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Expression, purification and characterization of a feruloyl esterase A from *Aspergillus flavus*

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ABSTRACT

Feruloyl esterases are key enzymes involved in the complete hydrolysis of hemicellulose. In the present study, the encoding sequence of putative feruloyl esterase A (*Af*FaeA) was cloned from genomic DNA from *Aspergillus flavus* and expressed in *Pichia pastoris*. The purified recombinant *Af*FaeA had apparent relative molecular mass of about 40,000 and had an optimum pH of 6.0, although it was stable at pH values ranging from 4.5 to 8.0. The optimum temperature for *Af*FaeA was 58 °C. *Af*FaeA had hydrolytic activity toward methyl caffeate, methyl p-coumarate, methyl ferulate and methyl sinapate. Substrate specificity profiling of *Af*FaeA demostrated it is a type-A feruloyl esterase. The good performance of *Af*FaeA to release ferulic acid from steam exploded corn stalk in concert with *Geobacillus stearothermophilus* xylanase mutant indicated it is a promising biocatalyst for biomass degradation.

Introduction

Feruloyl esterases[1] (FAEs) play an important role in biodegradation of lignocellulose, which could hydrolyze the ester bonds between hydroxycinnamate and arabinoxylans and certain pectin in hemicellulose. The hemicellulose could be completely hydrolyzed with the synergistical effort of feruloyl esterases and other related enzymes, such as endoxylanase, β-xylosidase α-L-arabinofuranosidase and α-glucuronidase [1]. As a promising biocatalyst, feruloyl esterases could perform the activities of hydrolyzation and transesterification and have been applied to biofuel production, pharmaceutical synthesis, organic food making and paper manufacturing [2].

The traditional enzyme screening plays an important role for biocatalyst discovery through identification of functional microorganisms with suitable substrates. Up to now, tens of FAEs have been functionally screened from microorganisms based on their hydrolytic properties. In recent years, with the ever-increasing genomic sequences of microorganisms available, searching the new enzymes from genome database has been developed to be a new efficient pathway [3]. According to survey of Genomes OnLine Database (www.genomesonline.org), the genome sequencing of over 4000 microorganisms have been completed and more than ten thousands of microorganism genome sequencing projects are under way. The huge genome sequence data would provide an abundant resource for biocatalyst mining. So far, several new feruloyl esterases have been cloned and characterized from microorganisms with the help of genome database [4].

In this study, a putative feruloyl esterase A (*Af*FaeA) encoding sequence from *Aspergillus flavus* was identified in the genomic data published. Then it was cloned and heterologously expressed in the methylotrophic yeast *Pichia pastoris*. The enzymatic properties of *Af*FaeA were characterized and it was determined as a type-A feruloyl esterase based on its substrate specificity profiling and primary sequence identity. The liberation of ferulic acid from steam-exploded corn stalk with *Af*FaeA and xylanase demonstrated its great potential for industrial application.

Materials and methods

Materials and chemicals

The substrate 2-chloro-4-nitrophenyl ferulate CNPF was synthesized as previously described [5]. Methyl esters of ferulic (MFA), p-coumaric (MpCA), caffeic (MCA) and sinapic (MSA) acids were purchased from Apin Chemicals Ltd. (Abingdon, UK). Steam exploded corn stalk was kindly donated by Professor Hongzhang Chen at the Institute of Process Engineering, Chinese Academy of Sciences [6]. Oligonucleotides were synthesized by Invitrogen Life Technologies (Shanghai, China) in PAGE-purified grade. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). DNA sequencing was carried out in Invitrogen (Shanghai, China). All other reagents were obtained from general commercial suppliers and used without further purification.

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[1] *Abbreviations used:* FAEs, feruloyl esterases; PCR, polymerase chain reaction.

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Genome mining and genetic manipulations

The 783 bp nucleotide sequence of a biochemically characterized feruloyl esterase A (*An*FaeA) (EMBL accession number: FJ430154.1) from *Aspergillus niger*[7] was used to perform a BLAST search in the NCBI genome database with the somewhat similar sequences (blastn) program. As a result, a putative feruloyl esterase A encoding sequence in the *A. flavus* NRRL3357 (locus tag NW_002477244) showed 73% sequence identity aligned with the encoding sequence of *An*FaeA.

The *A. flavus* CBE332.1 strain was conserved in our laboratory, and grown in 100 mL Czapek-Dox liquid medium (30 g/L sucrose, 3 g/L $NaNO_3$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 1 g/L KH_2PO_4, 55 mg/L $FeSO_4$) at 30 °C, 200 rpm for 5 days. The mycelia were harvested and genomic DNA was extracted with fungal genomic DNA extraction Kit (Sangon, Shanghai, China). The target DNA sequence was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Clontech, Takara BIO Company) and the specific primers: FAE-F, 5′-ATGCCATTAAGTACCCTTTCG-3′ and FAE-R, 5′-CCATGTACAGGCTCCGC-3′. The PCR was performed under the condition at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and followed by a final extension at 72 °C for 10 min. The signal peptide of the deduced amino acid sequence was predicted using web-based program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) [8] and PrediSi (http://www.predisi.de/) [9]. The intron sequence in *Af*FaeA encoding sequence was confirmed by aligned with feruloyl esterase A gene sequence from *A. niger*.

The mature *Af*FaeA encoding sequence was amplified by overlap extension PCR technique to remove the signal peptide encoding sequence and intron sequence with primers: FAE-F1, 5′-TAGGAGGT*GAATTC*GCAATCACTCAGGGGATC-3′, FAE-R1, 5′-CATCGACGCACCCA GG CTATGTCCAGTAATCACCAG-3′ and FAE-F2, 5′-CTGGTGATTA CTGGACATAG CCTGGGTGCGTCGATG-3′, 5′-TAGGAGGT*GCGGCCGC*C CATGTACAGGCTCCGC-3′. The overlap extension PCR was performed following to the procedures reported [10]. The *Eco*RI and *Not*I restriction recognition sites were added in the front and the end of *Af*FaeA encoding sequence, respectively. The resulting 783-bp DNA fragment was digested with *Eco*RI and *Not*I and ligated into the corresponding sites of pGAPZαA (Invitrogen, Carlsbad, CA, USA) with T4 DNA ligase, and then transformed into *Escherichia coli* DH5α strain. The transformants were grown on Luria–Bertani (LB) medium agar supplemented with zeocin (25 μg/mL) at 37 °C. The recombinant plasmid was designated as pGAPZaA-*Af*faeA, which was then linearized by *Bsp*HI digestion and transformed into *P. pastoris* X-33 (Invitrogen, Carlsbad, CA, USA) using MicroPulser Electroporator (Bio-Rad Laboratories, Hercules, CA, USA). The transformants were grown on YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar powder supplemented with 1 M sorbitol and 100 μg/ml zeocin at 30 °C for 3 days.

Expression and purification of AfFaeA

A single colony was picked and inoculated in 100 mL YPD medium in 250 mL Erlenmeyer flasks at 30 °C, 250 rpm for 2 days. Enzymatic activity towards CNPF in supernatant was measured after centrifugation at 8000*g* for 10 min as described previously [5]. The purification procedures of *Af*FaeA were followed the method as reported [7]. The purified *Af*FaeA was confirmed by both enzymatic activity assay and SDS–PAGE. Protein estimation was done with the BCA Protein Assay Kit (Beyotime, Shanghai, China) with bovine serum albumin as a standard.

Enzymatic activity assay of AfFaeA

The pH optimum of *Af*FaeA was measured at 40 °C in 100 mM sodium acetate buffer (pH 4.0–5.5) and phosphate buffer (pH 6.0–8.0) with substrate MFA. The pH stability of *Af*FaeA was measured by performing an activity assay in 100 mM sodium acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–8.0) and Tris–Cl buffer (pH 8.5–9.5) at 40 °C for 1 h and the residual activity was measured with MFA at pH 5.0. The optimal reaction temperature was determined at temperatures ranging from 40 °C to 68 °C at pH 5.0 for 10 min. The kinetic parameters of *Af*FaeA toward four model substrates MFA, MpCA, MCA and MSA were determined by measuring the corresponding hydroxycinnamic acid with HPLC using a Luna C18 reverse-phase column (Phenomenex, Torrance, CA, USA) as previously described [11]. 1 unit of FAE activity is defined as the amount of enzyme that releases 1 μmol of corresponding hydroxycinnamic acid per minute. All assays were performed in triplicate. Data were fitted to the Michaelis–Menten equation or substrate inhibition equation using Graph-Pad Prism v5.0 (GraphPad Software, San Diego, CA, USA) to generate estimates of K_m and k_{cat} values.

Release of ferulic acid from steam exploded corn stalk

The liberation of ferulic acid from steam exploded corn stalk was performed with *Af*FaeA and purified xylanase XT6 mutant from *Geobacillus stearothermophilus* designated as FC06T synergistically [12]. 1 mg xylanase and 0.5 mg *Af*FaeA were added into 50 mL 100 mM sodium acetate buffer (pH 5.0) containing 2 g of steam exploded corn stalk. The hydrolysis reactions were performed at 40 °C and monitored for 8 h. The amount of ferulic acid released from steam exploded corn stalk was analyzed following the method reported previously [13]. The control samples were performed in the same system without the addition of xylanase.

Results and discussion

Cloning and expression of AfFaeA

The open reading frame (ORF) of A putative feruloyl esterase A was found in Locus NW_002477244 from *A. flavus* NRRL3357, showing high sequence identity (72%) with known *An*FaeA. The encoding sequence containing a 100 bp intron was cloned from the genomic DNA of *A. flavus* CBE332.1 and the intron sequence was removed by overlap extension PCR. The ORF of *Af*faeA encodes proteins of 281 amino acid residues and the DNA sequence information was submitted to GenBank with accession number of KC748203. The both of web-based program, SignalP and PrediSi, were used to predict the signal peptide of putative *Af*FaeA and the results showed that the AfFaeA has a signal peptide of 21 amino acids. The encoding sequence of mature *Af*FaeA was finally obtained after the encoding sequence of the signal peptide was removed. The mature *Af*FaeA has 260 amino acid residues with a molecular weight of 28,164 Da. The deduced protein sequence of mature *Af*FaeA exhibits a high degree of sequence identity with *An*FaeA from *A. niger* and *At*FaeA from *Aspergillus terrus* (76.71% and 74.81%, respectively) and it also displays 27.08% sequence identity with lipase (*Tl*Lip) from *Thermomyces lanuginosus*, and 28.87% sequence identity with lipase (*Rm*Lip) from *Rhizomucor miehei* (Fig. 1). The consensus sequence near the active site serine of esterases and lipases is known to be Gly–Xaa–Ser–Xaa–Gly [14], and the motif (Gly–His–Ser–Leu–Gly) matches exactly among *Af*FaeA, *An*FaeA, *At*FaeA, *Tl*Lip and *Rm*Lip (Fig. 1). The encoding sequence of mature *Af*FaeA from *A. flavus* was successfully cloned and then transformed in *P. pastoris* X-33. The shuttle vector pGAPZaA was used to facilitate the expression extracellular recombinant proteins constitutively. Enzymatic activity towards *p*CNPF reached a maximum of 0.5 U/mL after incubated for 2 days. Further incubation would result in a decrease in enzymatic activity. *Af*FaeA was