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Cloning, expression of a feruloyl esterase from *Aspergillus usamii* E001 and its applicability in generating ferulic acid from wheat bran

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Abstract A cDNA gene (*AufaeA*), which encodes a mature polypeptide of the type-A feruloyl esterase from *Aspergillus usamii* E001 (abbreviated to AuFaeA), was cloned and heterologously expressed in *Pichia pastoris* GS115. One transformant, labeled as *P. pastoris* GSFaeA4-8, expressing the highest recombinant AuFaeA (reAuFaeA) activity of 10.76 U/ml was selected by the flask expression test. The expressed reAuFaeA was purified to homogeneity with an apparent molecular weight of 36.0 kDa by SDS-PAGE analysis, and characterized using the model substrate of methyl ferulate (MFA). The purified reAuFaeA was optimally active at pH 5.0 and 45 °C, and highly stable at pH 4.0–6.5 and 45 °C or below. Its activity was not significantly affected by metal ions tested and EDTA. The K_m and V_{max} of reAuFaeA towards MFA were 4.64 mM and 115.5 U/mg, respectively. High-performance liquid chromatography analysis showed that only 9.7 % of total alkali-extractable ferulic acid (FA) was released from de-starched wheat bran by reAuFaeA alone. The released FA increased to 36.5 % when reAuFaeA was used together with a recombinant *Aspergillus usamii* GH family 11 xylanase A, indicating a synergistic interaction between them.

Keywords Cloning and expression · Feruloyl esterase · *Aspergillus usamii* · Ferulic acid · *Pichia pastoris*

Introduction

Agricultural waste materials, such as wheat bran, corncob, rice or wheat straw, and cane bagasse, are ubiquitous, abundant, and renewable organic carbon resources [20]. Recently, more interest is being focused on obtaining high-value compounds in environmentally friendly and efficient ways from those biomaterials, which mainly consist of cellulose, hemicellulose, and lignin [17, 27]. Ferulic acid (FA, 3-methoxy-4-hydroxycinnamic acid), the most abundant hydroxycinnamic acid present in plant cell walls, is covalently cross-linked to polysaccharides by ester linkages and to components of lignin mainly by ether linkages [2, 28]. These cross-links contribute to the recalcitrance of plant tissues but largely limit the digestibility of biomaterials.

Feruloyl or ferulic acid esterases (FAEs, EC 3.1.1.73), which exist widely in various organisms, can catalyze the hydrolysis of ester linkages between FA and polysaccharides. FA generated from plant cell walls by FAEs has great commercial value as an antioxidant, photoprotectant, food preservative, antimicrobial agent, and anti-inflammatory

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agent [11, 18]. Besides, it can be enzymatically converted to various value-added products, such as vinyl guaiacol and vanillin that are used as flavoring agents in foods and perfumes [16]. FAEs deserve particular attention due to their wide-ranging applications in the food, pulp, feed, pharmaceutical, and biofuel industries [1, 29]. FAEs were initially classified into four types (types A–D) based on their primary structure homology, biochemical properties, and substrate specificity towards four synthetic substrates (methyl ferulate, methyl caffeate, methyl *p*-coumarate, and methyl sinapate) [5]. More recently, a descriptor-based computational analysis with pharmacophore modeling provided a novel approach for the classification of FAEs, with which 12 FAE families were proposed [26].

Thus far, various FAEs have been isolated and characterized from fungi and bacteria [4, 7, 24]. However, few FAEs have been applied in industries owing to their low catalytic activities and expensive production costs. Identification, cloning and expression, and utilization of novel FAEs still remain hot topics. In this study, the cloning and heterologous expression of the cDNA gene (*AufaeA*) in *Pichia pastoris* GS115, as well as the purification and biochemical properties of reAuFaeA are reported. Furthermore, the synergistic interaction of reAuFaeA with a recombinant *Aspergillus usamii* GH family 11 xylanase A (reAuXyn11A) in hydrolyzing destarched wheat bran (DSWB) to produce FA is presented.

Materials and methods

Strains, vectors, and culture media

Aspergillus usamii E001, isolated from the soil in China [12], was used as the source for total RNA extraction. The strain was cultured at 30 °C in a medium comprising the following (w/v): 1.0 % tryptone, 0.5 % yeast extract, 1.0 % glucose, and 1.0 % birchwood xylan (Sigma, St. Louis, MO, USA), pH 6.0. *Escherichia coli* JM109 and pUCm-T vector (Sangon, Shanghai, China) were used for gene cloning and DNA sequencing. *E. coli* DH5α and pPIC9K vector (Invitrogen, San Diego, CA, USA) were used for construction of the recombinant expression vector. *E. coli* JM109 and DH5α were cultured at 37 °C in a Luria–Bertani medium containing the following (w/v): 1.0 % tryptone, 0.5 % yeast extract, and 1.0 % NaCl, pH 7.2. *Pichia pastoris* GS115 (Invitrogen, USA) was cultured at 30 °C in a yeast extract peptone dextrose (YPD) medium. Its transformants were cultured and induced in the minimal dextrose (MD), geneticin G418-containing YPD, buffered glycerol complex (BMGY), and buffered glycerol/methanol (BMMY) media, which were prepared according to the manual of the Multi-Copy Pichia Expression Kit (Invitrogen, USA). One transformant expressing the highest reAuXyn11A activity of 912.6 U/ml, labeled as *P. pastoris* GSXyn2-9, was constructed by, and preserved in, the Lab of Biochemistry and Molecular Biology, Wuxi Medical School, Jiangnan University, China.

Cloning of the cDNA gene encoding AuFaeA

A pair of degenerate PCR primers was designed on the basis of the highly conserved N- and C-terminal regions, AS(/I)TQGISE and T(/A)SGACTW, which were located by multiple homology alignment of five fungal type-A FAE amino acid sequences. Both the forward (F) and reverse primers (R), synthesized by Sangon (China), were FAE-F: 5′-GAATTCGCTWYYACKCARGGSATCTC-3′ and FAE-R: 5′-GCGGCCGCYYACCADGTRCARKSTC-3′ (W = A/T, Y = C/T, K = G/T, R = A/G, S = C/G, D = G/A/T) with *Eco*RI and *Not*I sites (underlined), respectively.

The AuFaeA-encoding cDNA gene (*AufaeA*) was amplified using the reverse transcription-PCR (RT-PCR) and nested PCR techniques. An Oligo dT-Adaptor Primer, 5′-GTTTTCCCAGTCACGAC(dT_{18})-3′ provided with the RNA PCR Kit (TaKaRa, Dalian, China), was used for reverse transcription of the first-strand cDNA from the *A. usamii* total RNA, which was extracted using the RNA Extraction Kit (Sangon, Shanghai, China) according to the method reported previously [31]. Using the first-strand cDNA as the template, the first-round amplification of nested PCR was performed with primers FAE-F and M13 Primer M4 (identical to Oligo dT-Adaptor Primer except Oligo dT_{18}). Conditions for the first-round PCR were as follows: an initial denaturation at 94 °C for 5 min, 30 cycles of at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 60 s, and an extra elongation at 72 °C for 10 min. Then, the *AufaeA* was amplified from the first-round PCR product by the second-round amplification of nested PCR with primers FAE-F and FAE-R under the same conditions as stated above except 53 °C instead of 51 °C in 30 cycles. The amplified target band was purified using the EZ-10 Spin Column DNA Gel Extraction Kit (BBI, Markham, Canada), inserted into pUCm-T vector, and used to transform *E. coli* JM109. The proper recombinant T vector, designated pUCm-T-*AufaeA*, was confirmed by restriction enzyme analysis and DNA sequencing.

Analysis of the primary and three-dimensional structures of AuFaeA

The Protparam program (http://au.expasy.org/tools/protparam.html) was applied to identify the physicochemical properties of AuFaeA. The homology sequence search

at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) was carried out using the BLAST server. The multiple homology alignment of the amino acid sequences among fungal type-A FAEs was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2). The putative N- and O-linked glycosylation sites of AuFaeA were located using the NetNGlyc program 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively. The three-dimensional (3-D) structure of AuFaeA was homologically modeled using the MODELLER 9.9 program (http://salilab.org/modeller/) based on the crystal structure of a type-A FAE from *Aspergillus niger* (PDB code 1USW). The modeled 3-D structure of AuFaeA was visualized using the PyMOL software (http://pymol.org).

Construction and transformation of the recombinant expression vector

The gene *AufaeA* was released from pUCm-T-*AufaeA* by digestion with *Eco*RI and *Not*I, agarose gel-purified, and inserted into pPIC9K vector digested with the same enzymes, then used to transform *E. coli* DH5α. The proper recombinant expression vector, designated pPIC9K-*AufaeA*, was confirmed by restriction enzyme analysis and DNA sequencing. Then, the resulting pPIC9K-*AufaeA* was linearized with *Sac*I and used to transform *P. pastoris* GS115 by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction.

Screening and expression of the *P. pastoris* transformants

All *P. pastoris* transformants were primarily screened on the basis of their ability to grow on an MD plate, and then successively inoculated on geneticin G418-containing YPD plates at increasing concentrations of 1.0, 2.0, and 4.0 mg/ml for screening multiple copies of the integrated *AufaeA*. Expression of the *AufaeA* in *P. pastoris* GS115 was performed according to the instructions of the Multi-Copy Pichia Expression Kit (Invitrogen, USA) with slight modification. In brief, each single colony of the *P. pastoris* transformants, grown on YPD plates containing different concentrations of G418, was inoculated into a 30 ml of BMGY medium in a 250-ml flask, and cultured at 30 °C on a rotary incubator with 220 rpm until the OD_{600} reached 2–4. Then, the yeast cells were harvested by centrifugation, resuspended in a 30 ml of BMMY medium, and induced for the expression of reAuFaeA by adding methanol to a final concentration of 1.0 % (v/v) at 24-h intervals at 30 °C for 72 h.

Purification of the expressed reAuFaeA

After the *P. pastoris* transformant was induced by 1.0 % methanol at 30 °C for 72 h, the cultured medium was centrifuged at 8,000 rpm for 10 min to remove yeast cells. Solid $(NH_4)_2SO_4$ was added to 50 ml of the resulting supernatant with stirring to achieve 75 % saturation. The mixture was then stored at 4 °C overnight. The resulting precipitate was collected by centrifugation, dissolved in 4.0 ml of 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 6.0), and dialyzed against the same buffer overnight. The dialyzed sample solution was concentrated to 1.0 ml by ultrafiltration at 8,000 rpm using a 10-kDa cutoff membrane (Millipore, Billerica, MA, USA), and then loaded onto a Sephadex G-75 column (Amersham Pharmacia Biotech, Uppsala, Sweden; inner diameter 1.6 × 80 cm), followed by elution with the same buffer at a flow rate of 0.4 ml/min. Aliquots of 2.0 ml eluent only containing reAuFaeA were pooled and concentrated for subsequent studies. All purification procedures were performed at 4 °C unless stated otherwise.

Enzyme activity and protein assays

Feruloyl esterase (FAE) activity was assayed by measuring the amount of free ferulic acid (FA) released from methyl ferulate (MFA) as described previously [33] with appropriate modification. In brief, 0.1 ml of suitably diluted enzyme solution was mixed with 0.9 ml of 1.0 mM MFA (Sigma, St. Louis, MO, USA) in 100 mM Na_2HPO_4/citric acid buffer (pH 5.0). After incubation at 45 °C for 10 min, the reaction was terminated by adding 0.4 ml of glacial acetic acid. The released free FA was analyzed using an UltiMate-3000 high-performance liquid chromatography (HPLC) system (Dionex, Beijing, China) equipped with a ZW&A-C18 reversed-phase column (Chrom-Matrix, Wuxi, China). A mobile phase of 60 % methanol in water containing 1.0 % (v/v) acetic acid was used and monitored at 320 nm with a VWD-3100 detector (Dionex, China). One unit (U) of FAE activity was defined as the amount of enzyme liberating 1 μmol of free FA per min under the standard assay conditions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [15] on a 12.5 % gel. The isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, USA), and their apparent molecular weights were estimated using Quantity One software on the basis of the standard protein markers. The protein concentration was determined with the BCA-200 Protein Assay Kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard.

Characterization of the purified reAuFaeA

The optimum pH, a pH value corresponding to 100 % relative activity, of reAuFaeA was determined under the standard assay conditions, except with 1.0 mM MFA in 100 mM Na_2HPO_4/citric acid buffer at a pH range of 3.5–7.5. To estimate its pH stability, aliquots of reAuFaeA were preincubated at 35 °C and at different pH values (Na_2HPO_4/citric acid buffer, pH 3.5–7.5; Tris–HCl buffer, pH 8.0–9.0) for 1.0 h. The residual reAuFaeA activity was measured under the standard assay conditions. The optimum temperature, a temperature corresponding to 100 % relative activity, of reAuFaeA was assayed at the optimum pH and at temperatures ranging from 30 to 70 °C. To evaluate its thermostability, aliquots of reAuFaeA were preincubated in the absence of substrate at 45, 50, and 55 °C for 10, 20, 30, 40, 50, and 60 min.

To estimate the tolerance of reAuFaeA to metal ions (Na^+, Li^+, Ca^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Sn^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}) and EDTA, aliquots of reAuFaeA were preincubated at 35 °C with an array of metal ions and EDTA, at a final concentration of 2.0 mM in 20 mM Na_2HPO_4/citric acid buffer (pH 5.0) for 1.0 h. The residual reAuFaeA activity was determined under the standard assay conditions. The enzyme solution without adding any additive was used as the control.

The hydrolytic reaction rate (U/mg) of reAuFaeA was determined under the standard assay conditions, except with concentrations of MFA ranging from 0.6 to 6.0 mM. The reaction rate was plotted versus substrate concentration to confirm whether the hydrolytic mode of reAuFaeA conforms to the Michaelis–Menten equation. The kinetic parameters, K_m and V_{max}, were graphically determined from the Lineweaver–Burk plot.

Synergistic interaction between reAuFaeA and reAuXyn11A

Wheat bran was treated according to the method as described previously [19], dried at 105 °C to constant weight, and milled to a fine powder by passing through a 250-μm sieve. Then, the resulting DSWB was used as the substrate in the hydrolysis experiments. Aliquots of 30 ml DSWB suspension (16.7 mg/ml) in 100 mM Na_2HPO_4/citric acid buffer (pH 5.0) were mixed well with reAuFaeA and/or reAuXyn11A of different activity and incubated at 40 °C for 10 h on a rotary incubator at 120 rpm. The hydrolytic reaction was terminated by boiling for 10 min. The samples were cooled and clarified by centrifugation at 10,000 rpm for 5 min. The amount of released free FA in the supernatant was analyzed by HPLC. The buffer instead of enzyme(s) was used as the control. Hydrolysis experiments were performed in triplicate. To determine the total amount of FA in DSWB, an alkali-extractable hydrolysis was conducted as reported previously [32].

Results and discussion

Cloning of the cDNA gene *AufaeA*

The ratio of OD_{260}/OD_{280} of the total RNA extracted from *A. usamii* E001 was 1.94 and the 18S and 28S rRNA bands, characterized as eukaryotes, were specific on formaldehyde denatured agarose gel electrophoresis (data not shown). These analytical results indicated that the isolated total RNA has high purity and is not decomposed [30]. Using the first-strand cDNA transcribed reversely from the *A. usamii* total RNA as the template, an approximately 1.0-kb clear band and several faint bands were amplified by the first-round PCR with primers FAE-F and M13 Primer M4. On the basis of the principle of the nested PCR technique, each band was agarose gel-purified, and subjected to the second-round PCR with primers FAE-F and FAE-R. As a result, an approximately 800-bp specific band was amplified only using the 1.0-kb band as the template. The DNA sequencing result verified that the cloned cDNA gene *AufaeA* is exactly 797 bp in length (containing *Eco*RI and *Not*I sites). In this work, three independent clones were randomly picked out for DNA sequencing. The sequencing result was adopted when three inserted cDNA fragments were identical to one another, or else the experiment was redone.

Bioinformatics analysis of the primary structure of AuFaeA

The gene *AufaeA* encodes a mature polypeptide of 260 amino acids, i.e., AuFaeA (Fig. 1). The theoretical molecular weight and isoelectric point (pI) of AuFaeA were predicted to be 28.3 kDa and 4.3, respectively, using the Protparam program. The homology alignment between two amino acid sequences using DNAMAN 6.0 software showed that the identities of AuFaeA from *A. usamii* E001 with other five fungal type-A FAEs from *Aspergillus niger* (XP_001393337), *Aspergillus awamori* (BAA92937), *Aspergillus terreus* (XP_001217493), *Aspergillus flavus* (XP_002380173), and *Aspergillus oryzae* (XP_001818752) were 99.6, 94.6, 86.5, 75.8, and 74.6 %, respectively. In addition, the multiple homology alignment among six type-A FAEs using the ClustalW2 program showed that a pentapeptide G-H-S-L-G (location from Gly^{131} to Gly^{135}) was strictly conserved (Fig. 1), corresponding to the classical motif G-X-S-X-G (X = arbitrary amino acid residue) of esterase or lipase [21, 23]. Three strictly conserved amino acid residues, Ser^{133}, Asp^{194}, and His^{247}, constitute a

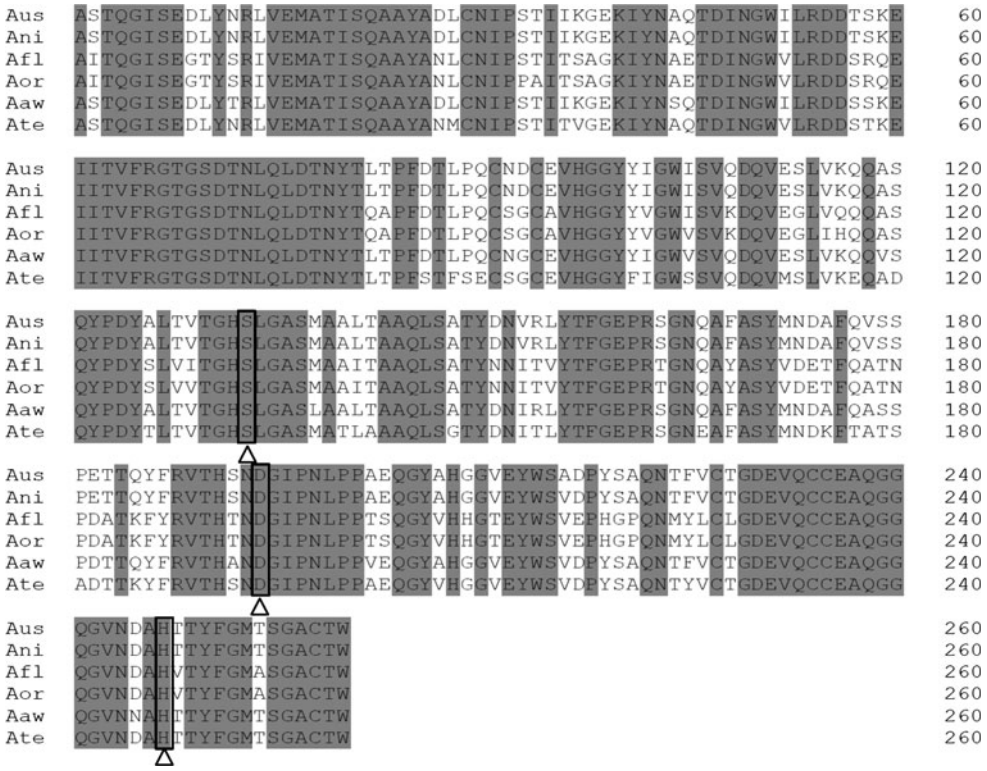

Fig. 1 Multiple homology alignment of primary structures between AuFaeA and five representative fungal type-A FAEs using the ClustalW2 program. Aus, *Aspergillus usamii* (in this work); Ani, *Aspergillus niger* (XP_001393337); Afl, *Aspergillus flavus* (XP_002380173); Aor, *Aspergillus oryzae* (XP_001818752); Aaw, *Aspergillus awamori* (BAA92937); Ate, *Aspergillus terreus* (XP_001217493). The identical amino acid residues among six type-A FAEs are marked in *gray*. The three *triangles* indicate the catalytic triad (Ser^{133}, Asp^{194}, His^{247})

typical esterase catalytic triad. All these primary structure features demonstrated that the AuFaeA is a type-A FAE.

Screening and expression of the *P. pastoris* transformants

The *P. pastoris* transformant that can grow well on a YPD plate with high concentration of geneticin G418 may have multiple copies of a heterologous gene, which can lead to the high-level expression of a protein as explained in the manual of the Multi-Copy Pichia Expression Kit (Invitrogen, USA). However, the protein expression level was not directly proportional to the concentration of G418 or the copy number of the heterologous gene [3, 23]. Owing to these reasons, three batches (ten transformants/batch) with gene *AufaeA* separately resistant to 1.0, 2.0, and 4.0 mg/ml of G418 were picked out for flask expression tests. They were labeled as *P. pastoris* GSFaeA1-1 to GSFaeA1-10, GSFaeA2-1 to GSFaeA2-10, and GSFaeA4-1 to GSFaeA4-10, respectively. *P. pastoris* GS115 transformed with pPIC9K vector instead of pPIC9K-*AufaeA*, labeled as *P. pastoris* GSC, was used as the negative control. After all *P. pastoris* transformants were induced by 1.0 % methanol for 72 h, the activities and proteins of the expressed reAuFaeA in the cultured supernatants were assayed. Among these tested transformants, one transformant expressing the highest reAuFaeA activity of 10.76 U/ml (Table 1), labeled as *P. pastoris* GSFaeA4-8, was chosen and used for subsequent studies. No FAE activity was detected in the cultured supernatant of *P. pastoris* GSC under the same expression conditions.

Purification of the expressed reAuFaeA

The yeast *P. pastoris* expression system had many advantages, one of which was that the purity of the expressed recombinant protein was very high according to the description of the Multi-Copy Pichia Expression Kit (Invitrogen, USA). This advantage could greatly facilitate or simplify the purification procedures. It was reported that the purity of the recombinant *Aspergillus sulphureus* β-mannanase expressed in *P. pastoris* X-33 reached 97 % [3]. In this work, the amount of reAuFaeA secreted into the cultured supernatant of GSFaeA4-8 accounted for more than 85 % of that of the total protein (Fig. 2, lane 2), which was assayed by protein band-scanning. Therefore, the expressed reAuFaeA was purified to homogeneity by a simple combination of ammonium sulfate precipitation, ultrafiltration, and Sephadex G-75 gel filtration (Fig. 2, lane 3). The specific activity of the purified reAuFaeA towards 1.0 mM MFA under the standard assay conditions was 44.9 U/mg. Its specific activity was much higher than those of the AoFaeB from *Aspergillus oryzae* (0.57 U/mg) [13], the AwFaeA from *Aspergillus awamori* (9.01 U/mg) [14], and the AnidFAE from *Aspergillus nidulans* (21.7 U/mg) [6].

Table 1 Screening and expression of *P. pastoris* GS115 transformants

G418 (mg/ml)	*P. pastoris* transformants	FAE activity (U/ml)	*P. pastoris* transformants	FAE activity (U/ml)
1.0	GSFaeA1-1	7.39 ± 0.15	GSFaeA1-6	7.89 ± 0.27
	GSFaeA1-2	6.87 ± 0.11	GSFaeA1-7	7.42 ± 0.13
	GSFaeA1-3	7.66 ± 0.21	GSFaeA1-8	8.03 ± 0.26
	GSFaeA1-4	7.72 ± 0.19	GSFaeA1-9	7.54 ± 0.35
	GSFaeA1-5	6.94 ± 0.32	GSFaeA1-10	7.12 ± 0.09
2.0	GSFaeA2-1	8.46 ± 0.25	GSFaeA2-6	9.27 ± 0.18
	GSFaeA2-2	8.59 ± 0.42	GSFaeA2-7	8.96 ± 0.29
	GSFaeA2-3	8.32 ± 0.15	GSFaeA2-8	9.45 ± 0.33
	GSFaeA2-4	9.12 ± 0.23	GSFaeA2-9	8.98 ± 0.21
	GSFaeA2-5	8.63 ± 0.31	GSFaeA2-10	9.25 ± 0.27
4.0	GSFaeA4-1	10.47 ± 0.18	GSFaeA4-6	9.96 ± 0.34
	GSFaeA4-2	10.15 ± 0.23	GSFaeA4-7	9.83 ± 0.47
	GSFaeA4-3	9.77 ± 0.37	GSFaeA4-8	10.76 ± 0.21
	GSFaeA4-4	10.28 ± 0.21	GSFaeA4-9	10.12 ± 0.33
	GSFaeA4-5	9.82 ± 0.13	GSFaeA4-10	9.86 ± 0.19

All values are mean ± SD from three independent experiments

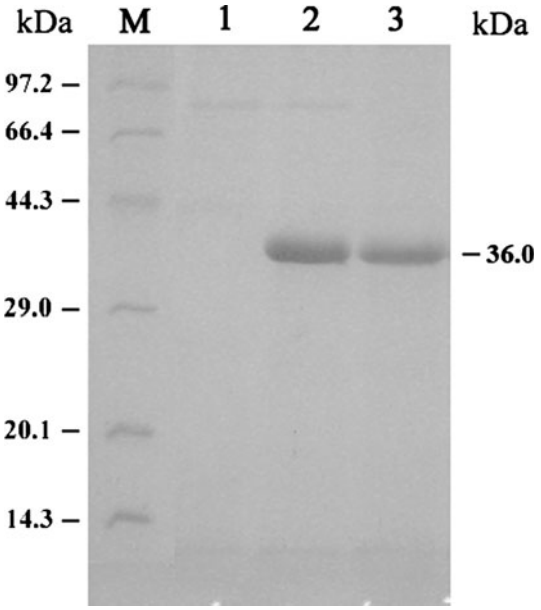

Fig. 2 SDS-PAGE analysis of the cultured supernatants of *P. pastoris* transformants (GSC and GSFaeA4-8) and the purified reAuFaeA. *Lanes M* protein marker, *1* the cultured supernatant of *P. pastoris* GSC, *2* the cultured supernatant of *P. pastoris* GSFae4-8, *3* the reAuFaeA purified by a simple combination of ammonium sulfate precipitation, ultrafiltration, and Sephadex G-75 gel filtration

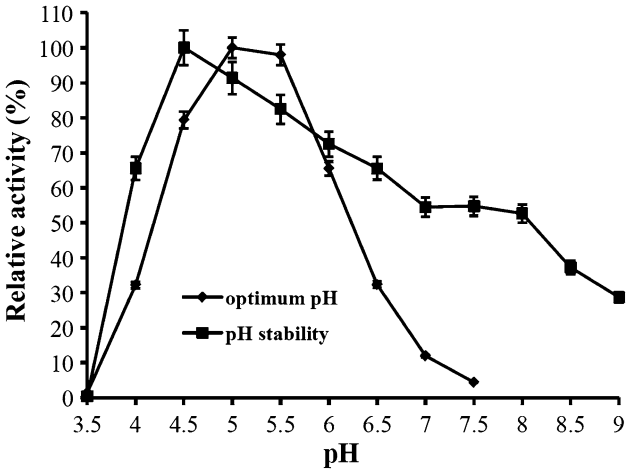

Fig. 3 Effects of pH value on the catalytic activity and stability of reAuFaeA. The optimum pH of the purified reAuFaeA was determined under the standard assay conditions, except with 1.0 mM MFA in 100 mM Na_2HPO_4/citric acid buffer at a pH range of 3.5–7.5. The pH stability of reAuFaeA was estimated by preincubating it at 35 °C and at different pH values (Na_2HPO_4/citric acid buffer, pH 3.5–7.5; Tris–HCl buffer, pH 8.0–9.0) for 1.0 h. The residual reAuFaeA activity was measured under the standard assay conditions. The *error bar* represents the standard deviation (SD) from three independent assays

SDS-PAGE analysis of the purified reAuFaeA displayed one single protein band with an apparent molecular weight of about 36.0 kDa (Fig. 2, lane 3), which was much larger than the theoretical one (28.3 kDa) of AuFaeA. *P. pastoris* enables expressed proteins to conduct some post-translational modifications, such as exclusion of signal peptide, assembly of disulfide bond, and N- and/or O-linked glycosylation. Bioinformatics analytical results demonstrated that there is one putative N-linked glycosylation site in the AuFaeA sequence, which may contribute to the increased molecular weight of reAuFaeA [27].

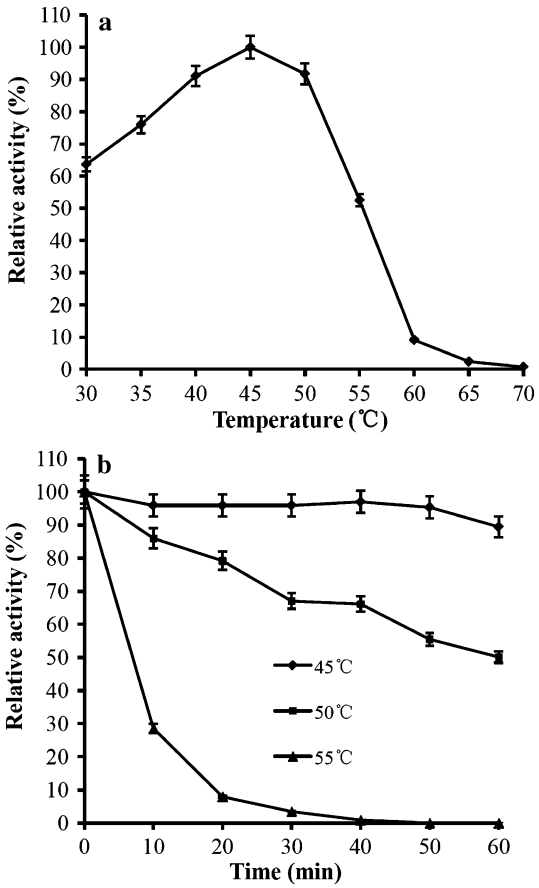

Fig. 4 Effects of temperature on the catalytic activity and thermostability of reAuFaeA. **a** The temperature optimum of reAuFaeA was assayed, at optimum pH and at temperatures ranging from 30 to 70 °C. **b** To evaluate its thermostability, aliquots of reAuFaeA were preincubated in the absence of substrate at 45, 50, and 55 °C for 10, 20, 30, 40, 50, and 60 min. The residual reAuFaeA activity was measured under the standard assay conditions. The *error bar* represents the standard deviation (SD) from three independent assays

Characterization of the purified reAuFaeA

The purified reAuFaeA exhibited higher relative activities at a pH range of 4.5–5.5 (measured at 45 °C). Its optimum pH (100 % relative activity) was at pH 5.0 (Fig. 3), which was equal to that of the AwFaeA from *A. awamori* [14]. Preincubated at 35 °C and at different pH values (3.5–9.0) for 1.0 h, the reAuFaeA was more stable at a pH range of 4.0–6.5, retaining more than 60 % of its original activity (Fig. 3). The optimum temperature (100 % relative activity) of reAuFaeA, measured at pH 5.0, was 45 °C (Fig. 4a), which was higher than those (37 and 30 °C) of the FAEs from *Penicillium expansum* and *Streptomyces olivochromogenes*, respectively [8, 9]. The reAuFaeA was highly thermostable (retaining over 90 % of its activity) when preincubated at 45 °C for 60 min, and retained 79.2 % of its activity at 50 °C for 20 min, but entirely lost its activity after 40 min at 55 °C (Fig. 4b).

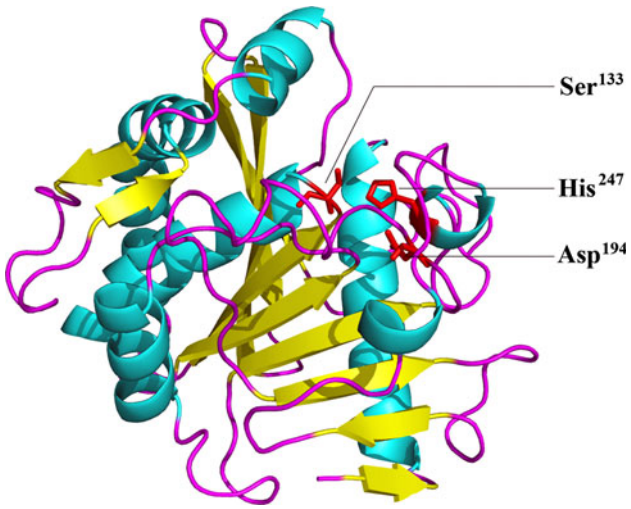

Fig. 5 Three-dimensional (3-D) structure of AuFaeA modeled homologically using the MODELLER 9.9 program and based on the known crystal structure of the *A. niger* type-A FAE (PDB 1USW). The modeled 3-D structure displays a globular shape with a catalytic triad Ser^{133}-Asp^{194}-His^{247}. It is composed mainly of one major nine-stranded β-sheet, two minor two-stranded β-sheets, and seven α-helices

Preincubated with an array of metal ions and EDTA at 35 °C for 1.0 h, the reAuFaeA activity was not significantly affected (less than 20 %) by all ions tested and EDTA (data not shown). The K_m and V_{max} values of reAuFaeA, towards MFA at pH 5.0 and 45 °C, were graphically determined to be 4.64 mM and 115.5 U/mg, respectively. The K_m of reAuFaeA was much lower than that (14.4 mM) of the AnFaeA from *A. niger* [34], which indicated that the reAuFaeA had a stronger substrate affinity.

Modeling and analysis of the 3-D structure of AuFaeA

Based on the known crystal structure of the *A. niger* type-A FAE (PDB code 1USW), which shared 98.5 % identity with AuFaeA in the primary structure, the 3-D structure of AuFaeA was predicted by homolog modeling using the MODELLER 9.9 program as shown in Fig. 5. The modeled 3-D structure displays a globular shape with a catalytic triad Ser^{133}-Asp^{194}-His^{247}. It is composed mainly of one major nine-stranded β-sheet, two minor two-stranded β-sheets, and seven α-helices. Although there were wide differences in their primary structures and biochemical properties, the AuFaeA and most lipases shared the similar core topology of the 3-D structure, known as the α/β-hydrolase fold [23].

Synergistic interaction between reAuFaeA and reAuXyn11A

The amount of free FA released from DSWB, hydrolyzed by reAuFaeA alone with activity ranging from 0 to 140 U,

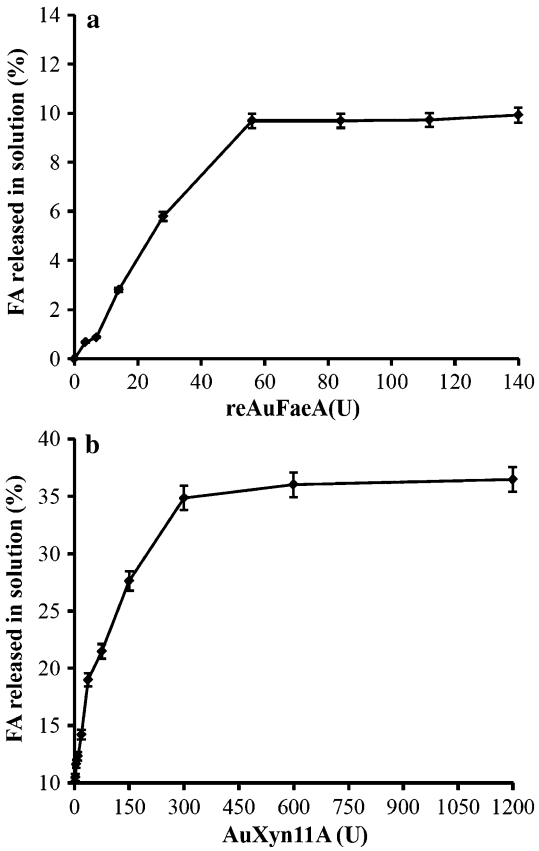

Fig. 6 Effects of activity of reAuFaeA and/or reAuXyn11A on the amount of free FA released from DSWB. **a** The amount of FA released from DSWB by reAuFaeA alone with activity ranging from 0 to 140 U at 40 °C for 10 h. **b** The amount of FA released from DSWB by reAuFaeA (56 U) and reAuXyn11A with activity from 0 to 1,200 at 40 °C for 10 h. The *error bar* represents the standard deviation (SD) from three independent assays

is shown in Fig. 6a. As the reAuFaeA activity rose, the amount of released FA gradually increased and was up to 9.7 % of total alkali-extractable FA at an activity of 56 U, after which no obvious increase was detected. This hydrolytic feature of reAuFaeA was similar to those of the fungal FAEs from *A. niger* [10] and *Fusarium oxysporum* [25].

No trace of FA was liberated from DSWB by reAuXyn11A alone with activity ranging from 0 to 1,200 U (data not shown). However, xylanases could interact synergistically with FAEs to significantly enhance the amount of free FA released from agricultural waste materials as reported previously [13, 22, 24]. In this work, 30-ml aliquots of DSWB suspension (16.7 mg/ml) were mixed with both reAuFaeA (56 U) and reAuXyn11A with different activity from 0 to 1,200 U, and then incubated at 40 °C for 10 h. The amount of FA greatly increased from 9.7 to 34.9 % when reAuXyn11A activity reached 300 U, but hardly increased from 300 to 1,200 U (Fig. 6b). The experimental result indicated that the synergistic interaction between reAuFaeA and reAuXyn11A played an important role in producing FA from DSWB. The produced FA has great potential for applications in the food, cosmetic, and pharmaceutical industries.

Conclusions

In this work, the AuFaeA-encoding cDNA gene (*AufaeA*) was cloned from *A. usamii* E001 and heterologously expressed in *P. pastoris* GS115. The expressed reAuFaeA displayed high specific activity, broad pH stability, and strong resistance to the metal ions tested and EDTA. In addition, the synergistic interaction of reAuFaeA with reAuXyn11A greatly enhanced the amount of FA released from DSWB. All these superior properties will make reAuFaeA a promising candidate for industrial processes. Considering the lower thermostability of reAuFaeA, more attention will be focused on improving its thermostability by genetic engineering, such as increasing the number of salt bridges, addition of a thermostabilizing domain, and introduction of a disulfide bridge.

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