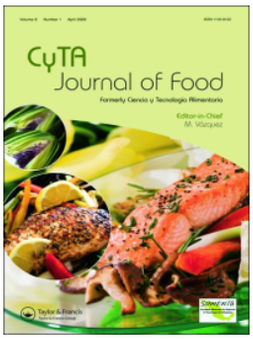

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Purification and biochemical characterization of an alkaline feruloyl esterase from *Penicillium sumatrense* NCH-S2 using rice bran as substrate

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ABSTRACT

Feruloyl esterases (FAEs) are essential accessory enzymes in the hydrolysis of plant cell wall structure. A novel FAE was obtained from *Penicillium sumatrense* NCH-S2. Enzyme purification was conducted by ultrafiltration, ammonium sulfate precipitation, anion exchange, and gel filtration chromatography. This FAE has a molecular mass of 36 kDa. Its optimum temperature and pH were 50°C and pH 9.0–10.0, respectively. The FAE demonstrated high pH stability at the pH ranging from 6.0 to 10.0. After enzymatic hydrolysis with FAE, the DPPH free radical scavenging capacity, ferrous ion chelating ability, and total phenolic content (TPC) of defatted rice bran (DRB) hydrolysate significantly increased. Furthermore, the amount of released FA from DRB under a synergistic interaction of FAE and hemicellulose increased by 18–21% in comparison with that of either enzyme acting alone.

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KEYWORDS
Alkaline feruloyl esterases; *Penicillium sumatrense*; enzyme purification; synergistic interaction

PALABRAS CLAVE
feruloil esterasa alcalina; *Penicillium sumatrense*; purificación enzimática; interacción sinérgica

Purificación y caracterización bioquímica de una feruloil esterasa alcalina obtenida de *Penicillium sumatrense* NCH-S2 utilizando como sustrato el salvado de arroz

RESUMEN

Las feruloil esterasas (FAE) son enzimas accesorias esenciales en la hidrólisis de la estructura de la pared celular de las plantas. Para el presente estudio se obtuvo una nueva FAE a partir de *Penicillium sumatrense* NCH-S2. La purificación de dichas enzimas se llevó a cabo mediante ultrafiltración, precipitación de sulfato de amonio, intercambio de aniones y cromatografía de filtración en gel. La nueva FAE obtenida tiene una masa molecular de 36 kDa. Su temperatura y pH óptimos fueron 50°C y 9.0-10.0, respectivamente. La FAE exhibió alta estabilidad de pH en el rango de 6.0 a 10.0. Tras la hidrólisis enzimática con FAE, la capacidad de eliminación de radicales libres del DPPH, la capacidad de quelación de iones ferrosos y el contenido fenólico total (TPC) del hidrolizado de salvado de arroz desgrasado (DRB) aumentaron significativamente. Además, la cantidad de FA liberado de DRB bajo la interacción sinérgica de FAE y hemicelulasa aumentó entre 18 y 21% en comparación con la de cualquiera de las dos enzimas actuando solas.

1. Introduction

Lignocellulosic biomass is one of the most abundant renewable raw materials on earth. It is able to be converted to many value-added products such as biofuels, enzymes, and fine chemicals (Xue et al., 2017). However, expensive cellulase enzymes are required in the industrialization of lignocellulose conversion. It was reported that the utilization of cheaper lignocellulosic substrates for enzyme production could lower production cost and environmental pollutants (Iqtedar et al., 2015).

Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) has been widely applied in food, pharmaceuticals, and cosmetic industries. It is the most abundant hydroxycinnamic acid in plants. It is often found to link covalently to functional groups like ester or ether bonds on polysaccharides as well as lignins. Many studies have reported that FA and its derivatives possessed a wide variety of bioactivities, such as antioxidation (Itagaki et al., 2009), anti-microbial action (Borges et al., 2013; Khatkar et al., 2015), anti-inflammation (Chmielowski et al., 2017; Nile et al., 2016), anti-diabetes (Adisakwattana et al., 2009), and anti-hyperlipidemia (Bumrungpert et al., 2018). The antioxidative activity of FA is primarily attributed to its ability to form stable phenoxyl radicals, thus FA could scavenge free radicals, bind transition metals (e.g., iron and copper), and prevent lipid peroxidation (Zduńska et al., 2018). However, the binding of FA to polysaccharides would reduce its bioaccessibility and consequently its bioavailability (Amaya Villalva et al., 2018). Enzymatic and chemical (e.g., alkaline) hydrolysis are the two main methods to extract FA and increase its bioaccessibility (Ares-Peón et al., 2016; Mandal et al., 2015; Nieter et al., 2016). The enzymatic method involves the use of microbial carboxylic acid esterases such as feruloyl esterases (also known as ferulic acid esterases; FAEs). As compared to chemical methods, enzymatic hydrolysis is relatively environment friendly because of its milder operation condition.

FAEs (E.C. 3.1.1.73), a subclass of the carboxylic ester hydrolases (E.C. 3.1.1), have been found to hydrolyze the ester linkages between hydroxycinnamic acids and

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polysaccharides in plant cell walls. Based on the substrate specificities against methyl esters of hydroxycinnamic acids (i.e. methyl ferulate, methyl caffeate, methyl *p*-coumarate, and methyl sinapate), growth substrate requirements of the microorganisms and protein sequence identity, FAEs could be classified into four types (A-D) (Crepin et al., 2004). FAEs have been produced and characterized from a wide range of microorganisms, including bacteria (e.g., *Actynomyces, Bacillus, Lactobacillus, Streptomyces*) and fungi (e.g., *Aspergillus, Fusarium, Penicillium, Talaromyces*) (Oliveira et al., 2019; Topakas et al., 2007). It was reported that FAEs were typically found when these microbes were cultivated on complex substrates. Agricultural by-products, such as wheat bran, corn bran, sugar beet pulp, and rice bran, contain high amounts of esterified FA. These by-products were efficiently used as the substrates for microbial production of FAEs (Goufo & Trindade, 2014; Liyana-Pathirana & Shahidi, 2006; Saulnier & Thibault, 1999).

Previous studies have mentioned that FAE worked synergistically with cellulases or hemicellulases to enhance plant biomass degradation. This was attributed to its ability to break down the lignin-cellulose-hemicellulose crosslinked network, thus increasing the accessibility of cellulases and hemicellulases to their respective substrates (Oliveira et al., 2019). Xue et al. (2017) revealed that the synergistic interaction between FAE and cellulase significantly increased the release of phenolic acids from rice straw, and further increasing its antioxidative activities. On the other hand, the release of FA and xylooligosaccharides from wheat bran under combined action of xylanase (AnXyn11A) and FAE (AnFaeA) were greatly enhanced, compared to using AnFaeA alone (Wu et al., 2017).

FAEs play an important role in the hydrolysis of lignocellulosic biomass and are applied in many industries. These wide applications involve various substrates, which require multiple FAEs with different specificities. The subtle differences in specificity are often neglected. This may be important in determining the optimum synergy between FAEs and hemicellulases. Therefore, the exploration of novel FAEs with different properties, such as substrate preferences and reaction conditions continues to be an active research area (Topakas et al., 2007). However, there are only few reports on FAEs production from *Penicillium* strains. In this study, we have isolated a new *Penicillium sumatrense* strain and evaluated its optimum medium for the production of FAE. The FAE produced from this strain was then purified and characterized. Subsequently, FAE and hemicellulases were used in combination with rice bran to evaluate its potentiality in improving the antioxidant activities and released FA of rice bran.

2. Materials and methods

2.1. Screening and cultivation of microorganisms

A screening assay was conducted according to the methods of Donaghy et al. (1998) with some modifications. Strains were cultivated using potato dextrose agar (PDA, Difco, USA). Before pouring plates, each agar plate (15 mL) was supplemented with 0.15 mL ethyl ferulate (10% v/v in dimethylformamide) and was ensured a homogeneous distribution (cloudy overlay) throughout the plate. After incubating at 30°C for 6 d, the formation of clear zone around the colonies indicated feruloyl esterase production. The colonies with positive reaction were subsequently picked and transferred to potato dextrose broth (PDB, Difco, USA) supplemented with 2% (w/v) rice bran to determine the amount of released FA.

2.2. Identification of strain

The strain was identified by Bioresources Collection & Research Center (BCRC, Hsinchu, Taiwan). Morphological characteristics (i.e. colony color, diameter, and conidiophore morphology) of the strain were observed on three differential media: Czapek yeast extract agar (CYA), malt extract agar (MEA), and yeast extract sucrose agar (YES). DNA sequencing of the strain was conducted by rDNA ITS1-5.8S-ITS2 sequence analysis and the sequences were compared with NCBI GenBank database.

2.3. Determination of ferulic acid

The liberated free FA was analyzed by high performance liquid chromatography (HPLC). Sample was filtered through a 0.22 μm membrane filter into HPLC vial. The filtrate was injected into an HPLC system (Hitachi Ltd., Japan) equipped with an autosampler (L-2200; Hitachi Ltd., Japan), a photodiode-array detector (L-2455; Hitachi Ltd., Japan), and a reverse phase column (Mightysil RP-18 GP 250 × 4.6 mm, 5 μm, Kanto Corporation, USA) according to the methods of Yuan et al. (2005). The mobile phase consisted of methanol/water/acetic acid (50:50:0.5, v/v/v), and eluted at a flow rate of 0.8 mL/min for 30 min. The absorbance of the eluate was monitored continuously at 320 nm. The concentration of FA was calculated using the standard curve of FA solutions.

2.4. Enzyme production

One mL of spore suspension with a concentration of 1×10^8 spores/mL was inoculated into a 500 mL Erlenmeyer flask containing 100 mL medium. The medium (containing 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7\ H_2O$, and 0.01 g $FeSO_4 \cdot 7\ H_2O$ in 1 L of distilled water as basal medium) was supplemented with 120 g rice bran, 40 g sucrose, and 10 g sodium nitrate. The flasks were then placed in an orbital shaker at 30°C, 120 rpm. After 6 days of incubation, the fermentation residues were removed from the culture broth by centrifugating at 12,000 × *g* for 20 min. The supernatant was collected and concentrated to one fifth of the original volume by ultrafiltration with Minimate Tangential Flow Filtration System (Pall Corporation, USA) equipped with Minimate TFF Capsules (10 kDa and 100 kDa molecular weight cut off). The retentate from the ultrafiltration was then further purified to obtain the enzyme.

2.5. Enzyme purification

The purification flow chart of FAE is described in Figure 1. After ultrafiltration, the crude enzyme was precipitated with 50–60% (w/v) ammonium sulfate. The precipitate was dialyzed against 20 mM Tris-HCl buffer (pH 7.0). After that, the enzyme sample was subjected to anion exchange chromatography, which was performed on a HiPrep DEAE FF 16/10 column (1.6 × 10 cm, GE Healthcare, USA) with ÄKTApurifier

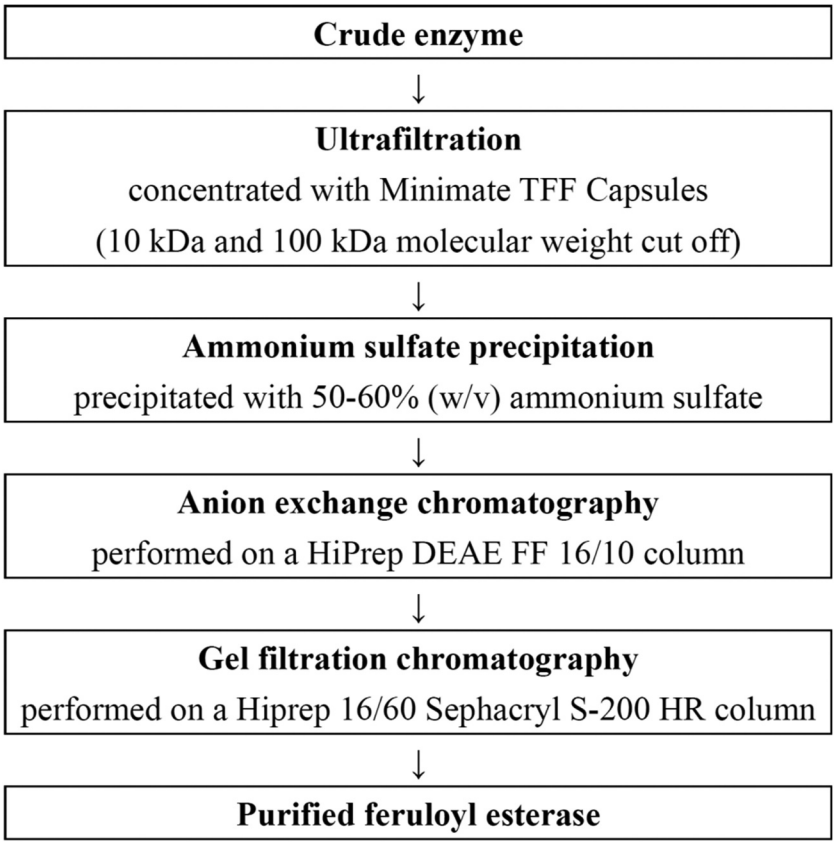

Figure 1. Purification flow chart of feruloyl esterase from *P. sumatrense* NCH-S2.

Figura 1. Diagrama de flujo de la purificación de la feruloil esterasa de *P. sumatrense* NCH-S2.

10 system (GE Healthcare, USA). The mobile phase consisted of Buffer A (20 mM Tris-HCl buffer, pH 7.0) and Buffer B (Buffer A containing 1 M NaCl). The enzyme sample was loaded onto a pre-equilibrated column. After washing with Buffer A, a linear gradient of 0–1 M NaCl was applied with a flow rate of 0.5 mL/min. Eluted fractions (3 mL each) were collected and analyzed for their FAE activity. The active fractions were loaded onto a Hiprep 16/60 Sephacryl S-200 HR column (1.6 × 60 cm, GE Healthcare, USA) equilibrated and eluted with 20 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.0). The fractions (2 mL each) were collected at a flow rate of 0.8 mL/min and FAE activity was measured for each fraction.

2.6. FAE activity

FAE activity was assayed by measuring the amount of FA released from methyl ferulate (MFA). The assay was carried out in 20 mM Tris-HCl buffer (pH 7.0) containing 4.8 mM methyl ferulate as substrate at 50°C for 10 min and the liberated free FA was analyzed by HPLC as the methods described in 2.3. One unit of FAE activity is defined as the amount of enzyme required to release one µmol FA per min.

2.7. Determination of molecular mass

Enzyme purity and molecular mass were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the methods of Laemmli (1970) with some modifications. SDS-PAGE was carried out using a 10% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. The electrophoretic mobility of the FAE was compared with prestained protein ladder (Thermo Scientific, USA). Gel filtration on a Sephacryl S-200 HR column, which was previously calibrated with molecular mass standards, was also employed to determine the molecular mass of the FAE from a calibration curve.

2.8. Determination of protein concentration

Total protein concentration was determined according to the Bradford method (Bradford, 1976), using Bio-Rad protein assay dye reagent. Bovine serum albumin was used as the standard. The protein samples were analyzed at 280 nm using a microplate reader (FLUOstar Omega; BMG LABTECH, Germany).

2.9. Characterization of FAE

The optimum temperature was studied by measuring the enzyme activity in 20 mM Tris-HCl buffer (pH 7.0) at different temperatures ranging from 30°C to 80°C. To determine thermostability, the enzyme was pre-incubated at different temperatures between 30°C and 80°C. The residual activity was determined at 50°C at one hour intervals.

The optimum pH was identified at 50°C in various buffers: 20 mM citrate buffer (pH 4.0–7.0), 20 mM Tris-HCl buffer (pH 7.0–10.0), and 20 mM Na_2HPO_4-NaOH (pH 10.0–11.0). In order to estimate pH stability, the enzyme was pre-incubated in various buffers (pH 4.0-pH 11.0) at 4°C for 24 h, and the residual activity was determined under the conditions (pH 7.0, 50°C).

Methyl caffeate (MCA), methyl *p*-coumarate (M*p*CA), and methyl sinapate (MSA) were used to evaluate the substrate specificity of the purified FAE. The enzyme activity was measured as described in 2.6.

2.10. Determination of antioxidant capacity

The fresh rice bran was sieved through a 20-mesh sieve. The sieved rice bran was subsequently defatted with n-hexane at a bran/solvent ratio of 1:10 (w/v) to obtain defatted rice bran (DRB). One mg of DRB was mixed with 10 mL Tris-HCl buffer (20 mM, pH 7). Then, 0.1 mL of purified FAE (1 mU) was added to the mixture and reacted at 50°C for 1 h. The residues were removed by centrifugating at 12,000 × *g* for 20 min. The antioxidant capacity of supernatant was determined by using the methods described as follows.

2.10.1. Determination of DPPH radical-scavenging capacity

The DPPH assay was conducted according to the methods of Shimada et al. (1992) with some modifications. First, 0.1 mL of sample or ethanol (control) was mixed with 0.4 mL Tris-HCl buffer (100 mM, pH 7.4). Then, 0.5 mL of 250 μM DPPH (1,1-diphenyl-2-picrylhydrazyl) in ethanol was added to the mixture prior being mixed evenly. The mixtures were allowed to stand in the dark at room temperature for 20 min before reading the absorbance at 517 nm. The scavenging capacity of the sample was calculated using the following equation:

$$\text{Scavenging capacity}\% = [1 - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

2.10.2. Determination of ferrous ion chelating ability

The ferrous ion (Fe^{2+}) chelating ability was performed according to the methods of Decker and Welch (1990) with some modifications. The sample (0.25 mL) was mixed with 0.8 mL distilled water and 0.025 mL $FeCl_2$ (2 mM). After 30 s, 0.05 mL of 5 mM ferrozine was added. The solution was allowed to stand for 10 min at room temperature before taking the absorbance of samples at 562 nm. The chelating ability of the sample was calculated using the following equation:

$$\text{Chelating ability}\% = [1 - (A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

2.10.3. Determination of total phenolic content

The total phenolic content (TPC) was determined using the Foline-Ciocalteu colorimetric method by Sato et al. (1996) with modifications. The sample (0.4 mL) was mixed with 0.4 mL Foline-Ciocalteu reagent. After reacting for 3 min, 0.04 mL of 10% sodium carbonate was added to the solution and allowed to stand in the dark for 1 h at room temperature. The samples were measured at 735 nm versus a blank using a spectrophotometer. The results were expressed as mg of gallic acid equivalent (GAE)/mL of sample.

2.11. Synergistic interaction between FAE and hemicellulase

One mg of DRB was mixed with 10 mL Tris-HCl buffer (20 mM, pH 7). Then, 0.1 mL of Hemicellulase Amano 90 (Amano Enzyme Inc., Japan) or FAE were added to the mixture and reacted at 50°C. Different enzyme addition sequences were investigated as follows: H5- only hemicellulase was added and reacted for 5 h; F5- only FAE was added and reacted for 5 h; HF5- hemicellulase and FAE were added together and reacted for 5 h; H4F1- add hemicellulase to react for 4 h, then add FAE to react for 1 h; F1H4- add FAE to react for 1 h, then add hemicellulase to react for 4 h; Control- enzymes were replaced with water and reacted for 5 h. The amount of released FA was determined by HPLC as the methods described in 2.2. The amount of released saccharides was analyzed by using a refractive index detector (L-2490; Hitachi Ltd., Japan) and a CarboSep CHO-682 column (Transgenomic, USA). The mobile phase was distilled water at a flow rate of 0.4 mL/min and the column was kept at 80°C.

2.12. Statistical analysis

Statistical differences among groups were determined through one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS, version 20 (IBM Corporation, USA). Values of $p < .05$ were considered statistically significant.

3. Results and discussion

3.1. Screening and identification of strains with FAE activity

During the screening process, S-02 showed an increasing trend in the amount of FA released during fermentation and a higher content (56.13 ± 0.62 μg/mL) on the sixth day. Therefore, S-02 was selected as the strain for further study. It was identified according to the morphological characteristics of colony culture and rDNA ITS1-5.8S-ITS2 sequence analysis (Figure S1) that this particular colony was *Penicillium sumatrense*. This strain was then named as *P. sumatrense* NCH-S2.

3.2. Optimization of FAE production

Rice bran has been widely used as a substrate to produce value-added products (Meselhy et al., 2020; Wang et al., 2020). *P. sumatrense* NCH-S2 was grown in a liquid culture for eight days using rice bran as the substrate. By the use of optimum medium (containing 12% rice bran, 4% sucrose, and 1% sodium nitrate), FAE activity increased with the incubation period. The maximum FAE production was observed on the sixth day of incubation and the production remained steady until the eighth day (Figure S2). The FAE activity and protein content of the crude enzyme were 8.98 ± 0.19 mU/mL and 0.17 ± 0.01 mg/mL, respectively. The FAE activity was about 49.8 times higher than that of the initial cultivation conditions (0.18 ± 0.04 mU/mL).

3.3. Purification of FAE

In this study, FAE was purified with ultrafiltration, ammonium sulfate precipitation, anion exchange, and gel filtration chromatography. The chromatographic profile of the FAE on anion exchange chromatography is shown in Figure 2(a). Fractions 27 to 29 were found to exhibit the majority of

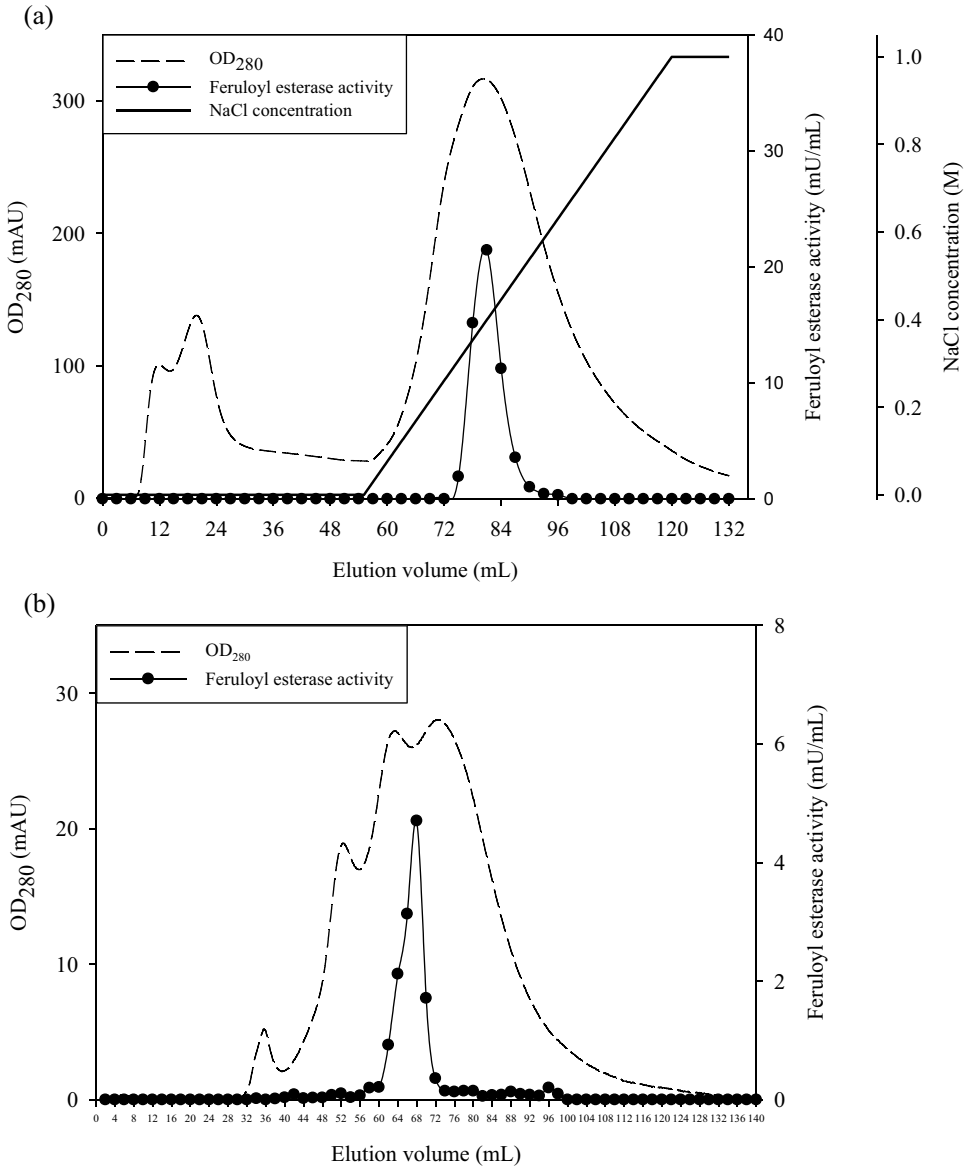

Figure 2. The elution profile of (a) anion exchange and (b) gel filtration chromatography of feruloyl esterase from *P. sumatrense* NCH-S2.

Figura 2. Perfil de elución de (a) intercambio de aniones y (b) cromatografía de filtración en gel de feruloil esterasa de *P. sumatrense* NCH-S2.

the FAE activity at 0.3–0.5 M NaCl concentration by anion exchange chromatography. During gel filtration chromatography, it was found that fractions 31 to 35 demonstrated higher FAE activity (Figure 2(b)). Fractions 33 and 34 with peaked activity were pooled and used for further characterization.

Changes in FAE activity throughout the purification process are shown in Table 1. After gel filtration chromatography, FAE activity was purified 13.92-fold. As demonstrated in Figure 3, only a single band was detected in the purified FAE by SDS-PAGE and the molecular mass was about 37 kDa. Concurrently, the molecular mass of the FAE estimated by gel filtration chromatography was 36 kDa (Figure 4), which means that the purified FAE is a monomer. Microbial FAEs in previous studies have molecular masses in a typical range of 11–210 kDa (Oliveira et al., 2019; Topakas et al., 2007).

Table 1. Purification of feruloyl esterase from *P. sumatrense* NCH-S2.

Tabla 1. Purificación de feruloil esterasa a partir de *P. sumatrense* NCH-S2.

Purification step	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Purification (fold)	Recovery (%)
Crude enzyme	2245.77	44.00	51.04	1	100
Ultrafiltration (10–100 kD MWCO)	643.16	11.69	55.01	1.08	28.64
$(NH_4)_2SO_4$ precipitation (50–60% saturation)	557.71	1.65	338.01	6.62	24.83
Anion exchange (DEAE FF)	113.67	0.31	366.68	7.18	5.06
Gel filtration (Sephacryl S-200 HR)	9.95	0.014	710.71	13.92	0.44

3.4. Characterization of the purified FAE

The optimum temperature of the purified FAE was observed at 50°C (Figure 5(a)); however, FAE activity dropped sharply after 60°C. In the evaluation of thermostability, FAE retained 98% and 80% of relative activity after incubating for 5 h at 30°C and 40°C, respectively (Figure 5(b)). At 50°C, the FAE activity decreased to 57% in 30 min, and 13% of residual activity was observed after incubation for 5 h. FAE activity

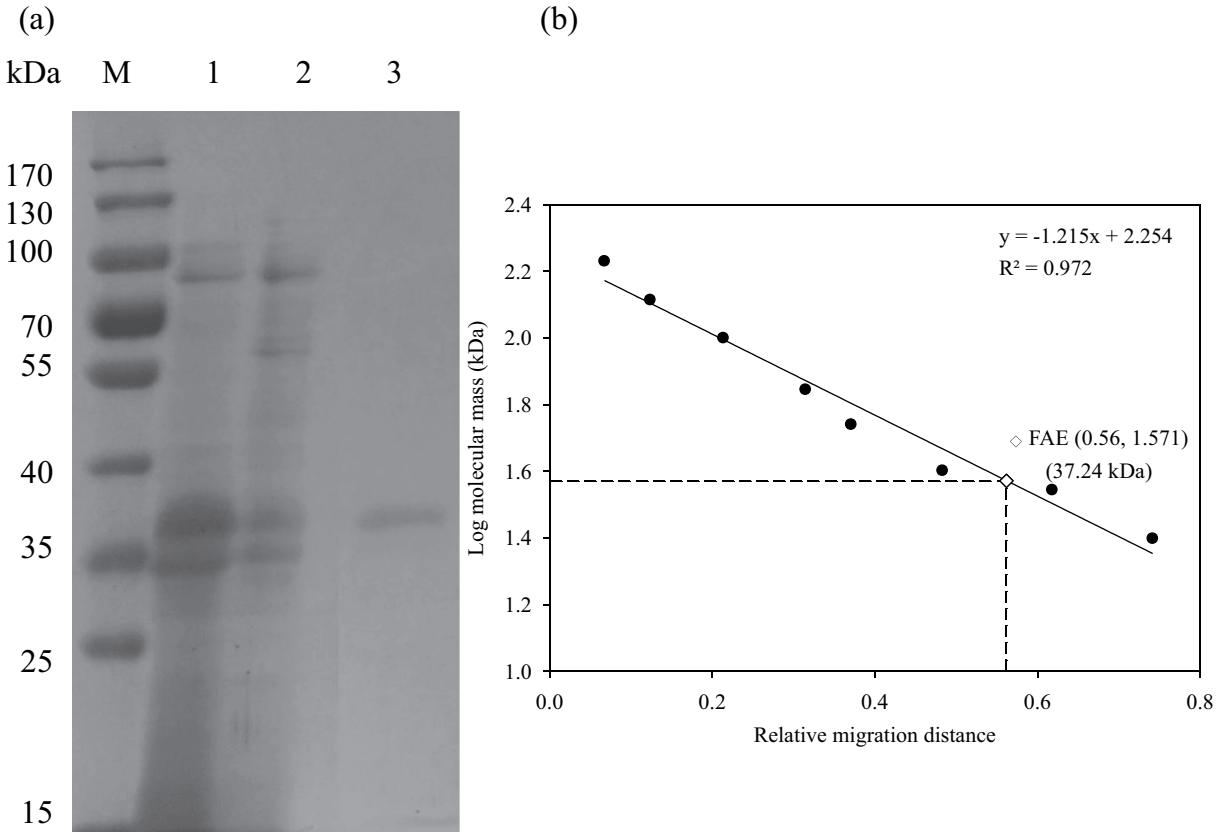

Figure 3. (a) SDS-PAGE and (b) determination of molecular mass of feruloyl esterase (FAE) from *P. sumatrense* NCH-S2. M: standard protein markers; lane 1: proteins of 50–60% ammonium sulfate saturation; lane 2: proteins of anion exchange chromatography; lane 3: protein of gel filtration chromatography.

Figura 3. (a) SDS-PAGE y (b) determinación de la masa molecular de la feruloil esterasa (FAE) a partir de *P. sumatrense* NCH-S2. M: marcadores de proteína estándar; carril 1: proteínas de saturación de 50–60% de sulfato de amonio; carril 2: proteínas de cromatografía de intercambio aniónico; carril 3: proteína de cromatografía de filtración en gel.

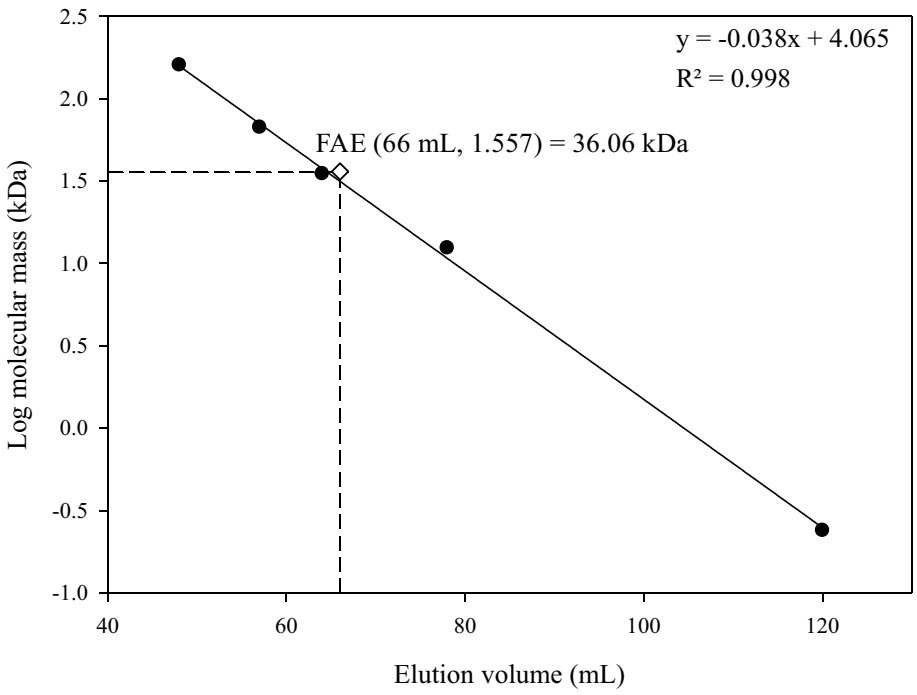

Figure 4. Determination of molecular mass of the purified feruloyl esterase (FAE) from *P. sumatrense* NCH-S2 by gel filtration chromatography.

Figura 4. Determinación de la masa molecular de la feruloil esterasa purificada (FAE) a partir de *P. sumatrense* NCH-S2 por cromatografía de filtración en gel.

was generally abolished in 30 min at temperatures greater than 60°C. The FAEs of *Aspergillus niger* (AnFaeB and AnFaeC), *Aspergillus oryzae* (AoFaeA), *Aspergillus terreus* (AtFaeA and AtFaeD), *Penicillium chrysogenum* (PcFAE1), and *Penicillium brasilianum* (FAE) had optimum esterase activity at 50°C (Oliveira et al., 2019; Phuengmaung et al., 2019). According to relative frequencies and medians, FAEs typically have a molecular mass of 36 kDa, optimum

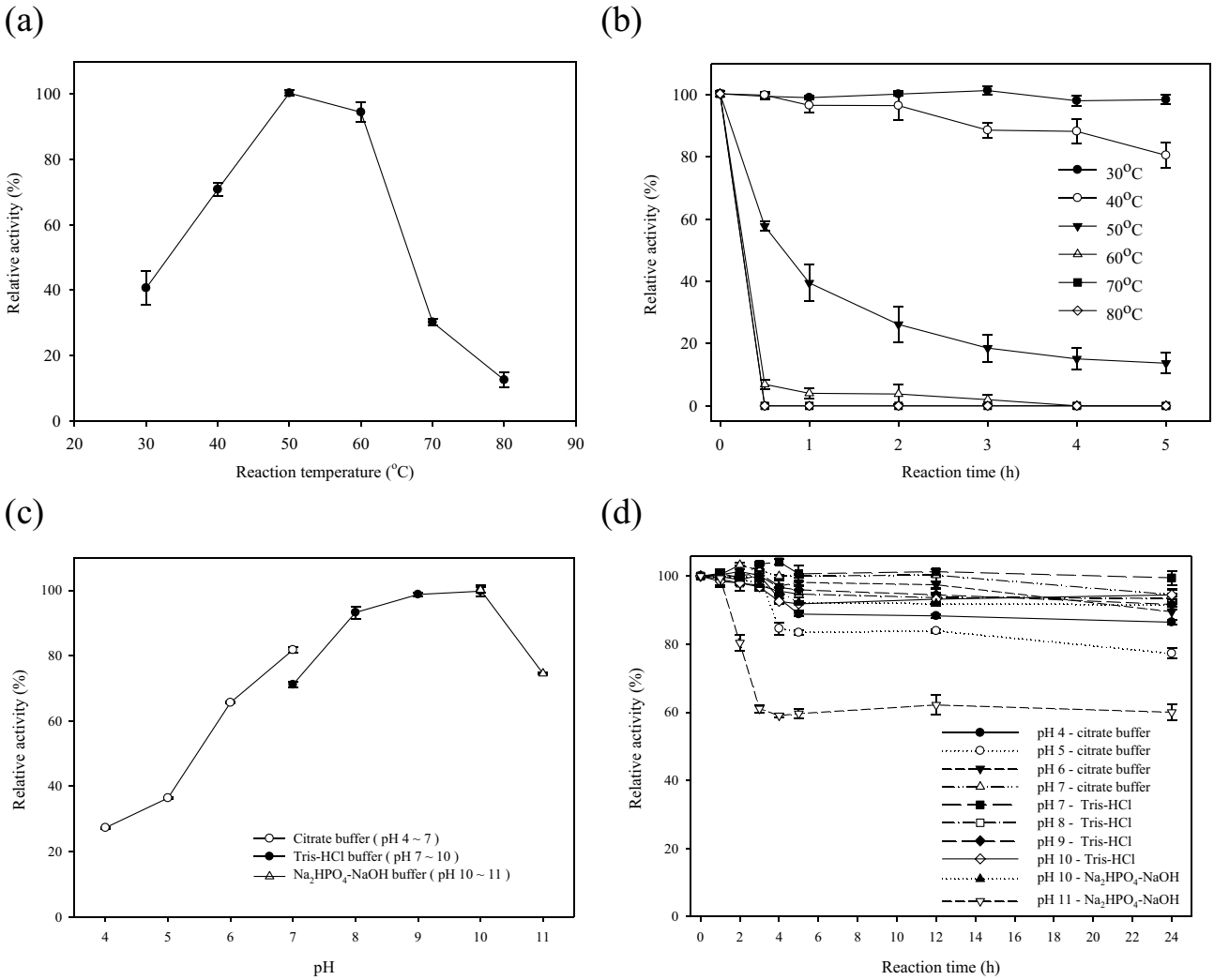

Figure 5. (a) Optimum temperature, (b) thermal stability, (c) optimum pH and (d) pH stability of the purified feruloyl esterase from *P. sumatrense* NCH-S2. Mean values and standard deviations from three replicate experiments are presented.

Figura 5. a) Temperatura óptima, b) estabilidad térmica, c) pH óptimo y d) estabilidad del pH de la feruloil esterasa purificada de *P. sumatrense* NCH-S2. Se presentan los valores medios y las desviaciones estándar de tres experimentos replicados.

temperature at 50°C, and pH at 6.5 (Oliveira et al., 2019). The molecular mass and optimum temperature of the FAE from *P. sumatrense* NCH-S2 were similar to many past identified FAEs.

The optimum pH of the FAE produced in this study was at 9.0–10.0 (Figure 5(c)). The FAE was more active in alkaline conditions. The relative activity decreased to 27% and 75% at pH 4.0 and pH 11.0, respectively. It was noteworthy to mention that the FAE displayed good pH stability between 6.0 and 10.0 (Figure 5(d)). The FAE was also stable at pH 4.0–5.0, which retained over 77% of its initial activity after storage at 4°C for 24 h. Many studies revealed that the majority of fungal FAEs demonstrated either acidic or neutral pH optima (Oliveira et al., 2019). Alkaline feruloyl esterases are useful in industrial applications, especially in the treatment of alkaline pulp. Nevertheless, there are scarce reports on such kinds of feruloyl esterases (Li et al., 2011). The good activity in alkaline conditions as well as the high stability in both acidic and alkaline conditions made the FAE produced by *P. sumatrense* NCH-S2 a potential candidate to apply in various industries.

Table 2 shows the substrate specificity of the purified FAE. The FAE is capable of hydrolyzing four methyl hydroxycinnamates. In particular, the highest FAE activity was observed with MFA (710.71 mU/mg), followed by those for MCA, MSA and M*p*CA. Phuengmaung et al. (2019) reported that PcFAE1 produced by *Penicillium chrysogenum* 31B had activity towards M*p*CA, MFA, and MCA, but not on MSA. PpFAE produced by *Penicillium piceum* showed enzymatic activity against four methyl hydroxycinnamates, and the highest activity was M*p*CA (Gao et al., 2016). The FAE produced by *P. sumatrense* NCH-S2 has higher relative activity for MFA comparing to other *Penicillium* species.

Table 2. Substrate specificity of feruloyl esterase from *P. sumatrense* NCH-S2.

Tabla 2. Especificidad del sustrato de la feruloil esterasa de *P. sumatrense* NCH-S2.

Substrate	Specific activity (mU/mg)	Relative activity (%)
MFA	710.71	100.00
MCA	101.37	14.26
M*p*CA	36.97	5.20
MSA	81.03	11.40

MFA: methyl ferulate; MCA: methyl caffeate; M*p*CA: methyl *p*-coumarate; MSA: methyl sinapate; Enzyme activity was determined according to FAE activity assay.

MFA: ferulato de metilo; MCA: cafeto de metilo; M*p*CA: *p*-cumarato de metilo; MSA: sinapato de metilo; la actividad enzimática se determinó de acuerdo con el ensayo de actividad de la FAE.

3.5. Improved antioxidant activities of DRB by the purified FAE

The DPPH radical-scavenging capacity, Fe^{2+} chelating ability, and total polyphenol content (TPC) were determined to evaluate the antioxidant activities of DRB after treating with purified FAE (Figure 6). DRB enzymatic hydrolysate had good DPPH scavenging capacity, which increased from 57.44% to 78.18%. The ability to chelate transition metal ions could reflect the antioxidant ability. Fe^{2+} chelating ability of DRB was increased from 73.56% to 89.78% by enzymatic hydrolysis.

As shown in Figure 6, TPC of rice bran was increased by 89% (from 2.8 mg as GAE/mL to 5.28 mg as GAE/mL) after the enzymatic treatment. Liu et al. (2017) revealed that enzymatic treatment altered the ratios of free and soluble conjugate forms of phenolics in rice bran extract. The amount of released FA was present at the highest levels, followed by protocatechuic acid. The increases in the phenolic compounds are mainly due to the release of insoluble bound phenolics, such as FA, protocatechuic acid, and quercetin.

The elevations in DPPH radical-scavenging capacity, Fe^{2+} chelating ability and TPC of the DRB hydrolysate were attributed to the higher amounts of free FA and other phenolic acids from enzymatic hydrolysis (Xue et al., 2017). The antioxidant potential of FA can be ascribed to the formation of a phenoxy radical from the phenolic nucleus. The stable resonance structure of the phenoxy radical could cease propagation of radical chain reactions (Paiva et al., 2013).

3.6. Synergistic interaction of the purified FAE

Table 3 demonstrates the variations in the amounts of released FA and different saccharides from DRB by enzymatic hydrolysis with different types of enzymes as well as additional sequences. The control, H5, and F5 treatments were able to release 106.93, 118.46, 115.93 μg/g of FA, respectively, from DRB. Both enzymes significantly ($p < .05$) enhanced the release of FA. It was interesting to note that the combination of hemicellulase and FAE showed a synergistic effect in which the FA

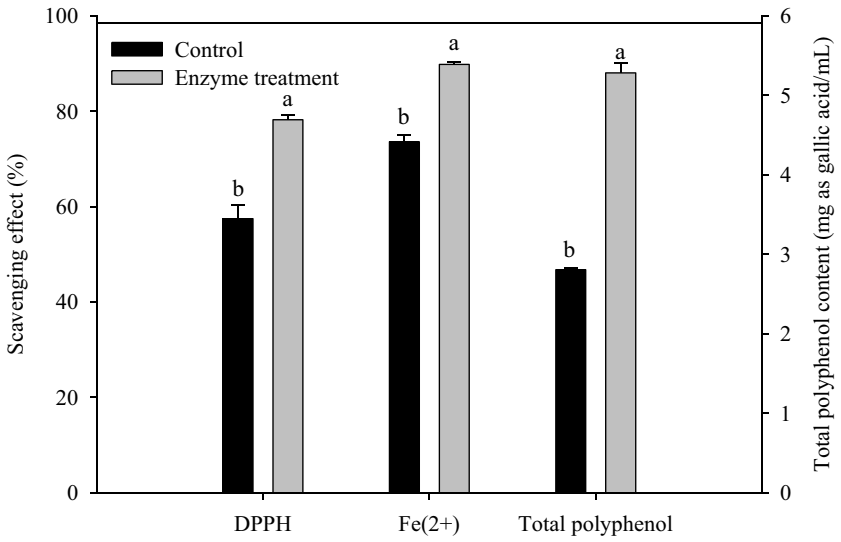

Figure 6. Antioxidant activity and total polyphenol content of defatted rice bran treated with purified feruloyl esterase. Control of each experiment was performed by replacing the purified feruloyl esterase with distilled water. Mean values and standard deviations from three replicate experiments are presented. Bars with different letters (a-b) are significantly different ($p < 0.05$).

Figura 6. Actividad antioxidante y contenido total de polifenoles del salvado de arroz desgrasado tratado con feruloil esterasa purificada. El control de cada experimento se realizó sustituyendo la feruloil esterasa purificada por agua destilada. Se presentan los valores medios y las desviaciones estándar de tres experimentos replicados. Las barras con letras distintas (a-b) son significativamente diferentes ($p < 0.05$).

Table 3. Variation in the amount of ferulic acid and saccharides released from defatted rice bran by enzymatic hydrolysis with different enzyme addition sequences.

Tabla 3. Variación de la cantidad de ácido ferúlico y sacáridos liberados del salvado de arroz desgrasado por hidrólisis enzimática con diferentes secuencias de adición de enzimas.

	Ferulic acid (μg/g)	Glucose (mg/mL)	Arabinose (mg/mL)	Galactose (mg/mL)	Xylose (mg/mL)	Xylobiose (mg/mL)
Control	106.93 ± 1.09^{d}	2.421 ± 0.030 ^{c}	0.021 ± 0.019^{b}	ND	ND	ND
H5	118.46 ± 3.66 ^{c}	3.737 ± 0.091^{a}	0.159 ± 0.037^{a}	0.024 ± 0.003^{a}	0.039 ± 0.001^{a}	0.013 ± 0.011^{a}
F5	115.93 ± 4.41 ^{c}	2.819 ± 0.112^{b}	0.048 ± 0.012^{b}	0.013 ± 0.004^{a}	ND	ND
HF5	144.72 ± 2.83^{ab}	3.872 ± 0.064^{a}	0.157 ± 0.022^{a}	0.054 ± 0.055^{a}	0.039 ± 0.003^{a}	0.010 ± 0.001^{a}
H4F1	147.28 ± 0.72^{a}	3.827 ± 0.024^{a}	0.159 ± 0.008^{a}	0.021 ± 0.001^{a}	0.038 ± 0.001^{a}	0.015 ± 0.005^{a}
F1H4	140.95 ± 1.35^{b}	3.718 ± 0.116^{a}	0.151 ± 0.016^{a}	0.024 ± 0.006^{a}	0.037 ± 0.002^{a}	0.016 ± 0.006^{a}

Mean values and standard deviations from three replicate experiments are presented. $^{a-d}$ Columns with different superscripts are significantly different ($p < 0.05$). ND: not detectable; Control: enzymes were replaced with water and reacted for 5 h; H5: only hemicellulase was added and reacted for 5 h; F5: only feruloyl esterase (FAE) was added and reacted for 5 h; HF5: hemicellulase and FAE were added together and reacted for 5 h; H4F1: add hemicellulase to react for 4 h, then add FAE to react for 1 h; F1H4: add FAE to react for 1 h, then add hemicellulase to react for 4 h.

Se presentan los valores medios y las desviaciones estándar de tres experimentos replicados. $^{a-d}$ Las columnas con distintos superíndices son significativamente diferentes ($p < 0.05$). ND: no detectable; Control: las enzimas se sustituyeron por agua y reaccionaron durante 5 h; H5: sólo se añadió hemicelulasa y reaccionó durante 5 h; F5: sólo se añadió feruloil esterasa (FAE) y reaccionó durante 5 h; HF5: se agregó hemicelulasa y FAE y reaccionaron durante 5 h; H4F1: se agregó hemicelulasa y reaccionó durante 4 h, luego se agregó FAE y reaccionaron durante 1 h; F1H4: se agregó FAE y reaccionó durante 1 h, luego se agregó hemicelulasa y reaccionaron durante 4 h.

yield increased by 18–21% compared to the F5 treatment. These results indicated that the synergistic interaction of FAE and hemicellulase was useful in extracting FA from DRB, which was consistent with the previous reports (Oliveira et al., 2019). More specifically, different addition sequences were found to affect the efficiency to hydrolyze DRB, with H4F1 treatment releasing the highest amount of FA (147.28 µg/g) among the other treatments. Oliveira et al. (2019) mentioned in his review that the sequence and co-incubation treatment with FAE and xylanase on lignocellulosic substrates such as wheat bran, sugar-beet pulp, and sugarcane bagasse also affected the concentration of FA released.

According to the report by Beloshapka et al. (2016), DRB contained 34.2% starch, 24.4% dietary fiber and 4.6% hemicellulose. The monosaccharide composition of hemicelluloses from DRB were xylose, arabinose, galactose and glucose (Shibuya & Iwasaki, 1985). To evaluate the activity of the FAE produced in this study, different saccharides, including glucose, arabinose, galactose, xylose, and xylobiose were quantified after hydrolyzing DRB. As shown in Table 3, apparently higher ($p < .05$) releases in glucose (3.718–3.872 mg/mL) and arabinose (0.151–0.159 mg/mL) were found with all combinations of hemicellulase and FAE as well as H5 treatments. It was observed that F5 treatment had lower efficiency to release glucose in DRB than that with hemicellulase and its combination treatment. On the other hand, there were no statistical differences in the amounts of galactose, xylose, and xylobiose released from the DRB. Parallel to the results of glucose, all combinations of enzymes (ranging from 3.947 mg/mL to 4.134 mg/mL) as well as H5 treatments (3.971 mg/mL) showed higher amount of total sugars than the other treatments (2.442–2.880 mg/mL).

4. Conclusion

The FAE produced by *P. sumatrense* NCH-S2 exhibited highest enzyme activity at 50°C. It has unique characteristics such as basophilicity (pH 9.0–10.0) and high stability in both acidic and alkaline conditions. The DPPH radical-scavenging capacity, Fe^{2+} chelating ability and TPC of DRB were enhanced by FAE hydrolysis. Moreover, the synergistic interaction between hemicellulase and FAE significantly increased the amount of released FA and antioxidant activity of DRB. The results of this study provide useful information for the characteristics of a new FAE produced by *P. sumatrense* NCH-S2. The enzyme could be a potential candidate to be applied in various industries due to its wide pH stability range and high resistance in alkaline.

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Author contributions

Conceptualization, C.S.C., and J.S.L.; Methodology, J.S.L, Y.Q.L. and T.Y.S.; Formal Analysis, J.S.L., and Y.Q.L.; Investigation, C.S.C., J.S.L., and T.Y.S.; Resources, C.S.C.; Data Curation, J.S.L., and Y.Q.L.; Writing – Original Draft Preparation, J.S.L.; Writing – Review & Editing, C.S.C., J.S.L., and Y.Q.L.; Visualization, C.S.C. and J.S.L.; Supervision, C.S.C.; Project Administration, C.S.C. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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