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Enzymatic action mechanism of phenolic mobilization in oats (*Avena sativa* L.) during solid-state fermentation with *Monascus anka*

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ABSTRACT

This work aims to investigate the effects of carbohydrate-hydrolysing enzymes on the release of phenolics in oat fermentation with *Monascus anka*. There were good correlations between phenolic content and α-amylase, xylanase and FPase activities. A high level of α-amylase activity (141.07 U/g) was observed, while xylanase (2.40 U/g), total cellulase (0.52 U/g) and β-glucosidase activities (0.028 U/g) were relatively low in the fermentation system. The phenolic content of oat powder treated with crude enzyme from fermented oats significantly increased, especially that of the ferulic acid in the insoluble fraction and the vanillic acid in the soluble fraction. The surface SEM morphology of the oats showed that the cell wall structure was damaged by the crude enzyme treatment, which led to the release of phenolics. This study could provide metabolic understanding for optimization of phenolic compounds which could more efficiently increase the nutrition of oat intended for functional food ingredients.

1. Introduction

Phenolic compounds in cereals have been extensively researched due to their potential use as functional foods and supplements in alternative therapies. Many studies of *in vitro* antioxidant assays found out that both the soluble and insoluble-bound phenolics have demonstrated high antioxidant capacity, but the insoluble phenolic bioavailability is rather low (Chandrasekara & Shahidi, 2010; Liyana-Pathirana & Shahidi, 2006). Most phenolic compounds in cereal grains are insoluble-bound phenolics that are covalently bond to the structural components of the cell wall, such as cellulose, hemicellulose, lignin, pectin and proteins (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Carbohydrate-hydrolysing enzymes, such as pectinases, cellulases, amylases, hemicellulases and glucanases, have been reported that they can effectively be used to release the insoluble-bound phenolics, transforming them into soluble-free phenolics (Zheng, Hwang, & Chung, 2009).

Solid-state fermentation (SSF) of cereal substrates by fungi has been employed to increase the phenolic content in cereal products (Bhanja, Kumari, & Banerjee, 2009; Cai et al., 2011; Razak, Rashid, Jamaluddin, Sharifudin, & Long, 2015; Singh, Singh, Singh, & Nautiyal, 2010; Xiao et al., 2015). Some carbohydrate-hydrolysing enzymes generated by the microorganism, such as α-amylase, cellulase, β-glucosidase, and xylanase have played key roles in breaking the cereal cell walls (Đorđević, Šiler-Marinković, & Dimitrijević-Branković, 2010; Razak et al., 2015; Wang, Wu, & Shyu, 2014), which leads to the liberation of insoluble phenolics (Bhanja et al., 2009; Singh et al., 2010). Moreover, many studies also reported that some carbohydrate-hydrolysing enzymes, such as α-amylase, α/β-glucosidase and lignocellulolytic enzymes, have important influence on the mobilization of soluble phenolics in soil-state fermentation (Ajila et al., 2012; Bhanja et al., 2009; McCue & Shetty, 2005). *Monascus* is widely used to produce fermented food and it can produce many metabolic products, including enzymes such as β-glucosidase and proteases (Dikshit & Tallapragada, 2015; Liang, Lin, Yen, Wang, & Wang, 2006). We also found that the yields of free, conjugated and bound phenolic contents in oats were significantly improved by SSF with *Monascus anka* and presented high bioactivity in scavenging DPPH and $ABTS^+$ radicals (Bei, Liu, Wang, Chen, & Wu, 2017).

The main aim of this research was to explore the action of the carbohydrate-hydrolysing enzymes in phenolic mobilization in oat during solid-state fermentation with *Monascus anka*. We investigated the relationship between the soluble and insoluble phenolic contents and the α-amylase, β-glucosidase, xylanase, and cellulase activities

Abbreviations: DAD, diode array detector; GAE, gallic acid equivalents; HPLC, high performance liquid chromatography; ND, not detected; PNPG, *p*-nitrophenol β-D-glucopyranoside; PDA, potato dextrose agar; SSF, solid-state fermentation; SEM, scanning electron microscope; SD, standard deviation; TPC, total phenolic content

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during the fermentation, which will lead to the more efficient strategy for improving phenolic antioxidants in oat.

2. Materials and methods

2.1. Materials

The compounds gallic acid (≥99.8%), chlorogenic acid (≥99.8%), *p*-hydroxybenzoic acid (≥99.8%), caffeic acid (≥99.8%), vanillic acid (≥99.8%), rutin (≥99.8%), *p*-coumaric acid (≥99.8%), sinapic acid (≥99.8%), ferulic acid (≥99.8%), and quercetin (≥99.8%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade phosphoric acid and acetonitrile and Folin-Ciocalteu's reagent were purchased from Merck (Darmstadt, Germany). The substrates used for the enzymatic assays were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and regents were analytical grade and purchased from the Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The oats (*Avena sativa* L.) were purchased from the supermarkets of Guangdong province, Guangzhou, China.

2.2. Oat fermentation

2.2.1. Strain and inoculum

The fungus *Monascus anka* (GIM 3.592) was obtained from the publicly accessible culture collection in the Guangdong Culture Collection Center of Microbiology (GDMCC/GIMCC, China). The *Monascus anka* was incubated on potato dextrose agar (PDA) medium for 7 days at 30 °C. The spore suspension used for fermentation was prepared by washing the fungal colonies in the PDA plate with 0.1% Tween 80. Three millilitres of the spore suspension (approximately 10^6-10^7 spores/ml) was added to a 250-ml Erlenmeyer flask containing 50 ml of seed medium. The seed medium, with a volume of 1 l, included 20 g of glucose, 3 g of yeast extract, 10 g of peptone, 4 g of KH_2PO_4, 0.5 g of KCl, and 0.01 g of $FeSO_4{\cdot}7H_2O$ at a neutral pH. The *Monascus anka* spore suspension was incubated at 30 °C while shaking at 180 rpm for 28 h.

2.2.2. Solid-state fermentation

Before being used for fermentation, the oats were soaked in water for 6 h, after being completely washed. The solid state fermentation substrate was made up by oats (20 g), glycerol (1.6 g), yeast extract (0.4 g) and water added to a 60% moisture content. The fermentation substrate was sterilized by autoclaving at 115 °C for 20 min, and then cooled prior to inoculation. The sterilized fermentation substrate was inoculated with *Monascus anka* spore suspension (0.1 ml of spore suspension per 1 g dry oats), and the culture was incubated at 30 °C for 14 days. During fermentation, the samples were aseptically taken out at days 0, 2, 4, 6, 8, 10, 12, and 14 for analysis. After drying at 60 °C overnight, the samples were milled with a food grinder and sieved through a 0.4-mm sieve for analysis.

2.2.3. Crude enzyme solution extracted from fermented oats

The crude enzyme solution were extracted by adding 50 ml of citrate buffer (50 mM, pH 5.5) to the fermented oats, followed by shaking the contents of the flasks at 180 rpm, 30 °C for 30 min to extract the enzymes into the solution. The contents were subsequently centrifuged at 4 °C and 10,000 ×*g* for 30 min. The supernatant was assayed for enzymes activity.

2.3. Enzyme activity assay

2.3.1. α-Amylase activity

The α-amylase activity was determined spectrophotometrically using a method described by Bhanja et al. (2009), with a little modification. Briefly, 500 µl of the appropriately diluted enzyme was mixed with 350 µl of 1% starch solution (w/v, in 20 mM sodium phosphate monobasic, 6 mM sodium chloride, pH 6.9). After 10 min of incubation at 50 °C, the concentration of liberated glucose by the α-amylase was estimated spectrophotometrically at 540 nm. One unit (U) of amylase activity is defined as the amount of enzyme that liberated one micromole of reducing sugar (glucose) per min under the assay conditions. The results were expressed as U/g.

2.3.2. Xylanase activity

The xylanase activity was assayed by a modified procedure based on the methods of Bhanja et al. (2009), and the results were express as U/g. It was determined by mixing 500 µl of the appropriately diluted enzyme with 500 µl of 1% xylan (w/v, in 50 mM citrate buffer, pH 4.8). The concentration of liberated xylose by the xylanase was estimate spectrophotometrically at 540 nm, after 30 min of incubation at 50 °C. One unit of enzyme activity was defined as the amount of enzyme that liberates one micromole of xylose per min under the assay conditions.

2.3.3. β-Glucosidase activity

The β-glucosidase activity was assayed using a modified procedure based on the methods of Hu et al. (2017), and the results were expressed as U/g. The reaction system contained 100 µl of enzyme solution, 800 µl of sodium acetate buffer (200 mM, pH 4.8), and 100 µl of 9 mM *p*-nitrophenol β-D-glucopyranoside (PNPG). After 10 min of incubation at 45 °C, the reaction was stopped by the addition of 500 µl of 0.1 M Na_2CO_3, and the absorbance was measured at 420 nm. One unit (U) of β-glucosidase activity was defined as the amount of enzyme that releases one micromole of *p*-nitrophenol per min under the assay conditions.

2.3.4. The total cellulase (filter paper cellulase, FPase) activities

Filter paper cellulase activity was reported in FP units per gram of dry substrate (U/g), using the IUPAC procedures recommended by Ghose (1987). One unit (U) of enzyme was defined as the amount of enzyme required to release one micromole of glucose per min under the standard assay conditions.

In each enzyme activity assay, inactivated crude enzymes solution was used as control.

2.4. Oat powder treatment with crude enzyme from fermented oats

The raw oats, which ground to 40-mesh using a micromill, were tempered to a moisture content of 60% by citrate buffer (50 mM, pH 5.5). The oat powders were mixed with crude enzymes solution to begin the reaction. The enzymatic reaction was carried out at 30 °C in the dark for 6 h. The oat powders with inactivated crude enzymes solution was used as control for the enzymatic treatment. The enzymatic reactions were terminated by being placed in a 105 °C oven for 5 min and reground to 40-mesh (Tang et al., 2016).

2.5. Soluble phenolic extraction

The soluble phenolic extraction procedure followed the method described by Qiu, Liu, and Beta (2010), with a little modification. One gram of oat powder was extracted twice with 25 ml 80% methanol (*v/v*). The mixture was incubated at 45 °C for 1 h before every extraction. The mixture was subsequently centrifuged at 25 °C and 8000 ×*g* for 5 min. The supernatant obtained from each extraction, were combined and concentrated by rotary evaporator at 37 °C. After using hexanes to extract the lipids in the concentrated solution, the solution was extracted three times with 70 ml ethyl acetate via liquid-liquid partitioning. The ethyl acetate extracts were combined and concentrated to dryness. The extract was redissolved in 5 ml of 50% methanol (*v/v*).

2.6. Insoluble phenolic extraction

Insoluble phenolic fraction was extracted from the soluble phenolic