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Expression of a novel feruloyl esterase from *Aspergillus oryzae* in *Pichia pastoris* with esterification activity

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ABSTRACT

A 783-bp gene (*AofaeA*) that encodes a novel type A feruloyl esterase (AoFaeA) was amplified from *Aspergillus oryzae* CICC40186 by reverse transcription-PCR (RT-PCR), and extracellularly expressed in *Pichia pastoris* GS115. The recombinant *A. oryzae* feruloyl esterase (re-AoFaeA) was purified and characterized. SDS-PAGE analysis of the purified re-AoFaeA displayed a single protein band with an apparent molecular weight of about 37.0 kDa, larger than the theoretical one (28.14 kDa) of AoFaeA. The re-AoFaeA showed its optimal activity at 50 °C and pH 5.0. It was stable at 50 °C or below for 6 h, and at a pH range of 4.0–6.0. Its activity was not significantly influenced by an array of metal ions tested and EDTA, but inhibited by Cu^{2+}. The K_m and V_{max} of re-AoFaeA, toward methyl ferulate, were 0.81 mM and 82.2 U/mg, respectively. The esterification yield of ferulic acid with glycerol by re-AoFaeA (200 U/g ferulic acid) reached 60.3%. In addition, 47.8% of the total alkali-extractable ferulic acid in wheat bran was released by synergistic action of re-AoFaeA (50 U/g wheat bran) with a recombinant *A. oryzae* GH family 11 xylanase (re-AoXyn11A, 600 U/g wheat bran), which was about 11.7-fold higher than that (4.1%) by re-AoFaeA alone.

1. Introduction

Hydroxycinnamic acid derivatives, such as *p*-coumaric acid and ferulic acid, are both ester-linked to polysaccharides (arabinoxylan, pectin, etc.) and ether-linked to lignins in plant cell walls. These resultant networks contribute to the integrity of cell walls and the low digestibility of biomasses [1,2]. Feruloyl esterases (EC 3.1.1.73), existing in various organisms and belonging to a subfamily of the carboxylic esterase family, cleave the ester linkages between polysaccharides and ferulic acid or other phenolic acids [3]. They have been widely applied in the food, feed, papermaking and pharmaceutical industries, and the production of biofuels [4]. Based on their homology alignment of primary structures and the specificity toward substrate aromatic moieties, feruloyl esterases have been classified into four types, namely, type A, B, C and D [5].

Ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) was initially prepared by chemical synthesis and alkaline/acidic hydrolysis from agricultural byproducts such as wheat bran and corncob [6,7], which are time-consuming and pollution-causing. Recently, its production by enzymatic hydrolysis, an efficient and environmentally friendly bioprocess, has drawn much attention [2,8]. Ferulic acid showed various biological activities, such as anti-oxidative, food preservative and anti-inflammatory effects. It also exhibited the therapeutic effects against diabetes, cardiovascular disease and cancer [9,10]. However, the applications of ferulic acid were bottlenecked by its low water solubility. Reportedly, the solubility of feruloyl glycerol ester or sugar ester, catalyzed by feruloyl esterase in the low-aqueous system, was much higher than that of ferulic acid [11]. Recently, Hydroxycinnamic acid glycerol esterases were synthesized by a type A feruloyl esterase from *Aspergillus niger* [12], and feruloylated oligosaccharides were synthesized by feruloyl esterase from *Humicola* spp. [13].

Although many feruloyl esterases have been produced and characterized from bacteria and fungi, their commercialization and

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applications are limited by the low catalytic activity and expensive cost. Therefore, more interest is being focused on the gene cloning and heterologous expression of novel feruloyl esterases with superior properties and higher specific activities [4,14]. Thus far, two feruloyl esterases from *Aspergillus oryzae* were cloned and expressed in *Pichia pastoris* that are classified in subclasses B and C [15]. In this work, the gene *AofaeA* that encodes a novel type A feruloyl esterase was amplified from *A. oryzae* by RT-PCR and functionally expressed in *P. pastoris*. Later, the expressed re-AoFaeA was purified and characterized. Furthermore, the esterification of ferulic acid with glycerol into feruloyl glycerol by re-AoFaeA and the hydrolysis of wheat bran releasing ferulic acid by synergistic action of re-AoFaeA with re-AoXyn11A were investigated, respectively.

2. Materials and methods

2.1. Microorganisms, plasmids and culture media

A. oryzae CICC40186, purchased from the Center of Industrial Culture Collection (CICC) of China, was used for total RNA extraction. *Escherichia coli* JM109 and plasmid pUCm-T (Sangon, Shanghai, China) were applied for gene cloning and DNA sequencing, while *E. coli* DH5α and pPIC9K (Invitrogen, San Diego, USA) for construction of the recombinant expression plasmid. *E. coli* JM109 and DH5α were cultured at 37 °C in the Luria-Bertani medium. *P. pastoris* GS115 and its transformants were cultured and induced as described in the manual of Multi-Copy Pichia Expression Kit (Invitrogen, USA). The *P. pastoris* transformant (GSAorX4-3) containing *Aoxyn11A*, a xylanase-encoding gene from *A. oryzae*, was constructed and preserved in our lab [16].

2.2. Amplification of the gene AofaeA

By multiple homology alignment of four *Aspergillus* type A feruloyl esterase sequences separately from *A. niger* (GenBank no. ADI70526), *Aspergillus awamori* (BAA92937), *Aspergillus terreus* (XP_001217493) and *Aspergillus tubingensis* (CAA70511), we discovered two highly conserved peptide segments, ASTQGISEDLY and TYFGMTSGA(/H)CTW, which were located at the N- and C-termini. A pair of degenerate primers Fae-F (5′-GAATTCGCCTCTACRCAGGGCATCTC-3′ with an *Eco*RI site, underlined; R = A/G) and Fae-R (5′-GCGGCCGCTYACCADGTRCARKSTCCGC-3′ with a *Not*I site, underlined; Y = C/T, D = G/A/T, K = G/T, S = C/G) was designed corresponding to ASTQGIS and SGA(/H)CTW, respectively. An Oligo dT-Adaptor Primer, 5′-GTTTTCCCAGTCACGAC(dT_{18})-3′ (TaKaRa, Dalian, China), was used for reverse transcription of the first-strand cDNA from the *A. oryzae* total RNA isolated by RNA Extraction Kit (Sangon, China). Using the resultant first-strand cDNA as a template, a gene *AofaeA* was amplified by PCR with primers Fae-F and Fae-R under conditions: at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s in 30 cycles. The target PCR product was gel-purified and inserted into plasmid pUCm-T. The resultant recombinant T-plasmid, designated pUCm-T-*AofaeA*, was transformed into *E. coli* JM109 and confirmed by DNA sequencing.

2.3. Analysis of the primary and three-dimensional (3-D) structures

Homology sequence search at NCBI website (http://www.ncbi.nlm.nih.gov/) was carried out using a BLAST server, while multiple homology alignment of primary structures using a ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Physicochemical properties were identified by Protparam program (http://au.expasy.org/tools/protparam.html). Putative N-linked glycosylation sites were located using a NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Using the crystal structure of *A. niger* type A feruloyl esterase (PDB code: 1UWC) as a template, the 3-D structure of AoFaeA was homologically modeled using a MODELLER 9.9 program (http://salilab.org/modeller/).

2.4. Enzyme activity and protein assays

Feruloyl esterase activity was assayed by mixing 0.1 mL of diluted enzyme with 0.9 mL of 1 mM methyl ferulate (Sigma, St. Louis, USA) in 50 mM Na_2HPO_4-citric acid buffer (pH 5.0) and incubating at 45 °C for 10 min [17]. The amount of enzyme liberating 1 μmol of ferulic acid per min was defined as one unit (U) of feruloyl esterase activity. Xylanase activity was assayed by measuring the amount of reducing sugars released from birchwood xylan (Sigma, USA) using the 3,5-dinitrosalicylic acid (DNS) method as described previously [16]. SDS-PAGE was carried out using the method of Laemmli [18]. The separated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, USA), whose apparent molecular weights were estimated using a Quantity One software based on the standard marker proteins. The protein and carbohydrate contents were measured with a BCA-200 Protein Assay Kit (Pierce, Rockford, USA) and a phenol–sulfuric acid method [19], respectively.

2.5. Expression and purification of re-AoFaeA

AofaeA was excised from pUCm-T-*AofaeA* by *Eco*RI and *Not*I, and inserted into plasmid pPIC9K digested by same enzymes. Then, the *Sal*I-linearized recombinant expression plasmid, pPIC9K-*AofaeA*, was electroporated into *P. pastoris* GS115. All *P. pastoris* transformants were successively inoculated on YPD plates containing geneticin G418 at increasing concentrations for screening multiple copies of integrated *AofaeA*. *P. pastoris* electroporated with pPIC9K was used as a control (*P. pastoris* GSFC). Expression of re-AoFaeA was performed as reported previously [20]. The expressed re-AoFaeA in the supernatant was salted out by adding $(NH_4)_2SO_4$ to 75% saturation. Then, the collected precipitate was dissolved in and dialyzed toward 20 mM Na_2HPO_4-citric acid buffer (pH 5.0). The dialyzed solution was concentrated by ultrafiltration using a 10-kDa cut-off membrane (Millipore, Billerica, USA), and loaded onto a Sephadex G-75 column (Amersham Pharmacia Biotech, Uppsala, Sweden; 1.6 cm × 80 cm), followed by elution with the same buffer at a flow rate of 0.4 mL/min. Aliquots of 2 mL eluent only containing re-AoFaeA were pooled.

2.6. Enzymatic properties of re-AoFaeA

The temperature optimum of re-AoFaeA was measured under the standard assay conditions stated above, except for reaction temperatures ranging from 30 to 65 °C. For estimating the thermal stability, aliquots of re-AoFaeA were treated at 48, 50 and 52 °C, respectively, for 6 h. The thermostability was defined as a temperature, at or below which the residual re-AoFaeA activity retained over 80% of its original activity. The pH optimum of re-AoFaeA was measured at the optimal temperature and toward 1 mM methyl ferulate in 50 mM Na_2HPO_4-citric acid buffer at a pH range of 3.5–7.0. For evaluating the pH stability, aliquots of re-AoFaeA were incubated (at 40 °C) at pH 3.0–7.5, respectively, for 1 h. The pH stability was defined as a pH range, over which the residual re-AoFaeA activity was more than 85% of its original activity.

For evaluating the effects of metal ions and EDTA on re-AoFaeA activity, aliquots of re-AoFaeA were incubated with an array of metal ions and EDTA, respectively, at a final concentration of 5 mM in 20 mM Na_2HPO_4-citric acid buffer (pH 5.0) at 40 °C for 1 h. Then, the residual re-AoFaeA activity was measured under the standard assay conditions. The enzyme solution without any additive was