Electronic Journal of Biotechnology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system

Evelyn Romero Borbón [a], Daniel Grajales Hernández [b], Mariana Armendáriz Ruiz [b], Lorena Ramírez Velasco [b], Jorge Alberto Rodríguez-González [b], Luis Alberto Cira-Chávez [a], María Isabel Estrada-Alvarado [a], Juan Carlos Mateos-Díaz [b,*]

[a] Departmento de Biotecnología y Ciencias Alimentarias, Instituto Tecnológico de Sonora, 5 de Febrero 818 Sur, Centro, Ciudad Obregón, Sonora, Mexico
[b] Departmento de Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Camino Arenero 1227 El Bajío del Arenal, Zapopan, Jalisco, Mexico

ARTICLE INFO

Article history:
Received 12 February 2018
Accepted 27 June 2018
Available online xxxx

Keywords:
Aspergillus ochraceus
Biocatalyst
Butanol specificity
Butanol
Butyl caffeate
Butyl hydroxycinnamates
Hydroxycinnamic acids
Hydroxycinnamoyl esterase
Solid state fermentation
Strain producer
Type C feruloyl esterase

ABSTRACT

Background: *Aspergillus ochraceus* was isolated from coffee pulp and selected as an interesting hydroxycinnamoyl esterase strain producer, using an activity microplate high-throughput screening method. In this work, we purified and characterized a new type C *A. ochraceus* feruloyl esterase (AocFaeC), which synthesized specifically butyl hydroxycinnamates in a ternary solvent system.

Results: AocFaeC was produced by solid state fermentation (SSF), reaching its maximal activity (1.1 U/g) after 48 h of culture. After purification, the monomeric protein (34 kDa) showed a specific activity of 57.9 U/mg towards methyl ferulate. AocFaeC biochemical characterization confirmed its identity as a type C feruloyl esterase and suggested the presence of a catalytic serine in the active site. Its maximum hydrolytic activity was achieved at 40°C and pH 6.5 and increased by 109 and 77% with Ca^{2+} and Mg^{2+}, but decreased by 90 and 45% with Hg^{2+} and Cu^{2+}, respectively. The initial butyl ferulate synthesis rate increased from 0.8 to 23.7 nmol/min after transesterification condition improvement, using an isooctane:butanol:water ternary solvent system, surprisingly the synthesis activity using other alcohols was negligible. At these conditions, the synthesis specific activities for butyl *p*-coumarate, sinapinate, ferulate, and caffeate were 87.3, 97.6, 168.2, and 234 U/μmol, respectively. Remarkably, AocFaeC showed 5 folds higher butyl caffeate synthesis rate compared to type B *Aspergillus niger* feruloyl esterase, a well-known enzyme for its elevated activity towards caffeic acid esters.

Conclusions: Type C feruloyl esterase from *A. ochraceus* is a butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system.

1. Introduction

Feruloyl esterases (Faes; E.C. 3.1.1.73) are a group of hydrolases broadly distributed in plants and microorganisms that catalyze the hydrolysis or condensation between hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and sinapinic acid) and an alcohol moiety. Their substrate specificity against four methyl hydroxycinnamates and their primary sequence identity allows the Faes to be classified into four types (A-D) [1]. Type A Faes show a preference for methyl ferulate and sinapinate, type B Faes for methyl *p*-coumarate and caffeate, while type C and D Faes have a broad specificity, hydrolyzing all methyl hydroxycinnamates.

Nowadays, Faes from several fungi of the *Aspergillus* genus are produced by solid state fermentation (SSF) with several advantages, such as a high volumetric productivity. Furthermore, many agro-industrial by-products containing hydroxycinnamic acids, may be used as supports/substrates for the induction of Fae activity [2,3]. For example, wheat bran and sugar beet pulp are inducers for type A (AnFaeA) and type B (AnFaeB) feruloyl esterases from *Aspergillus niger*, respectively [4,5], while maize bran induces a type C feruloyl esterase from *Aspergillus terreus* [6].

Faes have considerable roles in biotechnological processes in a wide variety of applications such as in biofuel, food, feed, pulp, paper, cosmetics, and pharmaceutical industries [7,8]. They have the ability to release ferulic acid and other hydroxycinnamic acids from plant biomass, which have been demonstrated to possess antioxidant, anti-inflammatory, antitumoral, antimicrobial and photoprotective activities [9] and are precursors of high economic value molecules

* Corresponding author.
E-mail address: jcmateos@ciatej.mx (J.C. Mateos-Díaz).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2018.06.004

Please cite this article as: Borbón ER, et al, Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system..., (2018), https://doi.org/10.1016/j.ejbt.2018.06.004

[10,11,12]. In addition, when the acidic moiety of a hydroxycinnamic acid is esterified with an alcohol (aliphatic or sugar), its biological activities might be enhanced or modified. Furthermore, the hydrophilic–lipophilic balance modification of hydroxycinnamic acids after esterification may also allow its incorporation into water or oil based formulations, which could be of interest in the cosmetic, food, agro-, and pharmaceutical industries [9].

During the last 15 years, several research works demonstrated that alkyl hydroxycinnamates biological activities show a cut-off effect between C_4 and C_8 alkyl chain length [13,14,15]. In this respect, butyl and octyl esters of *p*-coumaric and caffeic acid, showed the highest *in vitro* and *in vivo* anti-inflammatory activity through NF-κβ pathway inhibition, suppressing the secretion of PGE2 [16]. Moreover, short and medium alkyl caffeate esters (<C5) showed enhanced antitumoral activities against cervical and gastric cancer cells in comparison with the non-esterified acids [17], while the butyl ester of sinapinic acid is reported as a potent antioxidant inhibiting lipid oxidation [18]. Additionally, butyl esters of coumaric acid showed the highest antifungal activity against *Pyricularia grisea, Valsa mali* or *Botryosphaeria dothidea* [19]. Finally, it has been demonstrated that butyl ferulate is an important precursor of a novel prodrug, used as a lipophilic antioxidant [20]. All these previous works prove the biological importance of butyl hydroxycinnamates and the need of alkyl specific enzymes for their synthesis.

The derivatization of hydroxycinnamic acids with an alkyl alcohol has been performed by esterification or transesterification using Faes in a ternary solvent system [21,22,23,24]. This system contains a non-polar solvent, an alcohol (used as the substrate), and an aqueous phase containing the enzyme. It has been shown that the concentrations of the solvent, alcohol, and water in this reaction system strongly influence the Fae conversion yield [21,23,25]. The synthesis of hydroxycinnamic esters using Faes instead of chemical catalysts reduces the formation of secondary by-products, using environmentally friendly processes. Type C Faes possess a broad specificity during the synthesis of hydroxycinnamic acid esters. Hence, type C Faes from *Fusarium oxysporum* [26], *Myceliophthora thermophila* (formerly *Sporotrichum thermophile*) [25], and *Talaromyces stipitatus* [27] were successfully used in the synthesis of propyl, butyl and arabinosyl hydroxycinnamic acid esters reaching maximum yields of approximately 70% at mild temperatures (30–37°C). However, at our knowledge Faes alkyl specificity during butyl hydroxycinnamates synthesis has been poorly studied. Therefore, bioprospecting for alkyl specific Faes capable of synthesizing hydroxycinnamic butyl esters is still required.

In a previous work, *A. ochraceus* was selected among a fungal strain collection isolated from coffee waste and used to produce an enzymatic extract containing Fae activity, which released caffeic acid from coffee pulp [28,29]. In this work, a new type C feruloyl esterase from *A. ochraceus* (AocFaeC) was produced by solid state fermentation using maize bran as an inducer, purified, biochemically characterized and its biocatalytic potential was demonstrated for the alkyl specific synthesis of butyl esters of hydroxycinnamic acids, which own interesting biotechnological applications.

2. Materials and methods

2.1. Materials and strains

Bradford reagent, phenylmethanesulfonyl fluoride (PMSF), $MgSO_4$, K_2HPO_4, HCl, formic acid, urea, ammonium sulfate, hydroxycinnamic acids, methanol, ethanol, propanol, butanol, *tert*-butanol, octanol, dodecanol, β- mercaptoethanol, bacteriological agar, bovine serum albumin (BSA), Tween 80, *n*-hexane, ethyl acetate, petroleum ether, diethyl ether, acetonitrile, buffer salts, and metal ion salts were purchased from Sigma (Toluca, Mexico). The broad range molecular weight standard was purchased from Bio-Rad (Ciudad de Mexico, Mexico). Maize bran was kindly provided by Minsa® (Los Mochis, Sinaloa, Mexico). *A. niger* strains for type A and type B feruloyl esterase production were kindly donated by Dr. Record and Dr. Levasseur from INRA, France.

2.2. Fungal strain conservation and inoculum preparation

A. niger and *A. ochraceus* spores were maintained in 50% *v*/v glycerol at −80°C. The spore suspension used as inoculum was prepared in 250 ml Erlenmeyer flasks each containing 50 ml of the media reported by Record et al. [30] and Rodríguez et al. [31] for *A. niger* and *A. ochraceus*, respectively. For *A. ochraceus*, washed maize bran with a particle size of approximately 0.15 mm (40 g/l) was used as an inducer. Flasks were incubated for 4 d at 30°C, and spores were collected by stirring 50 ml of 0.01% *w*/*v* Tween 80 sterile solution.

2.3. Feruloyl esterase production in SSF

SSF was performed essentially as described by Rodríguez et al. [31]. Prior to fermentation, maize bran (substrate) was washed thoroughly with tap water to eliminate the soluble sugars. Then, it was dried at 30°C for 3 d and finally milled and sieved to achieve a particle size of approximately 0.42 to 0.59 mm. Polyurethane foam was cut into 0.5 cm cubes and employed as a support for the SSF. The impregnation media composition (g/l) was as follows: $MgSO_4$ (1); K_2HPO_4 (5); and urea (4). The support and substrate were mixed in a 1/3 (*w*/w) proportion, and then, each gram of mixture was added with 1.5-fold concentrated impregnation media at a pH of 6.5 and sterilized at 121°C for 15 min. The spore suspension was added to achieve 3×10^7 spores per gram of dry matter (gdm). The humidity and temperature were adjusted to 75% w/w and 30°C, respectively. After culture, the solid ferment of each column was placed in a plastic bag. The enzymatic extract was obtained after mixing the solid ferment with 2.5 mM MOPS at a pH of 7.2 in a 1/2 (w/v) proportion and applying slight manual pressure to each bag. The enzymatic extract was centrifuged at 14,000 ×g for 5 min to eliminate fungal residues and suspended matter. Two fermentation columns were removed every 24 h to measure the Fae hydrolytic activity in the enzymatic extracts using methyl ferulate (MF) as the substrate and the spectrophotometric assay described below. Average values and standard deviation were reported.

2.4. Synthesis of methyl and butyl hydroxycinnamates

Each hydroxycinnamic acid was dissolved in methanol or butanol to obtain a 0.5 M solution, and then, 4% (v/v) HCl was added. The reaction mixture was heated under reflux for 18 h. The reaction progress was followed by thin layer chromatography (*n*-hexane/ethyl acetate; 2/1) and after complete reaction, the solvent was evaporated under reduced pressure using a rotary evaporator. The obtained powder was purified by silica gel column chromatography using different petroleum ether/diethyl ether mixtures. Fractions with the corresponding ester were pooled and dried to obtain white crystals with a purity of >98%.

2.5. Feruloyl esterase hydrolytic activity assays

2.5.1. Spectrophotometric assay

The Fae activity was determined by measuring the hydrolysis of different methyl hydroxycinnamates (5 mM) in the presence of the pH indicator 4-nitrophenol (0.5 mM), as described by Ramírez et al. [28]. The substrate solution was prepared using *tert*-butanol and 2.5 mM MOPS at a pH of 7.2 in a 1/9 (v/v) proportion. The decrease in absorbance at 410 nm was continuously monitored in a microplate reader X-Mark from BioRad (Ciudad de Mexico, Mexico) at 30°C and a pH of 7.2 over 15 min. The reaction rate was calculated using the extinction coefficient obtained from standard curves (0 to 1 mM) for each hydroxycinnamic acid. Blank experiments were carried out using

Please cite this article as: Borbón ER, et al, Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system..., (2018), https://doi.org/10.1016/j.ejbt.2018.06.004