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A novel feruloyl esterase from a soil metagenomic library with tannase activity

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ABSTRACT

A gene (*tan410*) encoding a feruloyl esterase was isolated by screening a cotton soil metagenomic library. Sequence analysis revealed that *tan410* encodes a protein of 520 amino acids with a predicted molecular weight of 55 kDa. The gene was further expressed in *Escherichia coli* BL21 (DE3) using a pET expression system. The recombinant enzyme was purified and characterized. Its optimum temperature and pH were 35 °C and 7.0, respectively. Tan410 activity was enhanced by the addition of Mn^{2+}, Mg^{2+}, NH_4^+ and Ni^{2+}. Besides ethyl ferulate, methyl caffeate, and methyl *p*-coumarate, Tan410 can also hydrolyze methyl gallate, tannic acid, epicatechin gallate, and epigallocatechin gallate which makes Tan410 an interesting enzyme for biotechnological applications.

1. Introduction

Feruloyl esterases (or ferulic acid esterases, FAEs) [E.C. 3.1.1.73] are a subclass of the carboxylic ester hydrolases (E.C. 3.1.1.1) which cleave the ester linkage between polysaccharides and phenolic acids [1]. As they are able to liberate phenolic acids such as ferulic acid and *p*-coumaric acid from naturally occurring hemicelluloses and pectins, FAEs are widely used in the food, feed, and pharmaceutical industries as well as fuel production [2]. On the basis of their amino acid sequence and model substrate specificity, FAEs have been classified into four subclasses, type A, B, C and D [3,4]. Type A is active on methyl ferulate (MFA), methyl *p*-coumarate (M*p*CA), and methyl sinapate (MSA). Type B is specific against MFA, M*p*CA, and methyl caffeate (MCA), but not MSA. Type C and D act on all four hydroxycinnamic acid methyl esters. Type C enzymes do not release diferulic acid (diFAs) from model substrates, whereas type D enzymes are able to hydrolyze dimers [5].

Although many fungi and bacteria have been found to produce FAE such as *Dickeya dadantii*, *Fusarium proliferatum*, *Fusarium oxysporum*, *Burkholderia multivorans*, *Aureobasidium pullulans*, *Aspergillus niger*, *Aspergillus flavipes*, *Aspergillus awamori* and *Aspergillus oryzea* [6–15], and a number of FAE-encoding genes have been reported and cloned from *A. niger*, *Aspergillus tubingensis*, *Pseudoalteromonas haloplanktis* TAC 125, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Clostridium thermocellum* and *Cellulosilyticum ruminicola* [16–22], the properties of FAEs from these microorganisms do not always meet the requirements for a given application. In addition, these culturable microorganisms represent only a small fraction of the microbial diversity [23], which in turn, presumably limits the search for possible novel FAEs that may be useful for the bioprocess industry.

With the metagenomic approach, several novel FAEs have been isolated from leachate and termite gut [24,25]. In this study, a cotton field metagenomic library was constructed for screening FAE genes. A novel gene (*tan410*) encoding FAE was cloned and sequenced. Subsequently, Tan410 was expressed in *E. coli* BL21 (DE3), purified and characterized. The purified FAE had tannase activity, and could hydrolyze substrates such as methyl gallate (MG), tannic acid (TA), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), which indicating an novel FAE with a novel function was isolated from a cotton field metagenomic library.

2. Materials and methods

2.1. Materials and bacterial strains

The compounds assayed in this study including ethyl ferulate (EFA), MCA, MSA, M*p*CA, ferulic acid, coumaric acid, caffeic acid, sinapinic acid and 5-bromo-4-chloro-3-indolyl-β-D-indolylcaprylate (X-caprylate) were purchased from J&K Scientific (Shanghai, China). Bovine serum albumin was purchased from Solarbio (Shanghai, China). MG, TA, ECG, EGCG and gallic acid were purchased from Aladdin (Shanghai, China). *Bam*HI, *Hin*dIII, T4 DNA

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ligase and DNA polymerase were purchased from Takara (Dalian, China). E.Z.N.A® Plasmid Mini Kit and E.Z.N.A® Gel Extraction Kit were purchased from Omega (Norcross, USA). *E. coli* DH5α was used as the host for gene cloning and *E. coli* BL21 (DE3) (Novagen, Madison, USA) for protein expression. pUC118 (Takara, Dalian, China) and pET-28a (+) (Novagen, Madison, USA) were used to construct the metagenomic libraries and express the target protein, respectively.

2.2. Metagenomic library construction and screening of feruloyl esterase activity

DNA from a cotton field sample was extracted based on a method described previously with minor modifications [26]. About 4 g of soil sample was saturated with 13.5 mL of DNA extraction buffer (100 mM Tris–HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB, 2% SDS) and was completely homogenized after shaking at 220 rpm for 30 min. Then the mixture was incubated at 65 °C with mixing every 15–20 min. After 2 h incubation, the supernatants were harvested by centrifugation at 6000 × *g* for 10 min, and then were poured into a new tube containing 15 mL chloroform. After complete mixing, the aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation (16,000 × *g*, 20 min) at 4 °C, washed twice with cold 75% ethanol and suspended in an appropriate volume of sterile deionized water. To purify the crude DNA, low-melting-temperature agarose gels were prepared in 1× TAE buffer. After electrophoresis at 40 V for 10 h, the gel containing DNA fragments ≥20 kb was cut off and the DNA was purified using an E.Z.N.A® Gel Extraction Kit. The isolated DNA was digested with *Bam*HI. DNA fragments of 2.5–10 kb were ligated into *Bam*HI-digested pUC118 and the ligated products were transformed into *E. coli* DH5α. In order to screen for FAE activity, the transformed cells were plated onto Luria-Bertani (LB) agar plates containing 100 mM X-caprylate as a substrate. After incubation at 37 °C for 24 h, clones with blue color were further tested for the ability to hydrolyze EFA. The positive clones were reconfirmed and sub-cloned.

2.3. Sequence and phylogenetic analysis

DNA sequencing was carried out by using an ABI Prism 377 DNA sequencer (Applied Biosystems, Inc.). The deduced amino acid sequence analysis and open reading frame (ORF) search were performed with the BLAST program provided by NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Signatures, p*I*, and MW were analyzed using the EXPASY site (http://www.expasy.ch). Feruloyl esterase sequences for comparative study were retrieved from protein and nucleotide databases on the NCBI Entrez server at http://www.ncbi nlm. nih. gov/Entrez/. Sequence similarity searches were performed with the BLAST 2.0 program [27]. Amino acid sequence alignments of Tan410 with homologous proteins and phylogenetic analyses were performed with the Align X program [28]. DNA restriction analysis was performed with the DNAman5.2 program.

2.4. Subcloning and expression of the recombinant FAE

The positive FAE gene was amplified from the pUC118-*tan410* plasmid using the primers P1 (5′-CGCGGATCCATGCCCGCAAAAACCCGCCT G-3′) and P2 (5′-CCCAAGCTTATTCCCGTTAGTAAAGCCGTC-3′) which contained restriction enzyme sites (underlined) for *Bam*HI and *Hin*dIII. The amplifications were carried out in a total volume of 50 μL. The reaction mixture contained 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 1 unit of *Taq* polymerase, and 3 μL of DNA template. The reaction conditions were 4 min of initial denaturation at 95 °C followed by 30 cycles, 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, 1.5 min of extension at 72 °C and a final extension step of 10 min at 72 °C. Amplified DNA was digested by *Bam*HI/*Hin*dIII, ligated into the *Bam*HI–*Hin*dIII- linearized pET-28a (+), and then introduced into *E. coli* BL21 (DE3) cells. Transformed cells were grown in a 250-mL flask containing 50 mL LB at 37 °C until the cell concentration reached an OD_{600} of 0.7, and then they were induced with 0.5 mM IPTG. After incubation at 25 °C for 12 h with shaking at 220 rpm, cells were harvested by centrifugation at 6000 × *g* for 10 min at 4 °C. The cells were sonicated and the supernatant was collected by centrifugation (16,000 × *g*, 20 min) at 4 °C. The purification of FAE was carried out by using Ni-NTA His-Bind resin column chromatography according to the manufacturer's instructions.

2.5. Protein determination and SDS-page analysis

Protein concentrations were determined according to Bradford's method [29]. Bovine serum albumin (BSA) was used as the standard. SDS-PAGE analysis was carried out according to the method of Laemmli [30]. A 12% separating gel and 5% concentrating gel was used for this study. Gels were stained with the Coomassie brilliant blue (G-250) method. The molecular mass of native protein was determined by gel filtration on a Superose 12HR 5/30 column. Gamma globulin (160 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) were used as the reference proteins. Isoelectric point (p*I*) was estimated by PAGE with 6.25% ampholine (pH 3.5–10) in a gel rod (0.5 cm × 10 cm) using a kit for isoelectric focusing calibration (Pharmacia LKB) according to the recommendations provided by the supplier.

2.6. Enzyme assays

The FAE activity of the enzyme was measured by analyzing the free *p*-nitrophenyl released from *p*-nitrophenyl ferulate. The assay was carried out in 100 mM phosphate buffer (pH 7.0) containing 1 mM *p*-nitrophenyl ferulate at 35 °C for 20 min, and then the liberated free *p*-nitrophenyl was measured at 405 nm. One unit of FAE activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenyl in one minute under specific conditions.

2.7. Effect of pH and temperature on Tan410 activity

Purified enzyme was used to determine the optimum pH and temperature. In order to determine the optimal pH of Tan410 activity, the following buffers were used for the different pH ranges: 100 mM sodium acetate buffer (pH 4.0–6.0); 100 mM phosphate buffer (pH 5.5–8.0); 100 mM Tris–HCl buffer (pH 7.5–9.0); 100 mM glycine–NaOH buffer (pH 8.5–10.0). The effect of temperature on FAE activity was carried out at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C. Tan410 activity assays were performed as described above to determine the optimal pH and temperature. For the thermal stability measurements, the enzyme was incubated in 100 mM phosphate buffer (pH 7.0) at 30, 40, 50 and 60 °C for 15 min, 30 min, 1 h, 2 h, 4 h and 13 h. After incubation, the residual activity was measured as described above. Each experiment was done twice and measured in triplicate, and the standard deviation was less than 1% of the mean.

2.8. Effect of different chemicals on Tan410 activity

Various chemicals ($ZnSO_4$, $MgSO_4$, $(NH4)_2SO_4$, $NiSO_4$, $AlCl_3$, $CaCl_2$, $CuCl_2$, $MnCl_2$, $FeCl_3$, EDTA and urea) at final concentrations

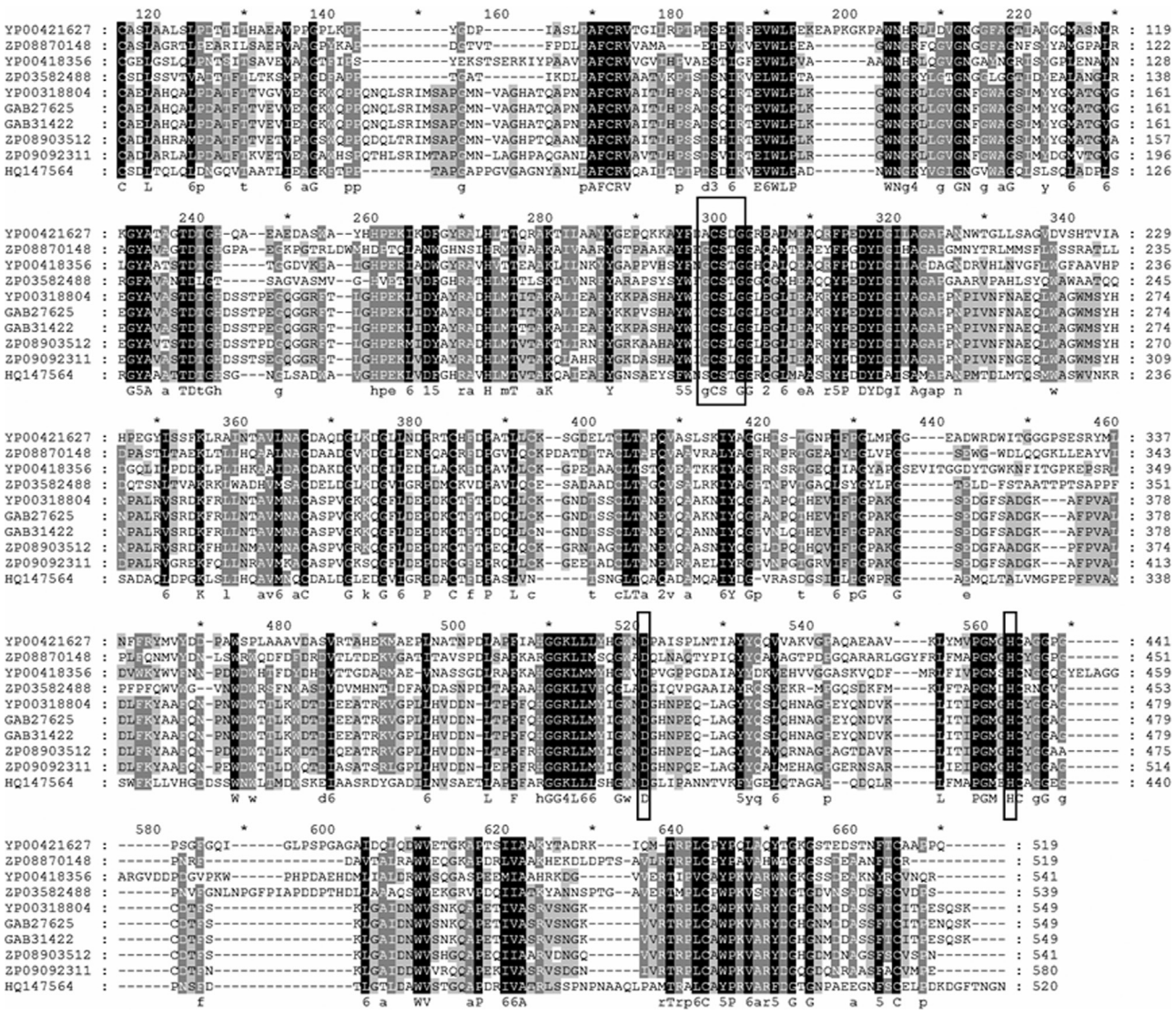

Fig. 1. Sequence alignment of Tan410 with other FAEs. Residues identical to the consensus are shaded. The aligned sequences are from the following organisms: feruloyl esterase (ZP09092311) from *Gluconobacter morbifer* G707, feruloyl esterase (ZP08903512) from *Gluconacetobacter europaeus* LMG18494, feruloyl esterase (GAB31422) from *Acetobacter pasteurianus* sp. LMG1262, feruloyl esterase (GAB27625) from *Acetobacter pasteurianus* NBRC101655, feruloyl esterase (YP003188040) from *Acetobacter pasteurianus* IFO3283-01, tannase (ZP03582488) from *Burkholderia multivorans* CGD1, tannase and feruloyl esterase (YP004183569) from *Terriglobus saanensis* SP1PR4, tannase and feruloyl esterase (ZP08870148) from *Azospirillum amazonense* Y2, tannase and feruloyl esterase (YP004216272) from *Granulicella tundricola*.

of 10 mM were added to the enzyme in 100 mM phosphate buffer (pH 7.0). The enzyme activity assays were measured under standard conditions after pre-incubation at 35 °C for 20 min. The Tan410 activity obtained in the absence of different additives was defined as the 100% level. Each experiment was done twice and measured in triplicate, and the standard deviation was less than 1% of the mean.

2.9. HPLC analysis of Tan410 on different substrates

The purified enzyme was tested for its ability to hydrolyze EFA, MCA, MSA, M*p*CA, MG, TA, ECG and EGCG by high performance liquid chromatography (HPLC). The enzyme sample (25 μg) with 0.5 mg/mL substrate in 100 mM phosphate buffer (pH 7.0) was incubated at 35 °C for 40 min. Aliquots (0.5 μL) of the reaction mixtures were loaded onto a HPLC system (HP1100, Hewlett Packard, USA) equipped with a 250 mm × 4.6 mm Diamonsil C18 column (Merck, Germany). The solvents were acetonitrile and phosphate buffer (20 mM, pH 3.5) (6:4, v/v). The flow was 0.8 mL/min. Signals were detected at 320 nm for ferulic acid and 280 nm for gallic acid.

2.10. Release of ferulic acid from crude wheat bran by Tan410

To measure Tan410 activity on natural substrate, the purified enzyme (25 μg) in the presence of 20 mg/mL wheat bran in 1 mL 100 mM phosphate buffer (pH 7.0) was incubated at 35 °C for 12 h. A total of 10 U xylanase from *Thermomyces lanuginosus* (Sigma, USA) was added into the reaction mixture to promote Tan410 catalysis. The obtained ferulic acid was quantitated by HPLC using the detection conditions as described above.

2.11. Nucleotide sequence accession number

The nucleotide sequence reported in this study has been submitted to GenBank under the accession number HQ147564.

3. Results

3.1. Screening the FAE from the metagenomic library

For this study, a previously constructed metagenomic library from a cotton field sample was used for screening FAE genes. The library consisted of approximately 92,000 clones with insert sizes ranging from 2.5 to 5.0 kb. Out of these clones, one clone with FAE activity was identified by its bright blue color. The insert DNA of the positive clone was sequenced. The sequence analysis revealed the presence of an ORF consisting of 1563 bp, encoding a protein of 520 amino acids with a predicted molecular mass of 54.88 kDa, which was belonged to the tannase superfamily. The amino acid alignment of Tan410 with the most homologous FAEs is presented in Fig. 1. The conserved active site motif of the pentapeptide GxSxG in most serine hydrolases with a serine acting as catalytic nucleophile was converted to SxSxG in Tan410. The deduced amino acid sequence of Tan410 was used to perform a BLAST search of the NCBI and SwissProt databases. This search revealed a relatively high similarity between Tan410 and other FAEs/tannases, including a FAE (GAB27625) from *Acetobacter pasteurianus* NBRC 101655 (42% identity), a FAE (GAB31422) from *Acetobacter pasteurianus* LMG 1262 (40% identity), a FAE (YP003188040) from *Acetobacter pasteurianus* IFO 3283-01 (39% identity), a tannase and feruloyl esterase (YP004216272) from *Granulicella tundricola* (39% identity), and a tannase (ZP03582488) from *Burkholderia multivorans* CGD1 (37% identity).

3.2. Expression of the recombinant Tan410 and SDS-PAGE analysis

For expression of the gene in *E. coli* BL21 (DE3), *tan410* was cloned into pET-28a (+) under the control of T7 *lac* promoter with an N-terminal 6×His tag. About 1 mg of Tan410 was purified from a 10 mL culture using affinity purification with the Ni-NTA resin. The molecular mass of the purified enzyme estimated by SDS-PAGE analysis was approximately 55 kDa (Fig. 2). The relative molecular mass of the native enzyme estimated by gel filtration chromatography was also about 55 kDa. Thus, it is assumed that Tan410 is a monomeric protein. The p*I* value was estimated to be 4.7.

3.3. Temperature and pH effect on Tan410 activity

The optimum activity of Tan410 was measured over the pH range of 4.0–10.0 and the temperature range of 20–70 °C with *p*-nitrophenyl ferulate as the substrate. As shown in Fig. 3, Tan410 showed its highest activity (100%) at pH 7.0, and the enzyme activity remained high when the pH was between 5.5 and 8.0. However, when the pH was outside of this range, Tan 410 had low activity. When the pH was below 4, the activity was completely lost. The effect of temperature on the activity of Tan410 is shown in Fig. 4. Tan410 displayed maximal activity at 35 °C and maintained high activity over the temperature range of 25–45 °C. The temperature stability study of Tan410 revealed that the enzyme was stable below 40 °C and it retained more than 90% of its total activity after incubation at 40 °C for 13 h. However, when the temperature increased, the enzyme activity decreased; it remained about 56% and 20% of total activity after pre-incubation for 13 h at 50 °C and 60 °C, respectively (Fig. 5).

3.4. Effect of different chemicals on Tan410 activity

The effect of various chemicals on Tan410 activity was examined by pre-incubating the enzyme with chemicals in 100 mM phosphate buffer (pH 7.0) for 20 min at 35 °C and then measuring its residual activity toward *p*-nitrophenyl ferulate as the substrate.

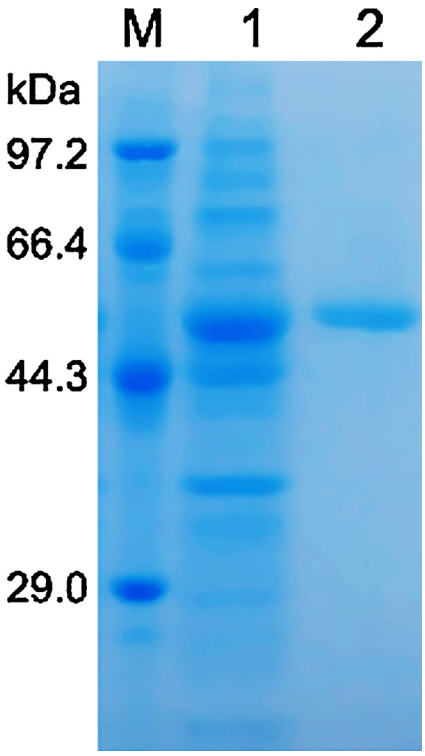

Fig. 2. SDS-PAGE analysis of recombinant Tan410. M, marker proteins; lane 1, unpurified Tan410; lane 2, purified Tan410.

The results (Table 1) showed that 10 mM Mn^{2+}, Mg^{2+}, NH_4^+ and Ni^{2+}enhanced Tan410 activity to 113.9%, 104.3%, 114.3% and 109.7%, respectively. Ca^{2+} and urea slightly inhibited Tan410 activity. The activity of Tan410 was decreased to 57.6%, 40.5%, 24.8%, 42.8% and 48.2% in the presence of 10 mM Zn^{2+}, Al^{3+}, Cu^{2+}, Fe^{3+} and EDTA, respectively.

3.5. HPLC analysis of Tan410 activity on different substrates

To examine the substrate specificities of the purified Tan410, EFA, M*p*CA, MCA and MSA were tested as substrates for feruloyl esterase activities. The tannase activity of the enzyme was quantitated by measuring the gallic acid released from MG, TA, ECG and EGCG. As shown in Fig. 6, all of the EFA, M*p*CA, MCA TA, ECG, EGCG

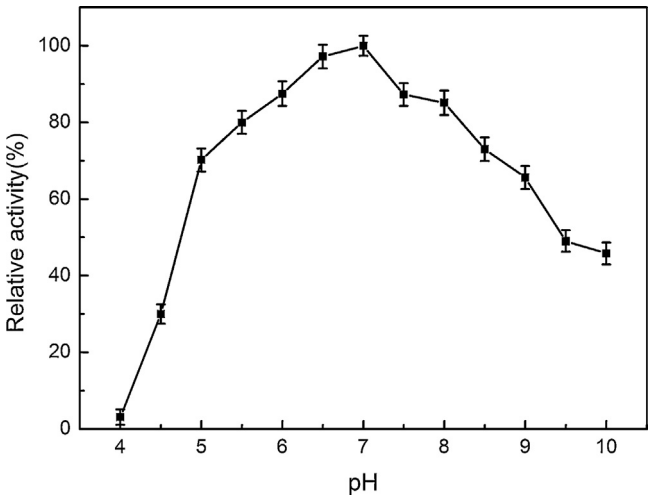

Fig. 3. Effect of pH on the activity of Tan410.

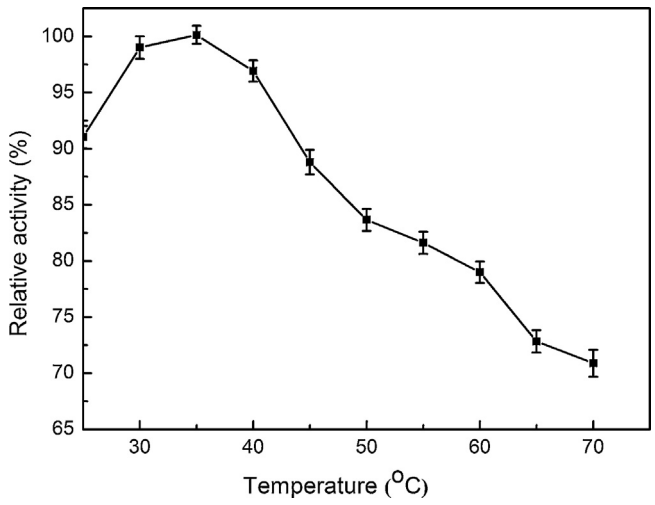

Fig. 4. Effect of temperature on the activity of Tan410.

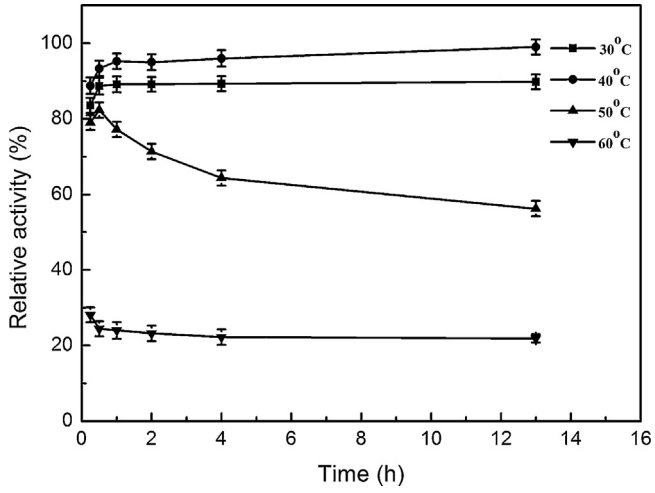

Fig. 5. Effect of temperature on the stability of Tan410.

and 46% of the MG were hydrolyzed within 40 min. However, MSA was not hydrolyzed in 40 min. These results indicated that Tan410 may be a new member of type B FAEs.

3.6. Release of ferulic acid from crude wheat bran

The purified Tan410 was tested for the release of ferulic acid present in wheat bran. In the absence of xylanase, Tan410 did not release ferulic acid from wheat bran. When the reaction was supplemented with xylanase, about 42% of ferulic acid was released from wheat bran, indicating a synergistic reactivity of the two enzymes.

Table 1
Effects of different chemicals on Tan410 activity.

Chemical (10 mM)	Relative activity(%)
Control	100
$ZnSO_4$	57.6
$MgSO_4$	104.3
$(NH4)_2SO_4$	114.3
$NiSO_4$	109.7
$AlCl_3$	40.5
$CaCl_2$	91.4
$CuCl_2$	24.8
$MnCl_2$	113.9
$FeCl_3$	42.8
EDTA	48.2
urea	93.8

4. Discussion

Metagenomics has been effectively used to isolate novel biocatalysts from the environment [31–33]. Its scale and scope have been expanded since it was first introduced [34]. In this work, a metagenomic library was constructed by using the vector pUC118, and a gene encoding feruloyl esterase was isolated from a cotton field environmental sample by functional screening. Analysis of the nucleotide and amino acid sequence revealed that *tan410* was a novel gene. Like most genes screened from metagenomic libraries, no information about a source microorganism could be obtained.

The gene *tan410* from the metagenomic library was cloned and expressed in *E. coli*, and its product was purified for characterization. As a monomeric 55 kDa protein, the molecular mass of Tan410 was higher than those of the analogous enzymes from *Lactobacillus acidophilus* (36 kDa) [35], *A. awamori* (31 kDa) [36], *Clostridium stercorarium* (33 kDa) [37] and *S. olivochromogenes* (29 kDa) [38], but was lower than that of the analogous enzyme from *A. niger* (75 kDa) [39]. The optimum pH of 7.0 for Tan410 activity was similar to that reported for an analogous enzyme from a termite gut metagenomic library (pH 7.0) [24], but higher than that recorded from *Lactobacillus acidophilus* (pH 5.6) [35]. The optimum temperature of 35 °C was comparable to that recorded for the enzyme from *Lactobacillus acidophilus* (37 °C) [35], but lower than that from *A. niger* (50 °C) [39]. It was reported that the FAE from the termite gut metagenomic library remained stable below 45 °C, and the enzyme activity was quickly lost when the temperature was higher than 50 °C [24]. The FAE from *Lactobacillus acidophilus* [35] was reported to be stable below 37 °C, and as much as 68% of its activity was lost after incubation at 45 °C for 5 min. However, the recombinant Tan410 retained more than 50% of its activity after incubation at 50 °C for 13 h. The thermostable Tan410 should prove beneficial for food industry applications.

The effects of metal ions on recombinant Tan410 activity were studied. NH_4^+, Ni^{2+} and Mn^{2+} had a stimulatory effect on Tan410 activity, indicating that these metals acted as cofactors to increase the catalytic activity of the enzyme. The inhibition of Tan410 activity by Zn^{2+}, Cu^{2+} and Fe^{3+} was consistent with the properties of FAE from *Clostridium stercorarium* [37] while for the FAE from *Penicillium expansum*, Zn^{2+} just slightly inhibited its activity [40]. However, Zn^{2+}, Cu^{2+} did not influence the activity of FAE from *Fromaspergillus awamor*i G-2 [41]. EDTA inhibited about 51.8% of Tan410 activity under the assay conditions. Kanauchi et al. reported that EDTA had no effect on FAE from *Fromaspergillus awamor*i G-2 [41]. The presence of Ca^{2+} slightly modified Tan410 activity which was consistent with the data reported for the FAEs from *Lactobacillus acidophilus* [35] and *Penicillium expansum* [40].

Tan 410 can hydrolyze EFA, M*p*CA and MCA which were used to determine the enzyme's feruloyl esterase activity, and can also hydrolyze MG, TA, ECG and EGCG which were used to determine its tannase activity. Its tannase activity was different to that of FAE from other microorganisms which were not reported to hydrolyze MG, TA, ECG and EGCG. To our knowledge, this is the first report of a FAE which can hydrolyze MG, TA, ECG and EGCG. Tannin acid, ECG, EGCG, caffeic acid and ferulic acid are widely spread in the plant kingdom. For example, EGCG, the major constituent of tea catechins, accounts for approximately 40% of the total catechin content in green tea [42]. A high concentration of ferulic acid was also found in several agroindustrial by-products. The ability to hydrolyze MG, TA, ECG, EGCG, EFA, M*p*CA, MCA and release ferulic acid from crude wheat bran is an important feature of Tan410. And this feature made Tan410 potential use in the instant tea industry,

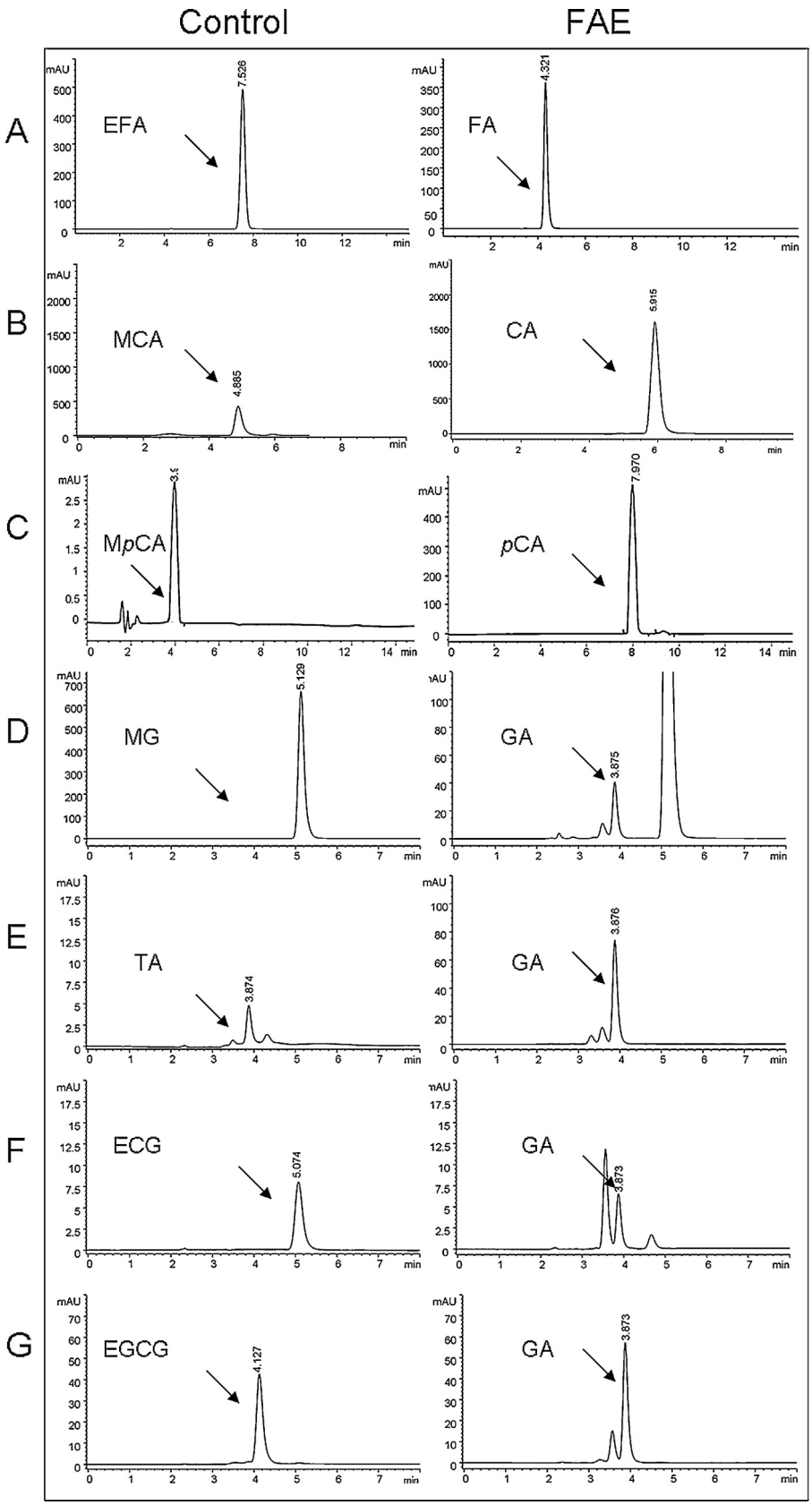

Fig. 6. HPLC analysis of different substrates hydrolyzed by Tan410. A, B, C, D, E, F and G stand for the chromatography of the reaction products obtained for the hydrolysis of EFA, MCA, M*p*CA, MG, TA, ECG and EGCG by inactive enzyme (control) and active enzyme, respectively.

for degradation of agroindustrial wastes and production of bioactive compounds such as caffeic acid, ferulic acid and gallic acid which have various biotechnological applications.

5. Conclusions

A novel gene encoding FAE was successfully isolated from a cotton field metagenomic library by functional screening and expressed in *E. coli* BL21 (DE3). The recombinant enzyme exhibited moderate thermostability and was able to hydrolyze MG, TA, ECG, EGCG, EFA, M*p*CA and MCA. Taken together, these properties make Tan410 an interesting candidate for biological applications.

Acknowledgements

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