Production and Antioxidant Properties of the Ferulic Acid-Rich Destarched Wheat Bran Hydrolysate by Feruloyl Esterases from Thermophilic Actinomycetes

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Ferulic acid is present at relatively high concentrations in the cell walls of several plants. Agricultural lignocelluloses are now used as bioresources in industry. This study attempted to increase the free ferulic acid content present in lignocellulose by using thermostable esterase produced from thermophilic actinomycetes to hydrolyze ester bonds. Destarched wheat bran was used as a carbon source for the production of esterases from the newly isolated thermophilic actinomycetes species *Thermobifida fusca*. After 96 h of cultivation, the esterase activity that accumulated in the culture broth was 946.0 U/mL. Two percent of the destarched wheat bran was then hydrolyzed by crude esterase preparation for 16 h. The ferulic acid was accumulated in the culture broth at a concentration of 310.0 μM. The hydrolysate had better radical-scavenging ability for both 1,1-diphenyl-2-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical-scavenging ability, as well as reducing power than ferulic acid. These results showed that the ferulic acid-rich destarched wheat bran hydrolysate had good antioxidant properties. It is suggested that this process can be advantageous for the industrial production of antioxidants derived from agricultural bioresources.

Keywords: Destarched wheat bran; Ferulic acid; Antioxidant; esterase; Thermobifida fusca

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INTRODUCTION

Lignocellulolytic bioresources comprise three major types of polymers: cellulose, hemicellulose, and lignin. These polymers are related to each other. Cellulose consists of D-glucose subunits that are linked by β-1,4 glycosidic bonds. Hemicellulose is a complex carbohydrate structure that consists of different polymers (Hendriks and Zeeman 2009). Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is covalently linked to polysaccharides by ester bonds and is a component of lignin (Scalbert *et al*. 1985). FA is present at relatively high concentrations in the cell walls of several plants, including monocots and dicots. Rice bran, wheat bran, bagasse, and corncobs are important agricultural wastes that are now used as bioresources in the bio-industry. Huang *et al*. (2011) used esterase and xylanase to hydrolyze these substrates and found that corncobs yielded the most ferulic acid.

FA esterases (FAEs, also known as feruloyl esterases, cinnamic acid esterases, or cinnamoyl esterases; EC 3.1.1.73) represent a diverse group of esterases that can release FA from the constituents of the plant cell walls to which they are bound. FAEs are key enzymes for cell wall hydrolysis and are increasingly used for the extraction of phenolic

acids from agricultural crops (Benoit *et al*. 2006; Wong 2006; Koseki *et al*. 2009). These enzymes have been found in and isolated from many microorganisms, including *Trichoderma* (Biely *et al*., 1988), *Aspergillus* (Khan *et al*. 1990; Poutanen *et al*. 1987), *Penicillium* (Chavez *et al*. 2006), *Schizophyllum* (Biely *et al*. 1988), *Rhodotorula* (Lee *et al*. 1987), *Fusarium* (Poutanen *et al*. 1987), *Streptomyces* (Dupont *et al*. 1996), *Fibrobacter* (McDermid *et al*. 1990), and *Bacillus* (Poutanen *et al*. 1987). These microorgansims can be classified as fungi and mesophilic bacteria.

FA is best known for its antioxidant and anti-inflammatory properties (Chawla *et al*. 1987). FA has the ability to increase the resistance of low-density lipoprotein (LDL) to peroxidation, thereby protecting LDL cholesterol from oxidation and preventing the oxidative modification of the LDL apoprotein B100 (Castelluccio *et al*. 1995). FA can be modified into vanillin, one of the most widely used aromatic molecules in the food, pharmaceutical, and cosmetic industries (Clark 1990). FA also can be converted into other valuable molecules, such as polymers, epoxides, alkylbenzenes, protocatechuic acid-related catechols, guaiacol, and catechol (Rosazza *et al*. 1995).

The radical-scavenging activity of ferulic acid and the polyamine conjugates of ferulic acid, diferuloylputrescine (DFP), and *p*-coumaroylferuloylputrescine (CFP), that had been isolated from corn bran were investigated previously. DFP showed potent DPPH (IC_{50} = 38.46 μM) and superoxide (IC_{50} = 291.62 μM) radical scavenging activities. CFP also exerted moderate DPPH, superoxide, and hydroxyl radical scavenging activities (Choi *et al*. 2007). FA derivatives, including feruloyl arabinose and FA dehydrodimer, have better antioxidant activity in the human low-density lipoprotein (LDL) oxidation system (Andreasen *et al.* 2001; Ohta *et al.* 1997).

The goal of this study was to isolate feruloyl esterase-producing thermophilic actinomycetes. This study attempted to increase the free ferulic acid content in ligno-cellulose by using thermostable esterase produced from the newly isolated thermophilic actinomycetes to hydrolyze the ester bonds of lignocellulose. The feasibility of this method was tested using lignocellulose hydrolysate instead of pure ferulic acid to increase the antioxidant properties.

EXPERIMENTAL

Microorganism

From the approximately 100 waste compost soil samples collected in Taiwan, 70 strains of thermophilic actinomycetes were isolated on CYC GELRITE plates consisting of 33.3 g Czapek-dox powder, 2.0 g yeast extract, 6.0 g casamino acids, 11.0 g GELRITE, and 0.1 g $CaCl_2$ per liter of distilled water (pH 8.0); the plates were stored at 65 °C in our laboratory (Yang *et al*. 2009).

Materials

Czapek-dox powder, yeast extract, casamino acids, and agar were obtained from BD Diagnostic Systems (Sparks, MD, USA). Ferulic acid, methyl ferulate, ethyl ferulate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,20-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS), gallic acid, Folin–Ciocalteau phenol reagent, gelrite gellan gum (GELRITE), xylan, and all other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). The bagasse sample was collected from Taiwan Sugar Company. Corncobs and

wheat bran samples were collected from a local market. Lignocellulolytic samples were washed thoroughly with tap water to remove dust and then air-dried.

Destarched wheat bran (DSWB) was prepared according to Mukherjee *et al.* (2007). Briefly, wheat bran was treated with 0.3% (w/v) potassium acetate at 95 °C for 30 min, followed by extensive washing with deionized water to remove starch.

These samples were then chopped and milled to 100-mesh size using a Cyclone Mill (Tecator AB, Hoganas, Sweden).

Isolation and Taxonomical Investigation of FA Esterase-producing Strains

The FA esterase-producing strains were screened on CYC GELRITE plates with xylan and ethyl ferulate at 50 °C. The formation of a clearing zone around the point of inoculation indicated ferulic acid esterase production. The selected strain was cultured in 50 mL of xylan-CYC medium in a 500-mL Hinton flask. For the methyl ferulate degrading assay, the culture supernatant was mixed with methyl ferulate to a final volume of 2.0 mL and incubated for 6 h at 50 °C.

After centrifugation, the ferulic acid and methyl ferulate contents were tested by HPLC. Identification was conducted according to Bergey's Manual of Determinative Bacteriology (William 1992). The whole-cell hydrolysate amino acid, sugar, fatty acid esters, and 16S rDNA sequence analyses were performed as previously described (Yang *et al*. 2009).

Esterase Activity Assay

To determine the esterase activity, *p*-nitrophenyl acetate was used as the substrate. Esterase activity was determined by measuring the released *p*-nitrophenol (Kademi *et al*. 1999). One unit of enzymatic activity is defined as the amount of the enzyme that releases 1 mmole *p*-nitrophenol per min at 25 °C. FA esterase activity was measured by monitoring the FA released from methyl ferulate (Abokitse *et al*. 2010).

HPLC Analysis

The FA and methyl ferulate retained in the broth were determined by HPLC (Agilent 1100 series, USA) using a PRE-PACKED XDB-C18 column (4.6 mm × 150 mm, Agilent, USA) and a 322-nm Variable Wavelength Detector (VWD, Agilent, USA). A mobile phase consisting of solvent A (0.05% acetic acid) and solvent B (99.0% methanol) and with the following elution profile: 0 to 5 min isocratic at 60% A and 40% B; 5 to 25 min linear from 40% to 60% B; 25 to 40 min isocratic at 60% B. Flow rate: 1.0 mL/min. Injection volume: 20 μL.

Hydrolysate Preparation

For enzymatic hydrolysis of lignocellulolytic agricultural bioresources for FA production, the reaction mixture contained 200 mg biomass and 1.0 U crude esterase solution/mg substrate in Tris buffer (50 mM, pH 8.0) in a final volume of 10.0 mL. The mixture was incubated with gentle shaking for 24 h at 45 °C. The reaction was stopped by placing the mixture in boiling water for 3 min. After centrifugation (10,000 $\times g$ for 15 min), the supernatant was used as the lignocellulolytic hydrolysate.

Total Phenolic Content Determination

The total phenolic content of the hydrolysate was determined using a modified Folin–Ciocalteau method (Kujala *et al*. 2000), with gallic acid as the standard. The

sample solution, 500 μL, was mixed with 500 μL of 1 N Folin-Ciocalteau reagent. The mixture was allowed to stand for 5 min, and then 1 mL of 20% Na_2CO_3 was added. After 10 min of incubation at ambient temperature, the absorbance of the supernatant was measured at 730 nm. The measurement was compared to a standard curve (the range of standard curve covers from 0 to 15 mg/mL) of prepared gallic acid solutions and expressed as gallic acid equivalents (GAE) in milligrams per gram of sample.

DPPH Radical-Scavenging Activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity of the hydrolysate was examined using the method reported by Tsai *et al*. (2011). First, 50 μL of the test sample in methanol (final concentrations were 1, 5, 10, and 50 μg/mL) were mixed with 450 μL of 50 mM Tris-HCl buffer (pH 7.4) and 1,500 μL of 0.1 mM DPPH-ethanol solution.

After 30 min of incubation at ambient temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. The inhibition ratio (percent) was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100\% \qquad (1)$$

ABTS Assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was conducted using a previously described method (Re *et al*. 1999). ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. The radical was stable in this form for more than two days when stored in the dark at room temperature. For the study of hydrolysate, the ABTS radical solution was diluted with PBS, pH 7.4, equilibrated at 30 °C. After addition of 1.0 mL of diluted ABTS radical solution to 10 mL of hydrolysate in PBS, the absorbance reading was taken at 30 °C exactly 1 min after initial mixing and up to 6 min.

Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants. The range of standard curve covered from 0 to 3.0 mM of FA. The inhibition ratio (percent) was calculated according to Eq. 1.

Reducing Power Assay

The 1.0 mL of hydrolysate was mixed with the same volume potassium ferricyanide [$K_3Fe(CN)_6$](1%), and then the mixture was incubated at 50 °C for 30 min. Subsequently, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 800 $\times g$ for 10 min. Finally, the solution was mixed with 0.5 mL of $FeCl_3$ (0.1%) solution, and the absorbance was measured at 700 nm (Yen and Chen 1995). Increased absorbance of the reaction mixture indicated increased reducing power. The range of standard curve cover from 0 to 3.0 mM of FA.

Statistical Analysis

All analytical measurements were performed at least in triplicate. Data are expressed as the means ± S.E (standard error).

RESULTS AND DISCUSSION

Isolation and Identification of the Thermostable Esterase-Producing Strain

Of the 70 strains of thermophilic actinomycetes isolated from the compost soil samples in Taiwan, strains No. 13-13 and No. 2-2 had the best methyl ferulate-degrading abilities; therefore, these strains were selected for further experimentation. The whole-cell hydrolysate of Strain No. 22 contained *meso*-diaminopimelic acid but no diagnostic sugars. The fatty acid pattern of the strain contained mainly iso- and anteiso- branched fatty acids. Small amounts of 10-methyl branched and unbranched fatty acids were also found. When the biological characteristics were compared with descriptions in Bergey's Manual of Determinative Bacteriology, both strains No. 13-13 and No. 2-2 resembled a number of thermophilic actinomycetes, especially the genus *Thermobifida*. Comparing the 16S rDNA sequence (Accession Number: JX 498938) with the GenBank database, the greatest similarities (99.8%) were found with *Thermobifida fusca*. However, strain No. 2-2 can grow well on CYC plate at 65 °C, while the strain No. 13-13 and the type strain (*T. fusca* ATCC 27730) cannot. The carbon sources utilizations of strain 2-2, strain 13-13, and the type strain were different. Strain No. 2-2 has been previously named *T. fusca* NTU22 (Liu and Yang 2002). Therefore, the newly isolated thermophilic actinomycetes strains No. 13-13 was identified as *Thermobifida fusca* PU13-13. Of these two strains, strain No. 13-13 had stable esterase-producing ability and was therefore selected for further analysis.

Production of Thermostable Esterase

T. fusca PU13-13 was able to grow in a basal salts-yeast extract medium containing various agricultural lignocelluloses, including xylan, bagasse, corncobs, and destarched wheat bran (DSWB). However, production of extracellular ferulic acid esterase by *T. fusca* PU13-13 was found to vary when different carbon sources were used. The enzyme production by *T. fusca* PU13-13 in basal salts-yeast extract media containing 0.5% (w/v) of various agricultural lignocelluloses is shown in Fig. 1. After 96 h of cultivation, the highest extracellular feruloyl esterase activity (830 U/mL; 770 U/mg protein) was found when *T. fusca* PU13-13 was grown in a medium containing DSWB. Xylan, bagasse, and corncobs were poor inducers. According to the result of Separose CL-6B gel-filtration chromatography, two major active fractions appeared. This was evidence that at least two esterases existed in the crude solution (data not shown).

Enzymatic Hydrolysate from Agricultural Biomass

The enzymatic hydrolysis conditions for the production of FA were investigated. When using DSWB as an agricultural-biomass substrate, 2% of the substrate was hydrolyzed by 1 U crude esterase solution/mg substrate after 16 h at 45 °C and pH 8.0. The HPLC chromatogram profile of the hydrolysate is shown in Fig.2. As shown in Fig. 3, FA accumulated slowly during the initial 4 h. After 4 h of incubation, FA continued accumulating rapidly. After 16 h of incubation, 310.0 μM FA had accumulated in the broth. The negative control (no enzymatic treatment) was considered in the experiments. Little ferulic acid was released in the broth with no enzymatic treatment experiment. A similar FA accumulation pattern was observed for enzymatic hydrolysis of corncob substrates. The highest FA accumulation was found in media containing DSWB.

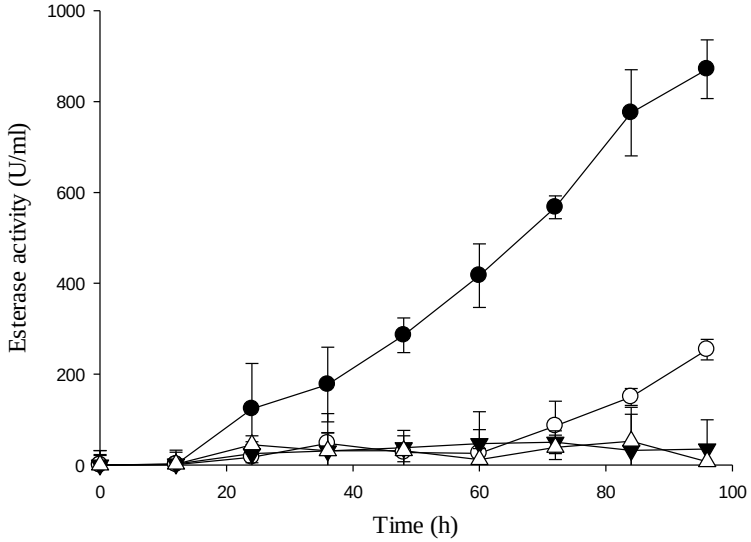

Fig. 1. Time course for the production of esterase by *T. fusca* PU13-13. Cells were grown aerobically in a 500-mL Hinton flask loaded with 50 mL of medium consisting of 0.5% lignocellulose, 0.4% ammonium nitrate, 0.05% yeast extract, and 0.68% potassium phosphate at pH 9.0 and incubated at 50 ^{o}C, 125 rpm for 96 h. Symbols: (●) destarched wheat bran, (○) corncob, (▼) bagasse, (△) xylan.

Bagasse and xylan were found to be poor substrates, with FA production of only 21.0 μM and 15.0 μM, respectively. The DSWB hydrolysate was further concentrated with freeze drying to a 3 mM FA concentration as the FA-rich DSWB hydrolysate. The total ferulic acid composition of DSWB after NaOH treatment was 1.89±0.21 mg/g. The production yield of ferulic acid was 15%.

Given the structural complexity of lignocellulose, the raw materials were conventionally pretreated before enzymatic degradation. In this process, raw lignocellulolytic agricultural waste was used to induce *T. fusca* PU13-13 for production of the feruloyl esterases. The crude esterase preparation can be used directly to hydrolyze the substrate and to produce the ferulic acid without pretreatment. It is not necessary to purify the enzyme further. As a result, the cost of the enzyme preparation can be reduced.

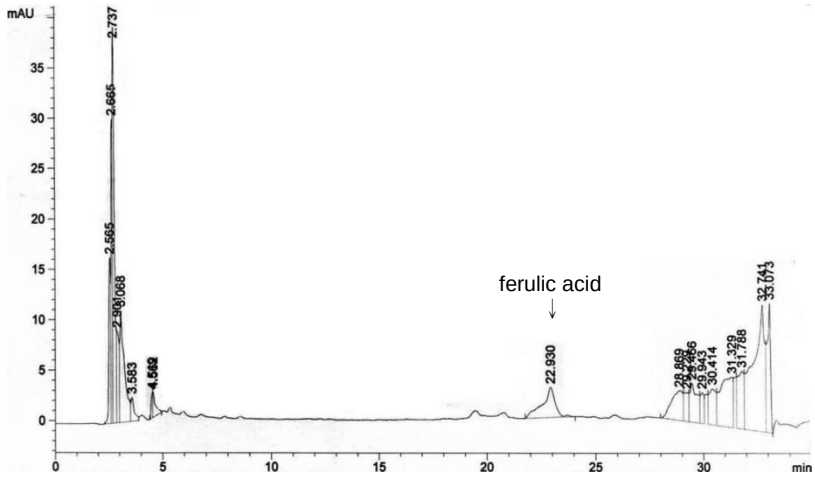

Fig. 2. The HPLC chromatogram profile of the hydrolysate

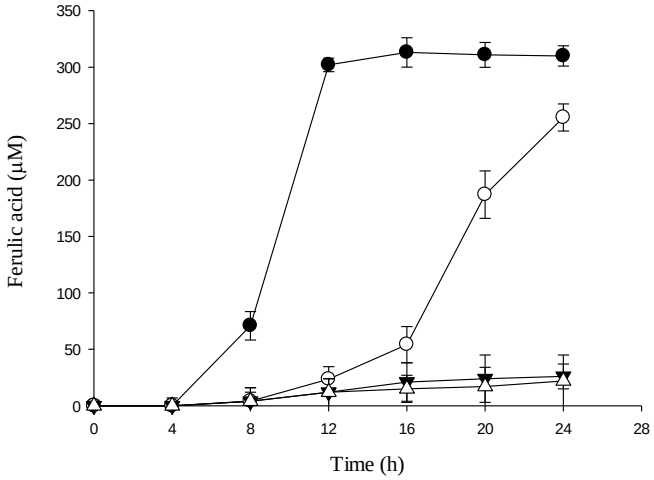

Fig. 3. Time course of ferulic acid accumulation from lignocellulolytic biomass. The reaction mixture contained 200 mg agricultural biomass and 1 U esterase/mg substrate in Tris buffer (50 mM, pH 8.0) in a final volume of 10 mL, which was gently shaken and incubated for 24 h. Symbols: (●) destarched wheat bran, (○) corncob, (▼) bagasse, (Δ) xylan.

Content of Total Phenolic Compounds in FA-rich DSWB Hydrolysate

The Folin-Ciocalteau method is commonly used to measure the total phenolic content. The total phenolic content in the FA-rich DSWB hydrolysate was 14.65 mg gallic acid equivalents/mL of hydrolysate solution. The FA concentration of DSWB hydrolysate was 0.58 mg/mL of hydrolysate solution. Many other phenolic compounds existed in the DSWB hydrolysate in significant quantities. Because many phenolic compounds exhibit potent antioxidant behavior as a result of their ability to scavenge radicals (Torres *et al*. 2002), the antioxidant properties of the hydrolysate solution were further investigated in the following experiments. Of the several approaches available to measure antioxidant properties, the DPPH radical-scavenging, ABTS radical-scavenging, and reducing power assays were selected for this study.

Assessment of Antioxidant Properties of the FA-rich DSWB Hydrolysate

The DPPH and ABTS radical-scavenging assays are widely applied to measure antioxidant activity. Awika *et al.* (2003) suggested that both methods could be equally useful for assessing the antioxidant activity of natural extracts at physiological pH and in situations in which color interference is not significant. As shown in Table 1, the FA-rich DSWB hydrolysate had better DPPH radical-scavenging ability and ABTS radical-scavenging ability than pure FA.

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*. 1995). The reducing power of FA-rich DSWB hydrolysate was 1.5 times greater than FA. When rye was hydrolyzed with commercial plant cell wall degrading enzyme preparations, ferulic acid, *trans-p*-sinapic, *trans-p*-coumaric, vanillic, *trans*-caffeic, protocatechuic, and p-hydroxybenzoic acid were found in the hydrolysate (Andreasen *et al.* 1999). These ferulic acid related compounds have good antioxidant properties (Kikuzaki *et al.* 2002). Such results can help to explain why the FA-rich DSWB hydrolysate had better antioxidant abilities than pure FA.

Table 1. Antioxidant Properties of FA-rich DSWB Hydrolysate

EC50 (μM)	FA-rich DSWB hydrolysate	FA
DPPH	179.21±0.43	298.09±0.07
ABTS	10.55±0.07	23.28±0.13

The antioxidant activities of putative antioxidants have been attributed to various mechanisms, including the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of hydrogen abstraction, and radical scavenging (Diplock 1997). According to the results of the total phenolic quantification, DPPH radical-scavenging assay, ABTS radical-scavenging assay, and reducing power assay, it was found that the FA-rich DSWB hydrolysate had greater antioxidant abilities than the same concentration of FA. Therefore, it is possible that there were other anti-oxidant compounds in addition to FA in the DSWB hydrolysate.

CONCLUSIONS

1. Of the xylan, bagasse, corncobs, and DSWB used as carbon sources for the production of feruloyl esterase, DSWB was found to be the best substrate in terms of extracellular esterase activity in the newly isolated thermophilic actinomycetes, *T. fusca* PU13-13.
2. After 96 h of cultivation with DSWB, the culture broth accumulated 946.0 U/mL of esterase activity.
3. The crude esterase preparation can be used directly to hydrolyze the DSWB and to produce the ferulic acid (310.0 μM) without pretreatment.
4. The FA-rich DSWB hydrolysate had better DPPH radical-scavenging ability, ABTS radical-scavenging ability, and reducing power than pure FA. The hydrolysate would have better antioxidant activity due to the presence of mixtures of phenolics.

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