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Partial purification and characterization of a thermostable mushroom tannase induced during solid state fermentation of *Toxicodendron vernicifluum* stem bark by *Fomitella fraxinea*

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ABSTRACT

A thermostable extracellular tannase induced during solid state fermentation of the stem bark of *Toxicodendron vernicifluum* (Stokes) F.A. Barkley (Anacardiaceae) with *Fomitella fraxinea* was homogeneously isolated through ammonium sulfate precipitation and purified by DEAE-cellulose, and Sephadex G–100 gel chromatography. It was then characterized for optimal reaction temperature and pH, thermal and pH stability, effects of metal ions as well as its hydrolysis patterns using 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) as a substrate. The enzyme was purified 103.9-fold with 2.92% recovery and a single protein band corresponding to it was observed at 44.9 kDa during SDS-PAGE analysis. Optimal temperature and pH were found to be 50–70 °C and 5.5, respectively. This enzyme was substantially stable at temperatures below 70 °C and pH values between 5.0 and 7.0. Its activity was inhibited by Fe^{2+} ion (66%) and Cu^{2+} (39%) at a concentration of 5 mM. It also showed a different hydrolysis pattern from that of commercial *Aspergillus oryzae* tannase when PGG was used as a substrate. The tannase purified from the fermented plant material in this study unusually produced oligomeric galloylglucoses such as digalloylglucose (DGG), trigalloylglucose (TGG) and tetragalloylglucose (TeGG) from PGG while the *Asp. oryzae* tannase produced gallic acid and methyl gallate as final products. Therefore, this enzyme can be more preferably applied for production of bioactive oligomeric galloylglucoses such as DGG, TGG and TeGG from PGG or tannic acid.

1. Introduction

Tannins are natural polyphenolic compounds that are widely distributed in several parts of vascular plants with molecular weights ranging from 500 to 3000 Da and high polyphenol contents (Aguilar et al., 2007; Rodríguez et al., 2008). Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes hydrolysis of ester and depside linkages of substrates such as gallotannins, ellagtannins, complex tannins, gallic acid esters, epicatechin gallates and epigallocathin gallate to release gallic acid, ellagic acid and glucose (Lekha and Lonsane, 1997; Banerjee and Mahapatra, 2012; Zhang et al., 2015). Gallic acid which is the major product during hydrolysis of tannin is a valuable compound used in pharmaceutical, dye making, food, leather and chemical industries (Lekha and Lonsane, 1997; Mukherjee and Banerjee, 2004; Hassan et al., 2018). Tannase is the key enzyme applied for commercial production of gallic acid from gallotannin and gallotannin-rich plant materials (Deschamps et al., 1983; Yao et al., 2014; Govindarajan et al., 2016). Tannases have also been widely applied in the food industry as clarifying agents in processing of fruit juices and coffee, flavoured soft drinks and manufacture of instant teas (Aguilar et al., 2007; Zhang et al., 2015; Mahmoud et al., 2018) as well as recovery of low-molecular weight gallotannins and biodegradation of anti-nutritional factors in agricultural residues (Da Luz et al., 2013; Si et al., 2014). Tannases are present in plants, animals and microorganisms while the commercially applicable tannases are mainly produced by the latter (Ayed and Hamdi, 2002; Belmares et al., 2004). Although a large number of microorganisms including fungi, yeasts, and bacteria have been studied to produce the enzyme (Banerjee and Mahapatra, 2012; Zhong et al., 2004; Kostinek et al., 2007) the most dominant tannase producers are *Aspergillus* sp. and *Penicillium* spp. of the fungi kingdom (Banerjee and

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Mahapatra, 2012).

However, it is still substantially attractive to search for new sources of tannase with more desirable characteristics in terms of catalytic function, stability and substrate specificity as well as product portfolio. Especially, mushrooms (wood-rot fungi) have potentials as good producers of extracellular tannases since they are capable of excreting various enzymes into the media (Buswell et al., 1996; Baldrian and Valášková, 2008). Few studies on the production and characterization of tannases from mushrooms have also been reported. For example, Hong et al. (1990) who examined tannase from various mushroom species such as *Pleurotus ostreastus*, *Lentinus edodes*, *Ganoderma lucidum* and *Lenzites betulina* under submerged fermentation conditions reported a higher tannase activity in *L. betulina* than the others. Sobal et al. (2003) reported degradation of tannin and production of tannase by edible mushrooms in coffee-husk solid culture medium and Da Luz et al. (2013) reported that *Pleurotus ostreatus* (oyster mushroom) can degrade tannins found in *Jatropha curcas* seed cake. A more recent study by Prendecka et al. (2016) reported induction of extracellular tannase when white-rot fungi such as *Phellinus pini, Fomes fomentarius* and *Tyromyces pubescens* were cultured in media containing tannic acid or rapeseed meal as carbon sources. Several studies have reported that production of microbial enzyme including tannase by solid state fermentation has interesting biotechnological advantages such as processing simplicity, simpler culture media, lower energy requirement, higher enzyme titers, increased yield and low risk of microbial contamination compared to submerged fermentation (Aguilar et al., 2001, 2008, 2008; Hölker et al., 2004; Aguilar et al., 2008; Castro and Sato, 2015). However, only limited studies on biotechnological features of tannases from mushrooms are available so far. In our previous study (Kim et al., 2019), we reported that the PGG present abundantly in the stem bark of *T. vernicifluum* was hydrolyzed to oligomeric galloylglucoses such as digalloylglucose (DGG), trigalloylglucose (TGG) and tetragalloylglucose (TeGG) rather than gallic acid during *F. fraxinea*–mediated fermentation. This sparked our interest to explore new biochemical properties of the mushroom enzyme through hydrolysis of PGG to commercially valuable components including galloylglucose derivatives. Therefore, this study aimed at purifying and characterizing tannase produced during solid-state fermentation of *T. vernicifluum* stem bark by the mushroom *F. fraxinea*, and investigating its hydrolytic characteristics using PGG as a substrate.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

Fresh stem bark of *T. vernicifluum* (Stokes) F.A. Barkley (Anacardiaceae) was purchased from a farm located near the area of Imsil-gun, Jeonbuk, Republic of Korea in October 2017. It was air-dried at 60 °C for 24 h and ground into coarse particles (approximately 6 mm × 2 mm) using household mixer and cutter (model HMF-3100S, Hanil Electric Co., Seoul, Republic of Korea) and stored at −20 °C until use.

2.1.2. Microorganism

F. fraxinea (Bull.) Imazeki (KACC 42289) used in this study was donated from the Korean Agricultural Culture Collection (KACC) of Rural Development Administration (Wanju, Jeonbuk, Republic of Korea). The strain was maintained by serial transferring of inoculum into a fresh medium from previous stock medium every three months and was kept on a slant of 2% saccharified malt extract agar at 4 °C.

2.2. Reagents

Tannic acid, gallic acid, methyl gallate, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), triethanolamine, diethylaminoethyl (DEAE)–cellulose, Sephadex G–100 and dialysis tubing cellulose membrane (14,000 cut–off molecular weight) were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). PGG was prepared from tannic acid according to the method of Chen and Hagerman (2004) with slight modification. The purified compound was identified through comparison of spectral data by nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectroscopy and retention time by high-performance liquid chromatography (HPLC) with that of an authentic compound (Kim et al., 2019). *Aspergillus oryzae* tannase (78 U/mg) was purchased from Wako Pure Chemical Co. (Osaka Japan). Mini-protean TGX precast gel and pre-stained protein molecular standards (10–250 kDa) and Coomassie brilliant blue R-250 for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA). All other reagents were of analytical grade from commercial sources.

2.3. Cultivation of mycelium culture

F. fraxinea strain was cultivated according to the procedure described in our previous paper (Kim et al., 2019). Briefly, the strain was pre-incubated onto potato dextrose agar (Becton, Dickinson and Company, Sparks, MD, USA) for 6 days at 25 °C. The pre-incubated strain was inoculated into germinated malt medium (11 Brix %), saccharified at 65 °C with four-fold tap water (v/v) for 8 h and then cultured for 2 weeks at 25–26 °C with gentle shaking (120 rpm) using an orbital shaker (model SK-600, Jeiotech Co., LTD. Daejon, Korea).

2.4. Induction of tannase by F. fraxinea in T. vernicifluum stem bark

2.4.1. Solid state fermentation

The coarse powder of air-dried *T. vernicifluum* stem bark (50 g) was placed in labeled Erlenmeyer flask (250 mL). Each flask was sterilized at 121 °C for 30 min and the day-0 (control) sample was freeze-dried immediately and stored at −20 °C until use. The remaining samples were inoculated with 2.5 mL starter culture of *F. fraxinea* liquid culture except for the unfermented (matched controls) and incubated at room temperature (25–26 °C) for 22 days without shaking. The sample for enzyme assay was withdrawn at regular intervals from a different flask for each period of fermentation and was freeze-dried immediately for 4 days, and then stored at −20 °C until use.

2.4.2. Enzyme extraction

The enzyme was extracted from the freeze dried sample with 5-fold (w/v) sodium acetate buffer (0.1 M, pH 5.5) by gentle stirring for 12 h at 4 °C. The resultant slurry was centrifuged at 4,500 × *g* for 20 min and the resultant supernatant was used for tannase assay.

2.5. Tannase purification

2.5.1. Preparation of crude enzyme extract

All procedures for enzyme purification were carried out at room temperature unless otherwise indicated. The freeze dried sample (100 g) after 10 days of fermentation was homogenized with 6-fold (w/v) sodium acetate buffer (0.1 M, pH 5.5) using an Omni mixer homogenizer (Omni International, Kennesaw, GA, USA) for 1 min at 4 °C. The slurry was squeezed through non-woven cloth and the filtrate was centrifuged at 10,000 × *g* for 20 min at 4 °C. Solid ammonium sulfate was added to the supernatant (500 mL), initially to 30% and eventually to 80% saturation. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the resultant precipitate was dissolved in 10 mM sodium acetate buffer (pH 5.5). After dialysis with a molecular weight dialysis tubing cellulose membrane (14000 cut-off) for 24 h, the solution was centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatant was lyophilized.

2.5.2. Ion exchange and gel chromatography

A lyophilized precipitate (80 mg) was dissolved in 10 mM sodium

acetate buffer (pH 5.5) and then loaded onto a DEAE–cellulose column (3 cm × 18 cm) pre-equilibrated with 10 mM sodium acetate buffer (pH 5.5). The column was washed with same buffer for 20 min. The enzyme was eluted with linear gradient condition of 0–0.5 M NaCl in 10 mM sodium acetate buffer (pH 5.5) with a flow rate of 1.0 mL/min and the eluate was collected 4 mL per tube. The active fractions were pooled, dialyzed against 10 mM sodium acetate buffer (pH 5.5) for 24 h at 4 °C and lyophilized. A lyophilized active fraction was dissolved in 10 mM sodium acetate buffer (pH 5.5) and was then loaded onto a Sephadex G–100 chromatographic column (1.8 cm × 70 cm) equilibrated with the same buffer. The column was eluted with 10 mM sodium acetate buffer (pH 5.5) at a flow rate of 1.0 mL/min. The fractions showing tannase activities were pooled and lyophilized.

2.6. Electrophoresis and molecular mass determination

SDS-PAGE of purified tannase was performed with a 12% mini-protean TGX precast gel at a constant current of 110 mA according to the method described by Laemmli (1970). The gel run time was 60 min. The gel was then stained with Coomassie brilliant blue R–250 and destained with a mixture of 10% methanol and 10% acetic acid in distilled water. Molecular weight of the purified tannase was determined by comparison of the migration distance with pre-stained protein standards (10–250 kDa) on SDS-PAGE.

2.7. Enzymatic activity assay and protein assay

Tannase activity was determined colorimetrically according to the method described by Mondal et al. (2001) with some modifications. The substrate reagent (0.15 mL) contained 3 mM of tannic acid in 0.2 M sodium acetate buffer (pH 5.5) and 0.2 mL of enzyme was added to it after which the reaction was incubated at 30 °C for 1 h. The enzymatic reaction was stopped by addition of 1.5 mL of BSA solution (1 mg/mL) which precipitated the remaining tannic acid. The reaction tube was centrifuged (4,500 rpm) for 20 min and the resultant precipitate was dissolved in 1.5 mL SDS-triethanolamine solution. Next, 0.5 mL of $FeCl_3$ reagent was added to each tube and it was kept at room temperature for 15 min to stabilize the color. The absorbance was then read at 530 nm against blank (same solution without tannic acid). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of tannic acid per 1 min. Protein concentration was assayed by the method of Lowry et al. (1951) using BSA as a standard.

2.8. Characterization of purified tannase

2.8.1. Optimal temperature and thermal stability

Tannase solution (0.2 mL) containing 2 U was mixed with 0.15 mL of 3 mM tannic acid in 0.2 M sodium acetate buffer (pH 5.5) and incubated at 20–90 °C for 30 min. Unreacted tannic acid was precipitated by adding 1.5 mL of BSA solution (1 mg/mL) followed by 15 min of incubation at room temperature. The mixture was then centrifuged and the amount of residual tannic acid quantified according to the method described above. Thermal stability of tannase was determined by maintaining 0.2 mL of enzyme solution at 40–90 °C (with 10 °C intervals) for 180 min with the addition of 0.15 mL substrate solution each time. The same procedures were then followed to precipitate and quantify residual tannic acid. The enzymatic activity was expressed as relative activity (%) based on the highest value after calculation.

2.8.2. Optimal pH and pH stability

Tannic acid dissolved in 0.1 M citrate buffer (pH 6.0–8.0), 0.1 M phosphate buffer (pH 9.0) and 0.1 M Tris buffer (pH 10.0) to a concentration of 3 mM and enzyme solution (0.2 mL) were added. After incubation for 30 min at 40 °C, 1.5 mL of the BSA solution (1 mg/mL) was then added to each reaction solution and the mixture was allowed to stand for 15 min at room temperature. After centrifugation for 10 min at 4 °C, the residual tannic acid was quantitatively determined in the same manner as in the optimum temperature. For pH stability, 0.2 mL of the enzyme solution was dissolved in a buffer solution having the same pH as in the optimum pH test, and then incubated at an optimum reaction temperature of 40 °C for 30 and 60 min, respectively. The remaining enzyme activity was measured as in the above method. The enzymatic activity was expressed as relative activity (%) based on the highest activity after the enzyme activity was calculated.

2.8.3. Effect of salts on enzymatic activity

The effects of salts on tannase activity were measured by a method described by Mukherjee and Banerjee (2006) with slight modifications. Briefly, each salts ($FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CuSO_4$, $CaCl_2$, $CoCl_2$) and ethylenediaminetetraacetic acid (EDTA) were added at 1 and 5 mM to 1.5 ml of the substrate solution containing 3 mM of tannic acid in 0.1 M sodium acetate buffer (pH 5.5) and then 0.2 mL of enzyme solution was added. The mixture was kept at 40 °C for 30 min. The reaction was terminated by adding 1.5 mL of BSA solution (1 mg/mL) to the reaction solution followed by maintaining the mixture at room temperature for 15 min. The amount of residual acid after centrifugation was then determined by the same method as in the optimum temperature assay above. The relative enzyme activity (%) after the addition of each salt was calculated against the blank (no salt).

2.9. Comparison of hydrolysis pattern of PGG by purified tannase

Hydrolysis patterns of PGG by the purified *F. fraxinea* tannase and a commercial *Asp. oryzae* tannase were compared as follows: 20 mL of reaction mixture containing 200 mg of PGG in 1.0 mL methanol and enzyme solution containing 100 U of tannase in 0.1 M sodium acetate buffer (pH 5.5) was incubated at 37 °C for 8 h with gentle shaking (80 rpm). 2 mL reaction mixtures were withdrawn at regular time intervals and the enzyme was inactivated by heating in boiling water bath for 10 min. The solution was filtered through membrane filter (0.4 μm) and was analyzed by HPLC.

2.10. HPLC analysis

HPLC analysis was conducted using Waters HPLC system (1525 binary pump, 717 plus autosampler and 996 photodiode array (PDA) detector and YMC–Pack Pro C18 RS (4.6 cm × 25 cm, 5 μm) column (YMC Co., Kyoto, Japan). The mobile phase consisted of 0.1% formic acid in deionized water (A) and 90% acetonitrile in deionized water (B). The ratio of A: B was maintained at 95:5 (2 min), 45:55 (2–25 min), 40:60 (25–30 min), and 95:5 (30–40 min) at a flow rate of 0.8 mL/min. The detector wavelength was set at 310 nm.

2.11. LC-MS analysis

LC-MS analysis was performed as described in our previous study (Kim et al., 2019).

2.12. Statistical analysis

All experiments were conducted in triplicate. All of the values were expressed as mean ± standard deviation (SD).

3. Results and discussions

3.1. Induction of tannase during fermentation of T. vernicifluum stem bark by F. fraxinea

In solid state fermentation process, the moisture content of substrate is considered to be one of the important factors. Too much moisture may compact the substrate, prevents oxygen penetration and facilitate contamination by fast growing microbes. Therefore, the moisture

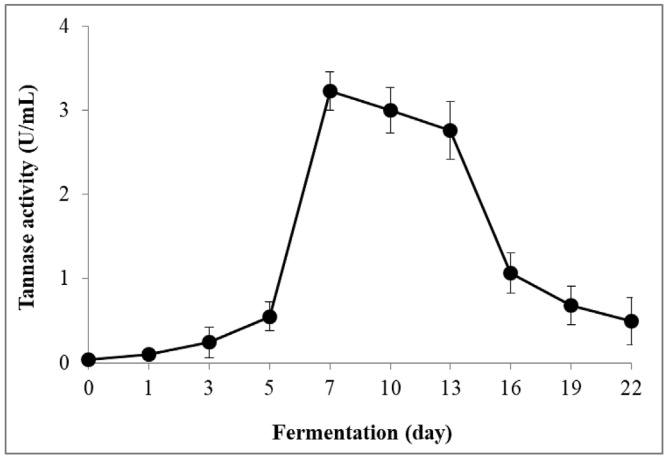

Fig. 1. Change of tannase activity during solid state fermentation of *T. vernicifluum* stem bark with *F. fraxinea*.

content of the substrate should not exceed the capacity of saturation of the solid bed in which the mushroom mycelia grow (Laukevics et al., 1984; Aguilar et al., 2008). Kumar et al. (2007) suggested that yield of tannase production from *Asp. ruber* using jamum (*Syzygium cunini*) leaves under solid state fermentation was maximum when substrate to water ratio was 1:2 (w/v). In this study, tap water was added to adjust the moisture content to approximately 65%. According to previous studies tap water is the best moisturizing agent for fungal enzyme production in solid state fermentation (Kumar et al., 2007; Manjit et al., 2008). The ability of microorganisms for enzyme production with tap water i.e. without addition of any minerals could also lead to overall cost reduction (Manjit et al., 2008). The changes in tannase activity during fermentation of *T. vernicifluum* stem bark with *F. fraxinea* are shown in Fig. 1. It can be seen that there was almost no tannase activity at the early stage of the fermentation. However, enzymatic activity rapidly increased from 5 days onwards and reached its maximum at 7–10 days of fermentation from which it started to decrease slowly until 13 days. After 13 days, there was a sharp fall in activity and only minimum tannase activity is observed from 20 days onwards. The reduction of enzyme activity is suggested to be caused by reduction of nutrients for the continued growth of mycelium, accumulation of the end product which hampers tannase production or due to appearance of toxic metabolites during fermentation (Kar et al., 1999; Manjit et al., 2008). Lal and Gardner (2012) reported that maximum tannase activity is observed at 7–9 days after inoculation of *Asp. niger* into Czapek's Dox medium supplemented with tannic acid as carbon source with 13 days of incubation. On the other hand, tannase activity could not increase in the matched control sample without mushroom inoculation incubated for the same period of time (data not shown). These results indicate that the *F. fraxinea* tannase is induced when the strain was cultured in the stem bark of *T. vernicifluum* containing high level of gallotannin.

It is well established that tannases are extracellular inducible enzymes produced in the presence of an inducer tannic acid. Tannin-rich plant materials are the most common inducers reported for tannase induction from fungi (Lekha and Lonsane, 1997; Das Mohapatra et al., 2006; Aguilar et al., 2007; Hassan et al., 2018). Many research findings indicated that some other chemical compounds like gallic acid, pyrogallol, and methyl gallate can also induce tannase expression (Bajpai and Patil, 1996, 1997). Aguilar et al. (2001) reported that the concentration of tannic acid adequate for tannase production was up to 20% in solid state fermentation, while it ranged from 0.1% to 10% in submerged fermentation. Many microorganisms capable of producing tannase have been reported to be able to use tannin as a carbon source (Yao et al., 2014). Moreover, our previous study (Kim et al., 2019) reported that PGG content decreased during fermentation of *T. vernicifluum* stem bark with *F. fraxinea* and several oligomeric

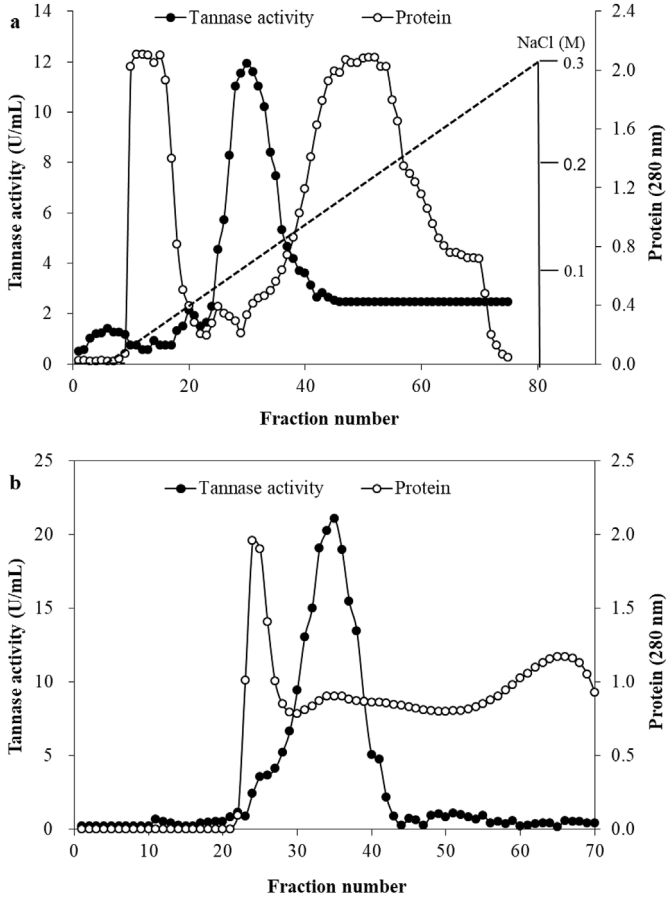

Fig. 2. Elution profile of tannase on DEAE-cellulose ion exchange (**a**) and Sephadex G-100 gel (**b**) chromatography.

galloylglucoses derived from PGG were newly detected. Therefore, it can be suggested that these results are attributed to the biocatalytic action of newly induced tannase from *F. fraxinea* mycelia during fermentation in gallotannin-rich medium. In this study, purification of tannase was attempted as described below to investigate enzymatic properties of the induced enzyme and the degradation characteristics of PGG as substrate.

3.2. Enzyme purification

Many studies so far have been reported on tannase purification by techniques such as ultrafiltration, solvent precipitation, ammonium sulfate precipitation, ion exchange and gel filtration chromatography (Yao et al., 2014). Extracellular tannases can be purified using the two chromatography techniques DEAE–cellulose anion exchange and gel filtration chromatography in chronological order. The enzyme was purified by DEAE–cellulose chromatography using ammonium sulfate precipitate (30–80%) prepared from fermented *T. vernicifluum* stem bark. A single peak with strong tannase activity was detected when eluted in a gradient mode using a buffer solution containing NaCl as an eluent (Fig. 2a). This result indicates that tannase produced by *F. fraxinea* is a single enzyme with no isozyme. Then, the active fraction separated by DEAE–cellulose chromatography was subsequently subjected to Sephadex G–100 gel chromatography. As shown in Fig. 2b, gel chromatography also enabled us to detect a major protein band with strong tannase activity. The general enzymatic characteristics of the tannase at each purification stage are shown in Table 1. It was able to be purified at 76.7 folds with a total recovery of 20.2% and specific activity of 94.3 U/mg protein. Although the purification fold or yield of tannase depends on the type of microorganism used and separation

Table 1
Purification of tannase from fermented *Toxicodendron vernicifluum* stem bark by *F. fraxinea*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yields (%)
Crude enzyme	2348.0	2880.0	1.2	1.0	100.0
$(NH_4)_2SO_4$ precipitate	336.0	1089.0	3.2	2.6	37.8
DEAE-cellulose	16.0	762.3	47.6	38.7	26.5
Sephadex G-100	6.16	580.6	94.3	76.7	20.2

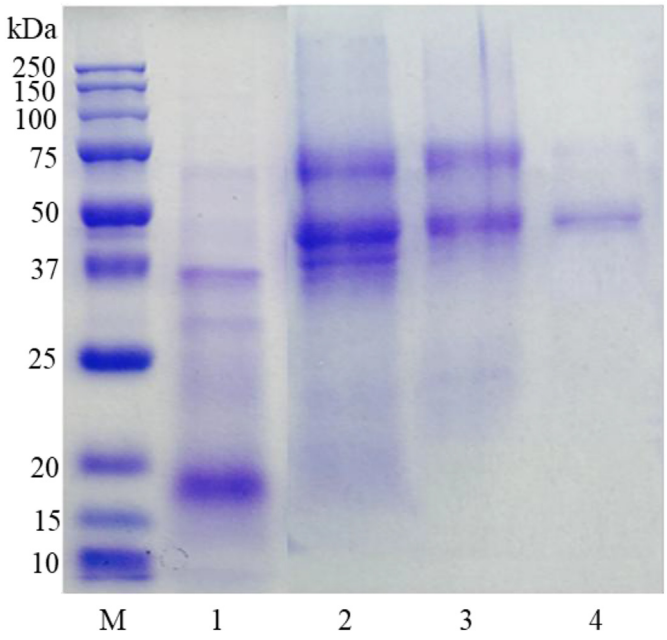

Fig. 3. SDS-PAGE analysis of tannase induced during solid state fermentation of *T. vernicifluum* stem bark with *F. fraxinea*. Lane M, protein markers; lane 1, ammonium sulfate (30–80%) precipitate from *F. fraxinea* culture cultivated in germinated malt medium; lane 2, ammonium sulfate precipitate from solid state fermented *T. vernicifluum* stem bark with *F. fraxinea*; lane 3, partially purified tannase by DEAE–cellulose chromatography; lane 4: purified tannase by Sephadex G-100.

techniques applied, purification fold of tannases from *Aspergillus* sp. have been reported to range from 2.33 to 135 fold with yields of 2.12–79.06% (Yao et al., 2014).

3.3. SDS-PAGE analysis

The gel patterns of the protein extract after 10 days of fermentation were compared by SDS–PAGE (Fig. 3). The results showed that the ammonium sulfate (30–80%) fraction resolved into several protein bands (lane 2 in Fig. 3) while a major protein band with a trace contamination was detected in the enzyme preparation after Sephadex G–100 gel chromatography (lane 4 in Fig. 3). On the other hand, protein band corresponding to tannase was not detected when *F. fraxinea* was cultured in germinated malt medium for 10 days under the same fermentation condition (lane 1 in Fig. 3). These results suggest that a new tannase enzyme can be produced from mushroom *F. fraxinea* by cultivation with the *T. vernicifluum* stem bark that contains gallotannin compounds such as PGG. Molecular weight of the tannase purified in this study was estimated to be 44.9 kDa by comparing its relative migration distance with those of protein standards based on the SDS–PAGE. All previously isolated tannase enzymes from fungi were reported to have molecular weights ranging from approximately 31 − 320 kDa (Banerjee and Mahapatra, 2012; Chávez-González et al., 2012; Yao et al., 2014; Aharwar and Parihar, 2018). Microbial tannases have different molecular weights depending on their origin. For example, bacterial tannases have low molecular weight ranging from 31–90 kDa (Mahendran et al., 2006; Curiel et al., 2009; Jana et al., 2013; Govindarajan et al., 2016), whereas fungal tannases can have relatively higher molecular weights ranging from 30–310 kDa (Yao et al., 2014; Aharwar and Parihar, 2018; Farag et al., 2018).

3.4. Enzyme characterizations (thermostability, pH stability, effect of solvated salt ions)

Thermal and pH stability are the most important factors for industrial applications of tannase. In order to determine the optimum temperature, enzymatic activity was investigated at reaction temperatures ranging from 20 to 90 °C for 30 min. The enzyme purified from the *F. fraxinea* fermented *T. vernicifluum* stem bark in this study exhibited strong activity at temperatures between 50 –70 °C and moderate activity at temperatures 70–80 °C (Fig. 4a). Generally, the optimum temperatures of tannases from fungi such as *Aspergillus* sp. and *Penicillium* sp. are in the range of 20–70 °C, while those of bacterial such as *Lactobacillus* sp., *Bacillus* sp. and *Paecilomyces variotii* are in the range of 40–70 °C (Belmares et al., 2004; Mukherjee and Banerjee, 2006; Banerjee and Mahapatra, 2012; Chávez-González et al., 2012; Yao

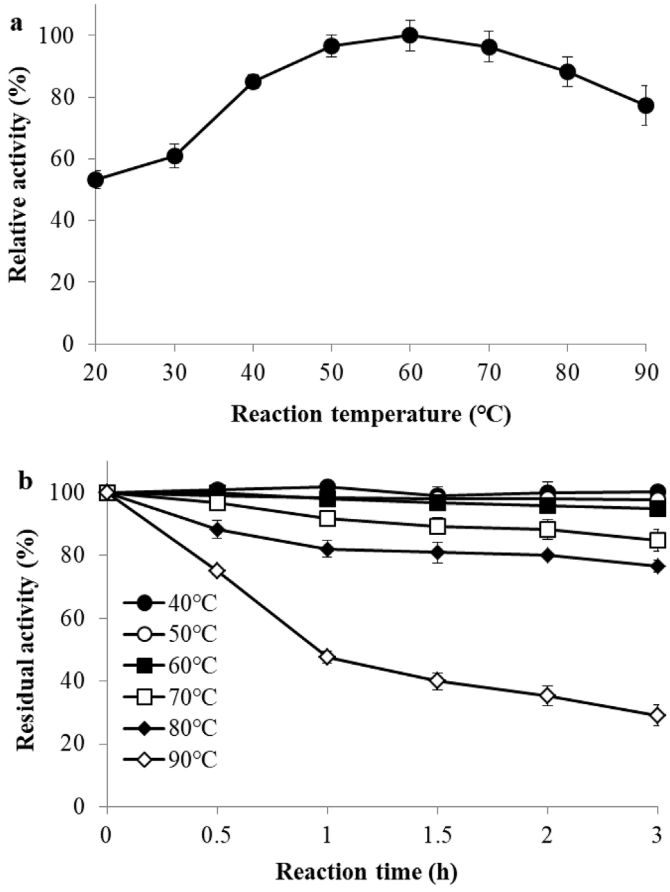

Fig. 4. Optimal temperature (**a**) and thermal stability (**b**) of purified *F. fraxinea* tannase.

et al., 2014; Aharwar and Parihar, 2018; Farag et al., 2018). The optimal temperature of the enzyme isolated in this study was considerably higher than those of tannases reported in previous studies which add to its novelty from thermostability point of view. However, optimal temperature of microbial tannases can reach higher values up to 70 °C (Ramírez-Coronel at al., 2003; Battestin and Macedo, 2007). To evaluate thermal stability of the purified enzyme at extended period of time, its activity was recorded at temperatures of 40–90 °C with 10 °C intervals after 180 min of incubations for each reaction temperature. There was no significant decrease in enzyme activity for temperatures below 70 °C at the end of 180 min while a decrease of about 20% was observed for reaction temperatures of 70–80 °C. As it can be seen from Fig. 4b, the enzyme showed maximum stability at 40–60 °C with a considerable stability up to 80 °C. It can also be observed that the enzyme exhibited maximum activity in a plateau phenomenon at temperatures from 50 to 80 °C rather than at a certain peak of narrow temperature range. Moreover, the induced tannase was found to be a thermophilic enzyme with an unusually strong activity at 70–80 °C rather than at the common low temperature for microbial tannases. In addition, a fungal plant pathogen, *Pestalotiopsis guepinii* URM 7114 was reported to have thermostability in a range from 30 to 90 °C (De Sena et al., 2014). Therefore, these results suggest that this enzyme is considerably thermostable compared to tannases isolated from other microorganisms so far.

Similarly, to investigate optimum pH and pH stability for the isolated tannase, it was tested at reaction pH values ranging from 3 to 10 as shown in Fig. 5. Enzymatic activity of the purified tannase in this

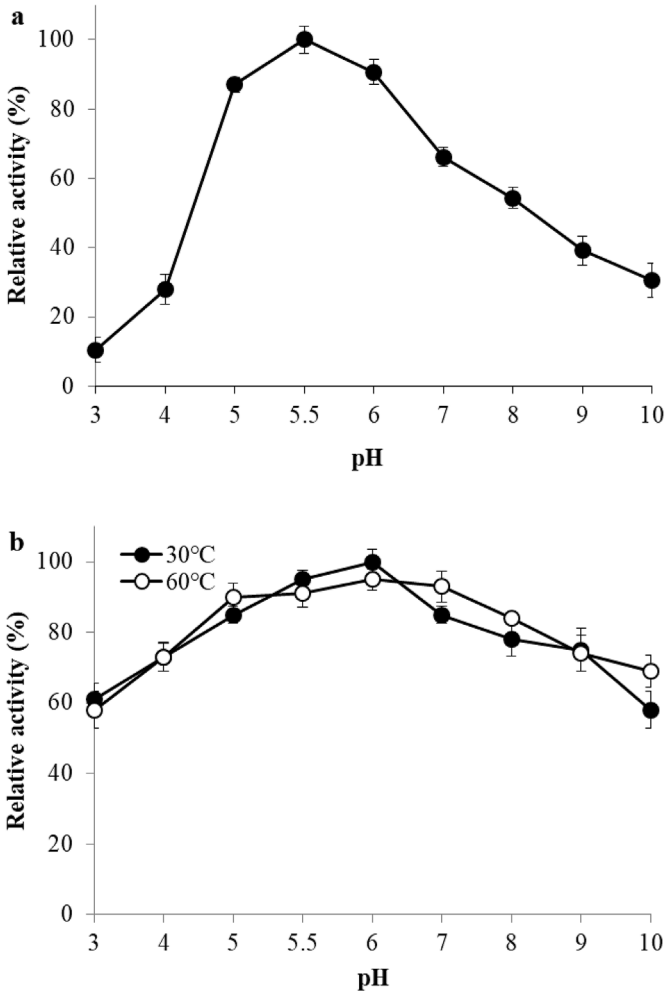

Fig. 5. Optimal pH (**a**) and pH stability (**b**) of purified *F. fraxinea* tannase.

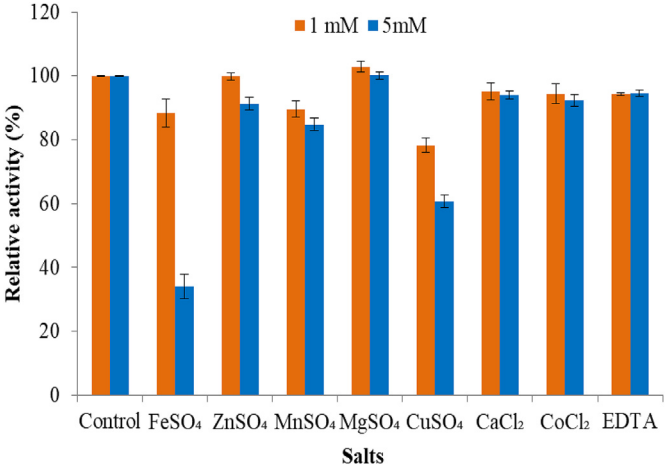

Fig. 6. Effects of salts on purified *F. fraxinea* tannase activity during hydrolysis of PGG.

study showed a sharp increase from pH above 4.0 with highest activity observed at a pH of 5.5 (Fig. 6a). Its activity markedly decreased at pH below 4.0 or above 7.0. These results are in agreement with previous studies that reported maximum activity of microbial tannases from *Aspergillus* sp., *Penicillium* sp. *Bacillus* sp. and *Rhizopus* sp. at pH 5.5 (Lekha and Lonsane, 1997; Banerjee and Mahapatra, 2012; Jana et al., 2013; Aharwar and Parihar, 2018). The enzyme was considerably stable at pH 5–7 when it was monitored at temperatures 30 and 60 °C (Fig. 5b). These results are in agreement with previous studies that reported fungal tannases showing pH stability in the range of pH 5.5–8.0 (Lekha and Lonsane, 1997; Banerjee and Mahapatra, 2012; Jana et al., 2013; Yao et al., 2014; Aharwar and Parihar, 2018).

In order to investigate the effect of solvated salt ions on the activity of purified tannase, Fe^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+} and Co^{2+} were tested at final concentrations of 1 mM and 5 mM. The results are summarized in Fig. 6. Fe^{2+} and Cu^{2+} at a concentration of 5 mM showed the strongest inhibitory effects of about 66 and 39%, respectively, compared with that of control. Both ions at 1 mM concentration also inhibited tannase activity to relatively less extents of about 12 and 21%, respectively. Zn^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} did not inhibit enzyme activity at both concentrations of 1 and 5 mM. The effects of salts on tannase activity were described by other previous reports (Lekha and Lonsane, 1997; Chávez-González et al., 2012; Aharwar and Parihar, 2018). It is well established that effects of salts on tannase activity may vary depending on fungal strain as well as metal ion (Banerjee and Mahapatra, 2012; Yao et al., 2014). Some research findings indicated that some metal ions increase tannase activity while some others decreased it. For example, Mg^{2+} at a concentration of 1 mM was able to enhance the activity of tannase. Magnesium-stimulated tannases have been reported in *Bacillus subtillus* PAB2, *Rhizopus oryzae*, *Asp. foetidus* and *Asp. awamori* (Yao et al., 2014). Generally, tannase activity was inhibited by metal ions such as Cu^{2+}, Hg^{2+}, Co^{2+}, Fe^{2+}, Ba^{2+}, Ca^{2+}, Zn^{2+} and Pb^{2+} (Gonçalves et al., 2012; Jana et al., 2013; Yao et al., 2014). In this study, Fe^{2+} and Cu^{2+} were able to inhibit *F. fraxinea* tannase activity while Zn^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} did not show any significant inhibitory effects. The strong inhibitory effect of Fe^{2+} and Cu^{2+} were suggested to be related to their binding to the active site of the enzyme. Particularly, Fe^{2+} has been reported to have the ability of binding with the thiol group of tannases and also interact with the carboxyl group of amino acids in the active site (Abdel-Naby et al., 2016). Effect of EDTA on tannase activity also varies depending upon microorganisms. EDTA significantly inactivated Tannase from *Asp. oryzae*, while it showed no inhibitory activity on yeast tannase (Banerjee and Mahapatra, 2012).

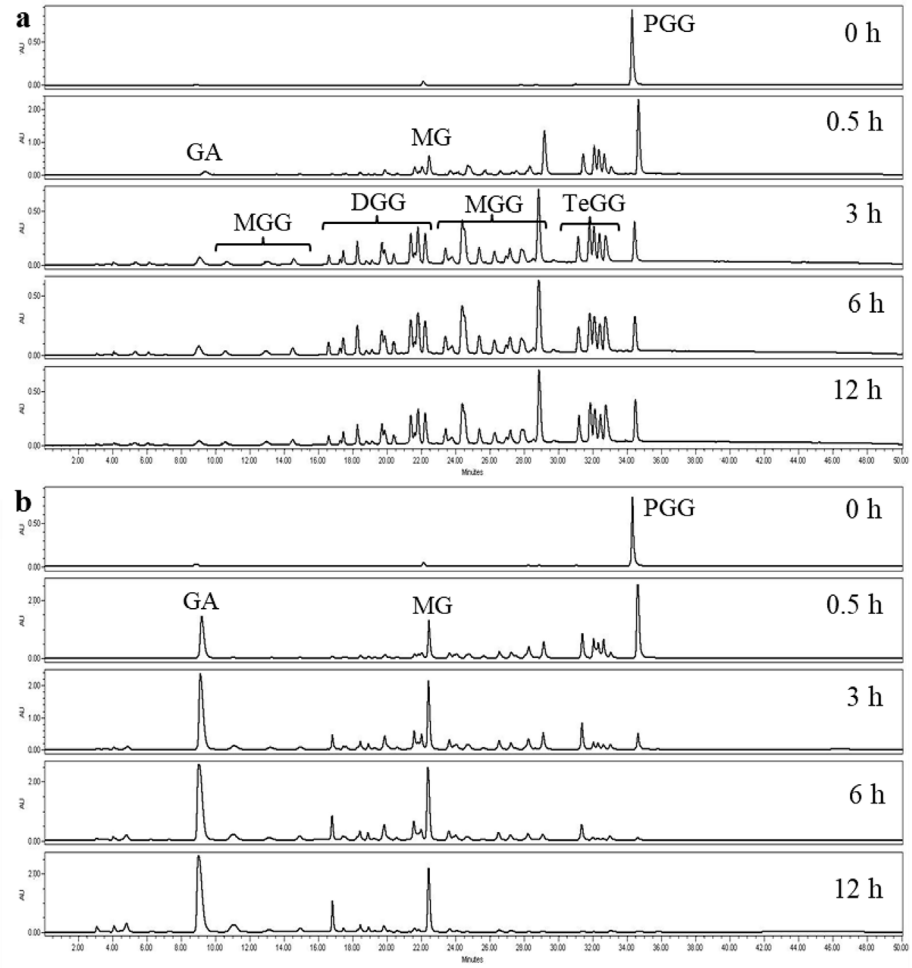

Fig. 7. Time course of PGG hydrolysis by purified *F. fraxinea* tannase (**a**) and commercial *Asp. oryzae* tannase (**b**). GA, gallic acid; MG, methyl gallates; MGG, monogalloylglucoses; DGG, digalloylglucoses; TGG, trigalloylglucoses; TeGG, tetragalloylglucoses; PGG, pentagalloylglucose (substrate).

3.5. Hydrolysis of PGG by purified tannase

Hydrolysis pattern of PGG by the tannase purified in this study was examined using commercial *Asp. oryzae* tannase as a positive control with regular sampling during the fermentation process and HPLC analysis. As the reaction time elapsed, large numbers of new peaks appeared while the peak of the PGG used as substrate gradually decreased (Fig. 7). These results indicate that PGG is hydrolyzed to form a large number of various new components during the reaction. The newly generated components are suggested to be MGG, DGG, TGG, TeGG and their positional isomers based on our previous LC-MS analysis results (Kim et al., 2019). Commonly, when galloyltannins such as PGG or tannic acid are reacted with microbial tannases, gallic acid is produced as a final hydrolysis product. However, in this study, most of the PGG was hydrolyzed to oligomeric galloylglucoses such as DGG, TGG and TeGG and their positional isomers (Fig.9a), whereas *Asp. oryzae* tannase produced gallic acid and methyl gallate as final metabolites (Fig. 9b) under same reaction conditions. This unusual hydrolysis pattern can be considered as a special feature of the tannase purified in this study. This feature can have of paramount significance in the production of oligomeric galloylglucoses such as DGG, TGG and TeGG from PGG. Some of these PGG hydrolysates have been previously found in *Eucalyptus nitens* wood (Barry et al., 2001), mango (Gómez-Caravaca et al., 2016), *Rhus coriaria* (Abu-Reidah et al., 2015), *Psidium guineense, Syzygium cumini, Pouteria macrophylla* (Gordon et al., 2011) and degradation products of tannin by *Aspergillus niger* (Abdel-Nabey et al., 2011; Chávez-González et al., 2014). These oligomeric galloylglucoses are known to play major roles in the expression of biological activities of the target plants. In addition, oligomeric galloylglucoses may have more potent antioxidant activities than PGG or ascorbic acid (Sugimoto et al., 2009). Generally, microbial tannases hydrolyze ester and depside bonds of hydrolysable tannins and PGG to produce gallic acid and methyl gallate as end products (Yao et al., 2014; Govindarajan et al., 2016). However, the tannase secreted from *F. fraxinea* in this study catalyzed hydrolysis of ester bonds of PGG to produce oligomeric galloylglucoses, mainly DGG, TGG and TeGG instead of gallic acid.

4. Conclusion

Tannase is an important enzyme extensively applied in diverse industries. Due to its wide range of industrial applications, there is always a quest for novel tannases with more suitable characteristics for each desired purpose. The present study reports enzymatic properties and hydrolytic patterns against PGG of extracellular tannase induced from a wood-rot fungus, *F. fraxinea* during fermentation of the gallotannin-rich plant material *T. vernicifluum* stem bark. The enzyme was found to display its optimum activity at temperatures between 50 – 70 °C and pH between 5 – 6. The purified enzyme was found to have a decent thermal and pH stabilities at temperatures below 70 °C and pH values 5–7 which are the most desirable characteristics for industrial applications. Furthermore, the enzyme was able to catalyze hydrolysis of ester bonds that are present in PGG to produce oligomeric galloylglucoses such as

mainly DGG, TGG and TeGG rather than the usual product gallic acid. These results suggest that the tannase induced from *F. fraxinea* in gallotannin-rich media can be applied in production of valuable oligomeric galloylglucoses from PGG or tannic acid, a higher galloylated PGG.

Conflicts of interest

The authors declare no conflict of interest.

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