efficient pretreatment method for cellulase action by increasing the surface area of wood cellulose (*24*), but we did not apply such high temperature because this may not be suitable for phenolic extraction from apple peel, because the yield of major phenolics of apple peel such as catechin and epicatechin were shown to be reduced at temperatures greater than 150 °C (*25*).

Since the cellulase activity measurement alone was not enough to evaluate the pretreatment conditions, the recovery of phenolics, which are rich functional ingredients in apple peel, was also investigated. Phenolic release without pretreatment was estimated to be 125 mg of gallic acid equiv per 100 g of apple peel. Phenolic release was not significantly affected by the acid treatment, whereas the combined treatment with pectinase and boiling significantly enhanced the phenolic release (**Figure 2**). It was hypothesized that phenolic release would be correlated to the cellulase activity, because the enhanced phenolic release was caused by the loosened cell wall by cellulase action. This correlation was observed only with pectinase and combined pretreatments, not with boiling and acid treatment alone. In the case of boiling pretreatment, the phenolic release was increased without cellulase treatment, and the increased cellulase activity did not affect the phenolic release for the acid pretreatment. The combined pretreatment could have further broken the nonspecific polysaccharide−phenolic complexes resulting in more phenolic extraction in a comparison to that of pectinase treatment alone. Thus, the most effective pretreatment option for maximum increase in cellulase activity and phenolic release turned out to be the combined pretreatments of boiling, acid, and pectinase.

Since the optimization of pretreatment conditions was performed using crude cellulase, we focused on pure cellulases produced by *T. fusca* to pinpoint which cellulase plays a specific role for the phenolic release. The individual cellulase was chosen based on the mode of action and the type of cellulase-binding module (CBM) to representative *T. fusca* cellulase (**Table 1**). Thus, Cel 6A and Cel 6B which represented endo and exo-cellulase, respectively, and Cel 9A which demonstrated family II/III CBM type unlike the other cellulases, were used. In addition, the mixture of Cel 6A + Cel 6B was used to test the synergistic effect of endo and exo cellulase and Cel 9A cd, which lacks in CBM, was studied to evaluate the cellulose-binding effect on the enzyme activity and phenolic release. As shown in **Figure 3**, overall individual enzyme activity increased with incubation time. It was expected that Cel 9A had the

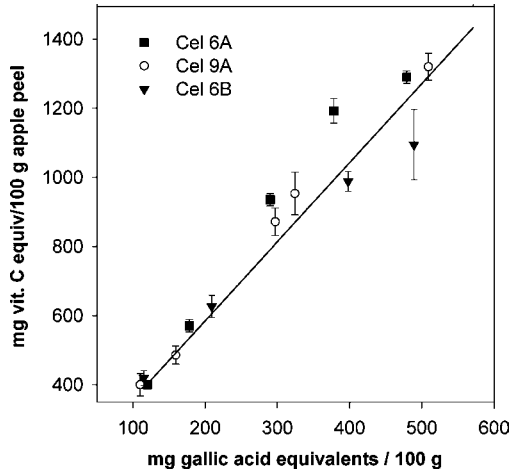

Figure 4. Correlation between phenolic contents and antioxidant capacity of Cel 6A, Cel 9A, and Cel 6B. Phenolic contents were expressed as mg of gallic acid equiv per 100 g of dry sample, and antioxidant activities were expressed on a dry weight basis as mg of vitamin C equiv per 100 g of sample from six replicates.

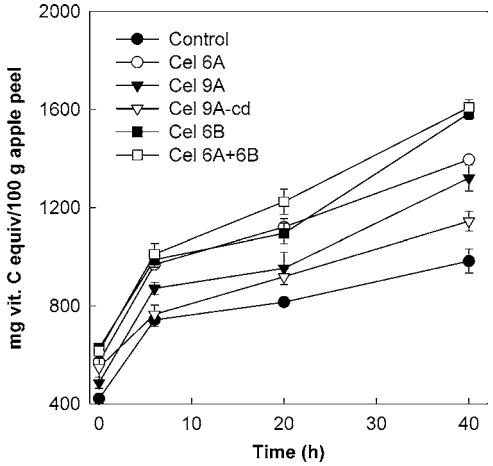

Figure 5. Effects of *T. fusca* cellulases on antioxidant capacity. Antioxidant activities were expressed on a dry weight basis as mg of vitamin C equiv per 100 g of sample from six replicates. Control: no cellulase treatment.

highest activity, since it is an unusual endocellulase which has typically higher specific activity on crystalline cellulose such as bacterial microcrystalline cellulose, plant cellulose, and filter paper (*18*, *26*). Cel 6A, an endocellulase, however, showed the highest activity on apple peel digestion among the tested enzymes at 40 h of incubation. In fact, plant cellulose is composed mostly of crystalline cellulose (*26*). These findings, however, were not surprising considering the size difference between Cel 6A and Cel 9A. As previously mentioned, the binding of the cellulase to its substrate is the critical and initial step for the cellulolysis, and the amount of the bound cellulase depends on the relative size of the cellulase. That is, the smaller, the more cellulases can access the cellulose interstice areas (*27*). Since it is reasonable to assume that there are various sizes of cellulose interstices which are produced by the combined pretreatment, the smaller Cel 6A, rather than Cel 9A, can access further into the cellulose matrix of the apple peel cell wall.

It was also notable that there was a difference in cellulase activity between Cel 9A and Cel 9A cd (**Figure 3**). As expected, Cel 9A cd, which lacked CBM, had lower enzyme activity than intact Cel 9A indicating that the binding of cellulase to the surface of cellulose was another important factor for enzymatic reactions. Cel 6B, an exocellulase, represented no significant effect on the apple peel digestion. Indeed, exocellulase has relatively less activity than endocellulases even though their role in cellulolysis is important (*17*). Since the cell wall has a complex and rigid structure, the combination of cellulases with different mode of action has been considered to be an efficient strategy to break down the CWM. In our study, the cellulase activity exhibited the highest value with the exo- and endocellulase combination as previously reported (*18*).

A correlation between an antioxidant capacity and phenolic content was observed ($r^2 > 0.95$) with all three individual cellulases; Cel 6A, 9A, and 6B (**Figure 4**). This suggests that the antioxidant capacity of apple peel is mainly derived from phenolics. In fact, phenolics are major antioxidant components in apple, especially rich in the peel (*5*, *28*). Among them, apple procyanidins are known to associate spontaneously and rapidly with the apple peel cell wall matrix, and the amount of bound procyanidins increased with their molecular weights (*26*). It was suggested that the binding of phenolics to the CWM could mask the enzyme-binding site by steric hindrance (*29*). Therefore, the cell wall structure itself, rather than the specific cell wall components, may be an important parameter in an association with phenolic compounds to the CWM. In fact, some phenolics, such as hydroxycinaminic acid and epicatechin, present in apple do not bind to the CWM (*26*). In addition, it was previously shown that the incubation at 40 °C for 48 h caused phenolic degradation during enzyme-assisted extraction from grape pomace (*30*). However, the yield of apple phenolics during digestion with *T. fusca* enzymes did not decline during the incubation for 42 h at 50 °C nor did the antioxidant capacity.

Higher antioxidant capacity was observed with Cel 9A than Cel 9A cd, indicating that binding of cellulase to apple peel may also be an important factor for the phenolic release and antioxidant capacity (**Figure 5**). As expected from the cellulase activity (**Figure 3**), Cel 6A showed more phenolic release than Cel 9A from apple peel. Exocellulase typically has a lower activity on crystalline cellulose compared to that of endocellulase (*31*), and this explains our results well in **Figure 3**, where the exocellulase Cel 6B showed lower cellulase activity than endocellulase Cel 6A. However, Cel 6B had relatively higher activity than the other tested endocellulases on phenolic extraction of apple peels at 40 h of incubation (**Figure 5**), suggesting that the exocellulase also played important roles in releasing phenolics from the apple peel CWM. It was assumed that exocellulase could break the glycosidic bonds between the sugar moiety and phenolic aglycones, and thus the availability of phenolics may have been increased.

On the basis of the high activity of Cel 6A in apple peel digestion and of Cel 6B in phenolic extraction, the combination of Cel 6A and Cel 6B was tested for synergism. The activity of Cel 6A was enhanced by simultaneous incubation with Cel 6B which clearly indicated some synergism existed between endo- and exocellulase in agreements with Weinberg et al. (*32*) who found that a cell-wall-degrading enzyme mixture is needed in order to rupture the cell wall and enhance the recovery of cell constituents. Although synergism between Cel 6A and Cel 6B was previously observed for cellulase activity (*18*), no synergism has been reported for the antioxidant capacity by phenolic release. A higher degree of both enzyme activity and antioxidant capacity was observed when both enzymes were present simultaneously, but a synergistic effect was not apparent in the antioxidant capacity (**Figures 3** and **5**). Indeed, some cellulases from *T. fusca* have shown the enhanced hydrolytic activity acting together especially endo−exo cellulase combinations. This possibly results from increased number of new cellulose chain ends by endocellulase action (*18*). However, the mechanism of

synergism for apple peel digestion and phenolic extraction by Cel 6A and Cel 6B was different from the synergism found with other cellulosic material.

In summary, pretreatments such as boiling, acid, and pectinase treatment generally increased both phenolic release and cellulase activity of crude *T. fusca* extract. Overall antioxidant capacity and phenolic recovery were parallel to the time-wise increase in individual *T. fusca* cellulase activity and further enhanced in an endo−exo cellulase combination. Moreover, cellulose size and cellulase binding to the substrate were thought to be important factors in cellulase activity on the apple peel cell wall. Since the plant cell wall is mainly composed of crystalline cellulose in its core structure which is covered by xyloglucan and paracrystalline cellulose, it is plausible that some cellulases from *T. fusca*, which actively digest crystalline cellulose, could be useful for phenolic recovery from apple peel. This is the first trial to investigate the effect of enzymes with different modes of action on the phenolic release and cellulolysis of apple peel. Moreover, we evaluated the phenolic release by the cellulase treatment with various pretreatment methods, and this would expand the chance for the application of cellulases in the recovery of phenolics from plant-derived byproducts. The rationale to use such techniques should be supported by further research for the optimization of the pretreatment procedures and the cell-wall-degrading enzymatic reactions to achieve maximal phenolic yields from apple peel and other byproducts from food processing.

ABBREBIATIONS USED

AAPH, 2,2′-azobis (2-amidino-propane) dihydrochloride; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); BSA, bovine serum albumin; CBM, cellulose-binding module; CD, catalytic domain; Cel, cellulose; CWM, cell wall matrix; IPTG, isopropyl-β-D-thiogalactoside; PAHBAH, *p*-hydroxybenzoic acid hydrazide; VCEAC, vitamin C equivalent antioxidant capacity.

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