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ARTICLE

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Isolation and Characterization of a Novel Tannase from a Metagenomic Library

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Supporting Information

ABSTRACT: A novel gene (designated as *tan410*) encoding tannase was isolated from a cotton field metagenomic library by functional screening. Sequence analysis revealed that *tan410* encoded a protein of 521 amino acids. SDS−PAGE and gel filtration chromatography analysis of purified tannase suggested that Tan410 was a monomeric enzyme with a molecular mass of 55 kDa. The optimum temperature and pH of Tan410 were 30 °C and 6.4. The activity was enhanced by addition of Ca^{2+}, Mg^{2+} and Cd^{2+}. In addition, Tan410 was stable in the presence of 4 M NaCl. Chlorogenic acid, rosmarinic acid, ethyl ferulate, tannic acid, epicatechin gallate and epigallocathchin gallate were efficiently hydrolyzed by recombinant tannase. All of these excellent properties make Tan410 an interesting enzyme for biotechnological application.

KEYWORDS: tannase, metagenome, tannin, gallic acid, chlorogenic acid

INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of the ester bonds in hydrolyzable tannins to release gallic acid and glucose. It is widely applied as clarifying agent in the manufacturing of instant tea, beer, fruit juices and some wines.[1,2] It is also used to treat tannin-polluting industrial effluents and agricultural wastes.[3] In addition, tannase plays an important role in the production of gallic acid,[4] which is an important substrate to synthesize antibacterial drugs and propyl gallate.

During the last two decades, some bacterial strains have been reported to possess tannase activity such as *Pseudomonas aeruginosa* isolated from tannery soil,[5] *Streptococcus* spp. from koala feces,[6] *Citrobacter* sp. from tannin rich environment,[7] *Enterobacter* sp. from soil,[8] *Staphylococcus lugdunensis* from human feces,[9] *Bacillus cereus* from soil[10] and a number of *Lactobacillus* strains from animal feces and fermented foods.[11−13] However, the bacterial tannase genes were only cloned from *Enterobacter* sp.,[8] *Staphylococcus lugdunensis*[9] and *Lactobacillus plantarum*.[14,15] To date, all tannases reported were isolated by the tranditional screening technique, and the disadvantage of this approach is that it only focuses on the culturable microorganisms in the environment. In fact, it is estimated that more than 99% of microorganisms are not cultivable.[16] Therefore the unculturable microorganisms are not an accessible resource for discovering novel tannases. The metagenomic strategy could be applied to screen for biocatalysts with novel characteristics for biotechnological applications without requiring the cultivation of microorganisms.[17] Various industrial biocatalysts such as lipase/esterase,[18,19] amylase[20] and protease[21] have been isolated from metagenomic libraries.

In this study, a cotton field metagenomic library was constructed for screening tannase genes. The gene encoding tannase was cloned and sequenced. Subsequently, Tan410 was expressed in *Escherichia coli* BL21 (DE3), purified and characterized. To our knowledge, this is the first report on tannase gene isolated from unculturable bacteria.

MATERIALS AND METHODS

Bacterial Strains and Materials. *E. coli* DH5α was used as the host for gene cloning and *E. coli* BL21 (DE3) (Novagen, Madison, USA) for protein expression. The pUC118 (TaKaRa, Dalian, China) and pET-28a (+) (Novagen, Madison, WI, USA) were used to construct metagenomic libraries and express the target protein, respectively. *E. coli* transformants were grown at 37 °C in Luria−Bertani (LB) broth with appropriate antibiotics. *Bam*HI, *Hin*dIII, T4 DNA 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). Methyl gallate (MG), tannic acid (TA), epicatechin gallate (ECG) epigallocatechin gallate (EGCG), chlorogenic acid (CGA), rosmarinic acid (RA), ethyl ferulate (EFA), ferulic acid (FA), gallic acid (GA) and caffeic acid (CA) were purchased from Aladdin (Shanghai, China).

DNA Extraction from Environmental Samples. For the construction of a metagenomic library, an environmental sample was collected from a cotton field. The total DNA was extracted based on a method described previously[22] with minor modification. Soil sample (4 g of wet weight) was mixed 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) and 1.5 mL of 20% SDS. The mixture was incubated in a 65 °C water bath for 2 h with gentle inversion every 15 to 20 min. The supernatants were collected after centrifugation (6000*g*, 10 min) at room temperature and transferred into 50 mL centrifuge tubes. An equal volume of chloroform was added and gently mixed. 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 (16000*g*, 20 min) at 4 °C, washed twice with cold 75% ethanol and suspended in an appropriate volume of sterile deionized water.

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Construction of a Metagenomic Library and Screening of Tannase Genes. To construct the metagenomic library, the purified DNA was partially 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. The transformed cells were plated onto LB agar plates containing 50 μg/mL ampicillin, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μM 5-bromo-4-chloro-3-indolyl caprylate (X-caprylate). After incubation at 37 °C for 24 h, clones with blue color were selected. Then clones with blue color were further tested for the ability to hydrolyze propyl gallate. Only one transformant with tannase activity was obtained and reconfirmed. Then the recombinant plasmid (pUC118-*tan410*) was sequenced on ABI 377 DNA sequencer.

Sequencing and Analysis of Tannase Gene. DNA sequencing was performed using a BigDye sequencing kit and ABI 377 DNA sequencer. The deduced amino acid sequence analysis and open reading frame search were performed with BLAST program provided by NCBI. The phylogenetic tree was constructed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis 3.1 software (MEGA, version 3.1).

Expression and Purification of the Recombinant Tannase. The putative tannase gene was amplified by PCR with the pUC118-*tan410* as template using primers P1 (5′-CGCGGATCCATGCCCG-CAAAAACCCGCCT G-3′) and P2 (5′-CCCAAGCTTATTCCCGT-TAGTAAAGCCGTC-3′) which contained restriction enzyme sites (underlined) for *Bam*HI and *Hin*dIII. Amplified DNA was digested by *Bam*HI/*Hin*dIII, ligated into pET-28a (+) which was linearized by *Bam*HI/*Hin*dIII, then transformed into *E. coli* BL21 (DE3) cells. *E. coli* cells transformed with this plasmid were plated onto LB agar containing 50 μg/mL kanamycin. Transformed cells were grown in a 250 mL flask containing 50 mL of LB (50 μg/mL kanamycin) at 37 °C until the cell concentration reached OD_{600} of 0.7, then induced with 0.5 mM IPTG. After incubation at 25 °C for 12 h with shaking at 220 rpm, cells were harvested by centrifugation (6000*g*, 10 min) at 4 °C and suspended in binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 7.9). The cells were disrupted by sonication, and the supernatant was collected by centrifugation (13000*g*, 10 min) at 4 °C. The sample was loaded onto a Ni-NTA His·Bind column pre-equilibrated with binding buffer. Then the column was washed with binding buffer and washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl, pH 7.9). Finally, bound protein was eluted with eluting buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The fractions containing the recombinant protein were collected and stored at −20 °C.

Determination of Molecular Mass of Tan410. The molecular mass of denatured protein was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Proteins were stained with Coomassie brilliant blue G-250. The molecular mass of native protein was determined by gel filtration on a Superose 12HR 5/30 column. Gamma globulin (160,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da) and carbonic anhydrase (30,000 Da) were used as the reference proteins.

Analysis of Tannase Activity. Tannase activity was determined according to the method described previously[23] with minor modification. The reaction mixture containing 25 μg of purified tannase and 200 μL of 30 mM propyl gallate in 100 mM phosphate buffer (pH 6.4) was incubated at 30 °C for 5 min. Then 200 μL of a methanolic rhodanine solution (0.667% w/v rhodanine in 100% methanol) was added to the mixture. After incubation at 30 °C for 5 min, 200 μL of 0.5 M KOH was added and the mixture was incubated for another 5 min at 30 °C. Then 1.4 mL of distilled water was added to dilute the mixture. After an additional incubation of 5 min at 30 °C, the absorbance was measured at 520 nm using a spectrophotometer. One unit of tannase activity was defined as the amount of enzyme required to release 1 μmol of gallic acid in 1 min under specific conditions.

Effect of pH and Temperature on Tannase Activity. The optimum pH of tannase was determined in a pH range of 4.0 to 11.0. The following buffers were used: 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) and Na_2HPO_4−NaOH buffer (pH 10.0−11.0). The pH stability was determined by incubating the purified enzyme at 30 °C for 0.25 h, 0.5 h, 1 h, 2 h, 4 and 12 h in the above different buffers. The optimum temperature was determined by measuring the tannase activity in pH 6.4 at a temperature range of 20 to 60 °C. The thermostability of tannase was investigated by incubating the enzyme in 100 mM phosphate buffer (pH 6.4) at 25, 35, 45, 50, and 55 °C for 0.25 h, 0.5 h, 1 h, 2 h, 4 and 12 h. Then the residual activity was measured as described above.

Effects of Various Chemicals on Tannase Activity. The effects of various metal ions ($CaCl_2$, $MgCl_2$, $MnCl_2$, $CuCl_2$, $ZnSO_4$, $CdCl_2$, $AlCl_3$, $CoCl_2$, $AgNO_3$, $Cr(NO_3)_2$ and $Hg(NO_3)_2$), Triton X-100, Tween 80, EDTA, urea, β-mercaptoethanol and phenylmethylsulfonyl fluoride (PMSF) on tannase activity were investigated. The enzyme was incubated with 1 mM chemicals at 30 °C for 15 min. Then the residual activity was assayed under standard conditions.

Salt Tolerance of Tannase. The enzyme was incubated with various concentrations of NaCl (1 to 5 M) at 30 °C for 24 h. Then the residual activity was measured under standard conditions.

High Performance Liquid Chromatography Analysis. Tan410 was tested for hydrolysis of methyl gallate, tannin acid, ECG, EGCG, chlorogenic acid, rosmarinic acid and ethyl ferulate by high performance liquid chromatography (HPLC). The enzyme samples (25 μg) with 0.5 mg/mL substrates in 100 mM phosphate buffer (pH 6.4) were incubated at 30 °C for 40 min. Aliquots (0.5 μL) of the reaction mixtures were loaded onto a HPLC system (HP1100, Hewlett-Packard, USA) using a 250 × 4.6 mm Diamonsil C18 column (Merck, Germany). The solvents were acetonitrile and phosphate buffer (0.02 M, pH 3.5) (6:4, v/v). The flow was 0.8 mL/min. Signals were detected at 260 nm for gallic acid and 320 nm for caffeic acid and ferulic acid.

Nucleotide Sequence Accession Number. The nucleotide sequence reported in this study has been submitted to the GeneBank data under the accession number HQ147564.

RESULTS

Construction and Screening of the Metagenomic Library. A metagenomic library containing about 92,000 clones was constructed for obtaining tannase genes. Its quality was analyzed by restriction digestion of plasmids from 22 randomly chosen clones. Restriction analysis showed that the inserted DNA fragments of these selected clones ranged from 2.5 to 5 kb with distinct restriction patterns, and the average insert size of these clones was estimated to be about 3.5 kb. The metagenomic library represented about 332 Mb of soil microbial genomic DNA. Out of approximately 92,000 clones, one positive clone with tannase activity was isolated.

Sequence Analysis of Tannase. The complete insert DNA sequence of pUC118-*tan410* was determined. The length of the insert DNA was 5,356 bp. BLAST analysis revealed the presence of an open reading frame consisting of 1,563 bp, encoding a full-length tannase gene (*tan410*). The gene *tan410* encoded a protein of 521 amino acids with a predicted molecular mass of 54.88 kDa. The deduced amino acid sequence of Tan410 was used to perform a BLAST research of the NCBI and SwissProt databases. This search revealed that Tan410 belonged to the tannase superfamily and it did not match perfectly with any known proteins in the databases, suggesting that Tan410 is a novel enzyme originating from a microorganism that has not

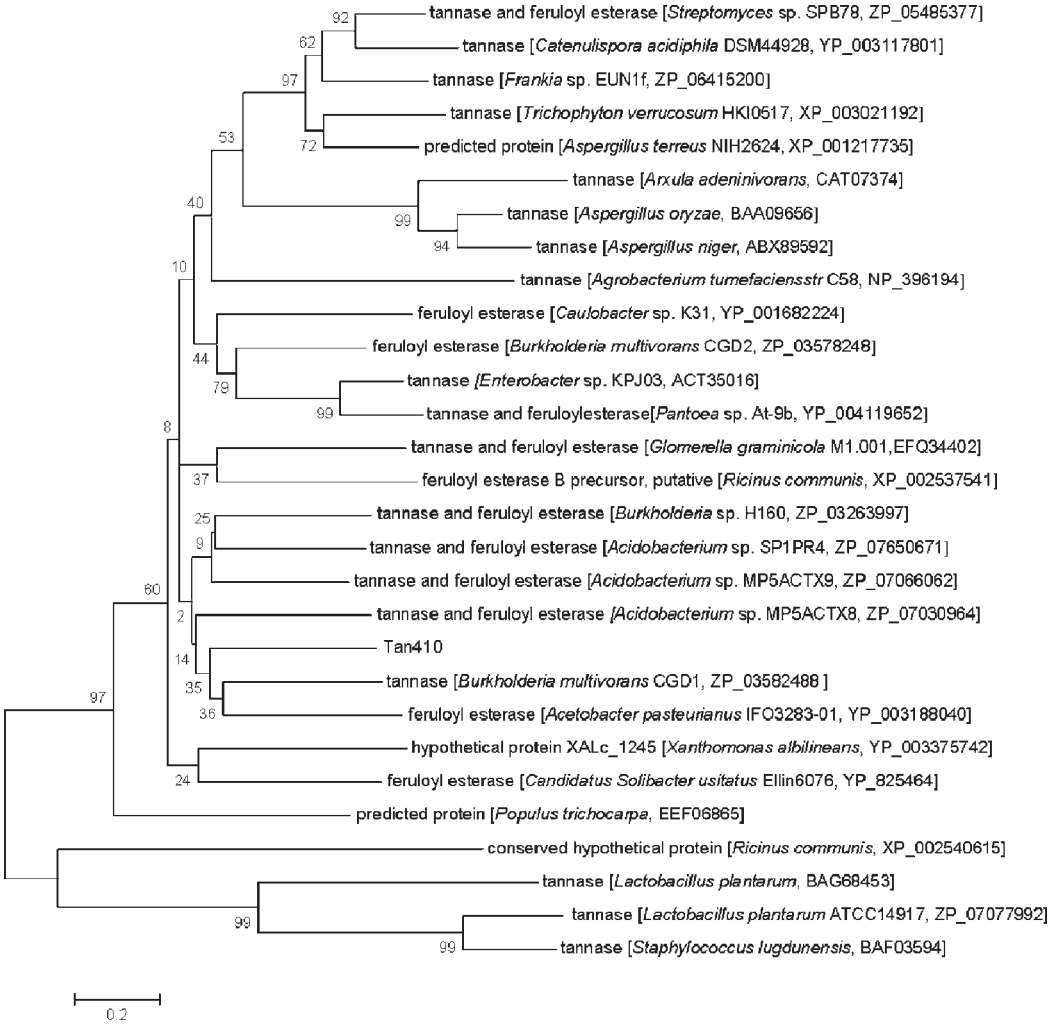

Figure 1. Phylogenetic tree analysis of tannase superfamily homologous to Tan410 by neighbor joining method.

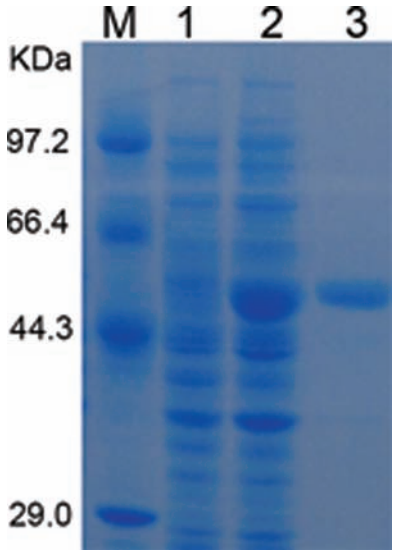

Figure 2. SDS−PAGE analysis of the purified recombinant Tan410. M, marker proteins; lane 1, extracts of IPTG-induced *E. coli* BL21 (DE3) (pET-28a (+)); lane 2, unpurified Tan410; lane 3, purified Tan410.

been identified or even cultured. The phylogenetic tree based on amino acid sequence was constructed to verify the evolutionary relationship of the Tan410 to other known tannase/feruloyl esterase, and 28 tannase/feruloyl esterase proteins were selected for the phylogenetic tree analysis. As shown in Figure 1, Tan410 is not closely related to other members of tannase superfamily, suggesting that Tan410 is a new member of tannase superfamily.

Expression and Purification of the Recombinant Tan410. To characterize the biochemical properties of Tan410, *tan410* was expressed as an N-terminal His-tag fusion protein using pET-28a (+) expression system under the control of T7 *lac* promoter in *E .coli* BL21 (DE3). The cells were harvested and disrupted by sonication on ice. No inclusion bodies were found in cell lysates, which suggested that the recombinant Tan410 was expressed in a soluble form. After purification with the Ni-NTA column, the molecular mass of the purified enzyme estimated by SDS−PAGE analysis was approximately 55 kDa (Figure 2). The relative molecular mass of native enzyme estimated by gel filtration chromatography was about 55 kDa. Thus, it is assumed that Tan410 is a monomeric protein.

Effect of pH and Temperature on Activity and Stability of Tan410. The effect of pH and temperature on tannase activity was measured at a pH range of 4.0 to 11.0 and a temperature range of 20 to 60 °C with propyl gallate as the substrate. Tan410

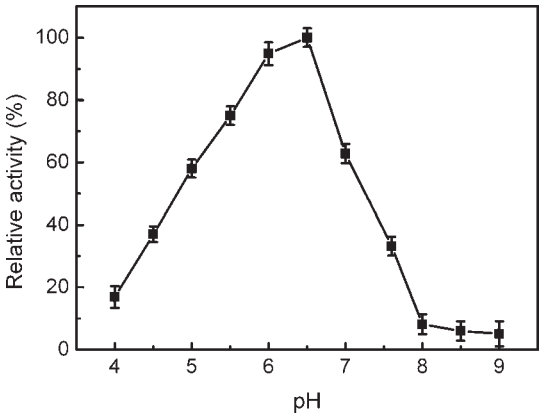

Figure 3. Effect of pH on activity of Tan410.

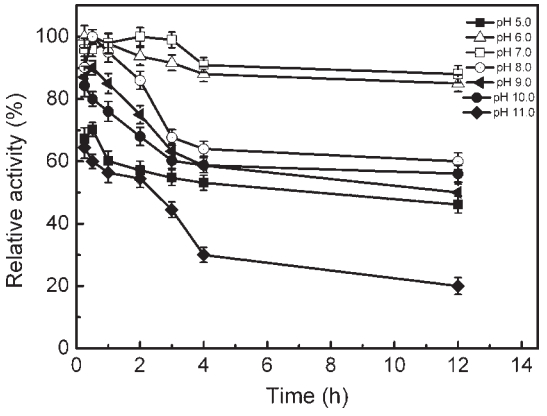

Figure 4. Effect of pH on the stability of Tan410.

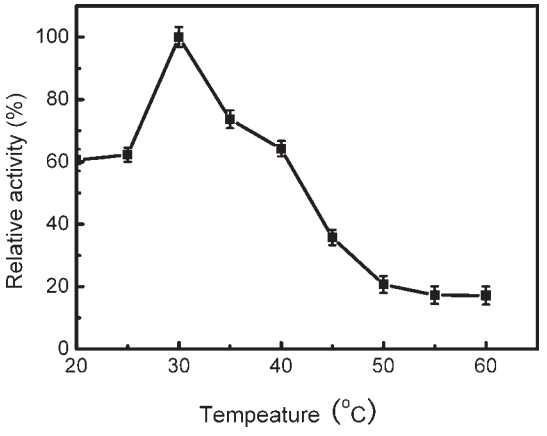

Figure 5. Effect of temperature on activityof Tan410.

showed its highest activity at pH 6.4 (Figure 3), and the enzyme was stable at a pH range of 6.0−7.0 (Figure 4). The optimum temperature of Tan410 was 30 °C (Figure 5). The enzyme was stable below 40 °C, as after prolonged incubation time it did not show obvious loss of activity under the standard conditions. However, its activity was drastically reduced over 40 °C. It kept only 47% of its activity after incubation at 50 °C for 12 h (Figure 6).

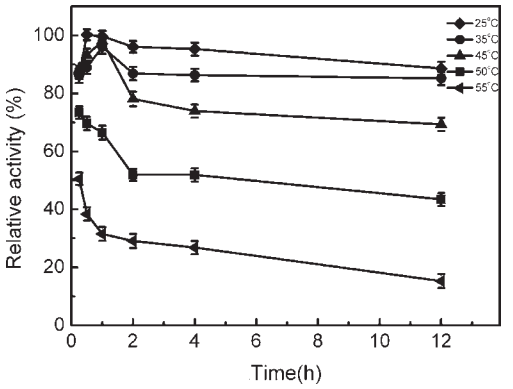

Figure 6. Effect of temperature on the stability of Tan410.

Table 1. Effects of Various Chemicals on Relative Activity of Recombinant Tannase

chemical (1 mM)	rel act. (%)
control	100
$CaCl_2$	126
$MgCl_2$	120
$MnCl_2$	90
$CuCl_2$	108
$ZnSO_4$	100
$AlCl_3$	97
$CdCl_2$	121
$CoCl_2$	100
$AgNO_3$	51
$Hg(NO_3)_2$	0
$Cr(NO_3)_2$	52
EDTA	86
urea	106
β-mercaptoethanol	63
PMSF	32
Triton X-100	100
Tween 80	47

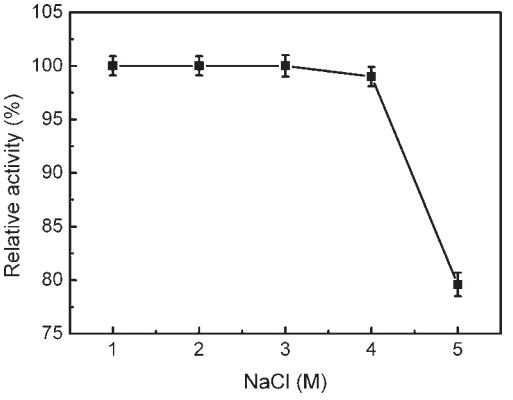

Figure 7. The stability of Tan410 at various concentrations of NaCl.

Effects of Different Chemicals on Tan410 Activity. The effects of various chemicals on Tan410 activity were examined by preincubating the enzyme with 1 mM chemicals at 30 °C for

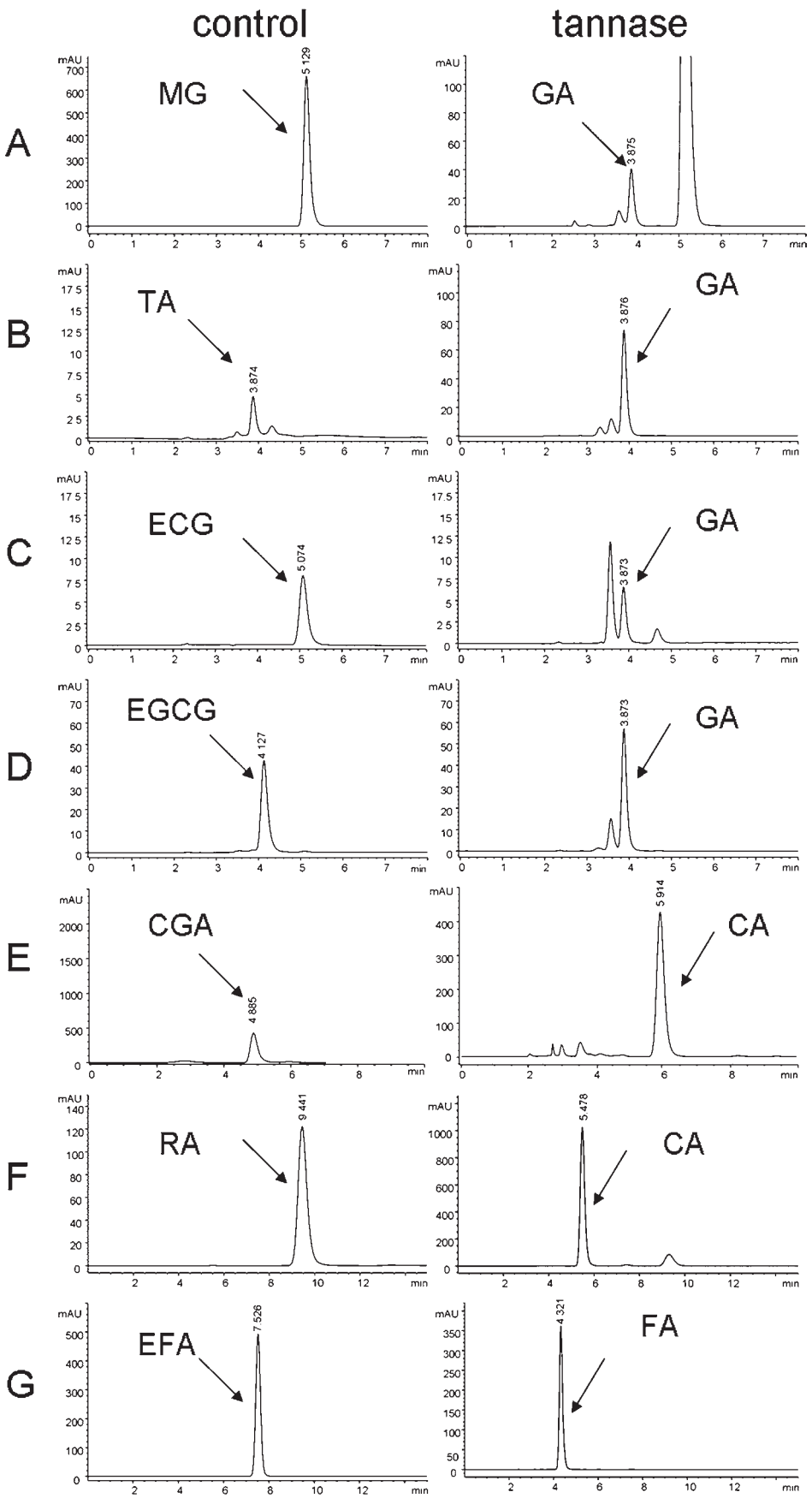

Figure 8. HPLC analysis of different substrates hydrolyzed by Tan410. A, B, C, D, E, F and G stand for the hydrolysis chromatography of MG, TA, ECG, EGCG, CGA RA and EFA by inactive enzyme (control) and active enzyme, respectively.

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15 min. The results (Table 1) showed that Ca^{2+}, Cd^{2+} and Mg^{2+} increased the Tan410 activity to 126%, 121% and 120%, respectively. Tan410 activity was slightly affected by Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Al^{3+}, EDTA, urea and Triton X-100. The activity of Tan410 was decreased to 51%, 52%, 63%, 47% and 32% by the presence of Ag^{+}, Cr^{2+}, β-mercaptoethanol, Tween 80 and PMSF, respectively. Hg^{2+} completely inhibited the activity of Tan410.

Tan410 remained stable in the presence of 4 M NaCl and retained 80% of its activity at 5 M NaCl (Figure 7), suggesting that Tan410 was a halotolerant enzyme.

Hydrolysis of Different Substrates. Tan410 was tested for hydrolysis of different substrates using HPLC. Figure 8 showed that tannic acid, ECG, EGCG, ethyl ferulate and chlorogenic acid were totally hydrolyzed within 40 min. In the same time, 88% of the rosmarinic acid and 46% of methyl gallate were hydrolyzed. This indicated that Tan410 showed higher affinity for tannins, ethyl ferulate, chlorogenic acid and rosmarinic acid than methyl gallate.

DISCUSSION

It is well-known that the soil is one of the main reservoirs of the microbial diversity on the planet, providing a vast resource for discovering biocatalysts. However, more than 99% of the microorganisms in the soil cannot be cultured.[16] It limits the discovery of novel biocatalysts. To overcome this limitation, metagenomics can be an alternative to access to the unculturable microorganisms. In this study, we constructed a metagenomic library with the sample from cotton field to explore novel tannase. Out of approximately 92,000 clones, one positive clone containing a tannase gene was isolated. The analysis of the nucleotide and amino acid sequence revealed that it was a novel gene. Like most other genes isolated from environmental metagenome libraries, no information about a source microorganism of this gene could be found. The reason for low yield of new tannase genes obtained from metagenomic libraries was attributed to different factors such as library construction (sources of DNA, method used for DNA extraction and purification, average size of insert metagenomic DNA and number of clones), difficulties of expression in heterologous host (toxicity of expression protein to the host, the stability and quantities of the expression protein in host), the formation of inclusion bodies and the choice of host organism. This suggests that discovery of novel tannases from the metagenome is technology challenging.[24]

The gene *tan410* from metagenomic library was cloned and expressed in *E. coli*, and its product was purified to be characterized. As a monomeric 55 kDa protein, Tan410 was different from some known tannases which were generally multimeric proteins with high molecular masses ranging from 186 to 300 kDa.[25−28] Optimum pH of 6.4 for Tan410 activity was similar to that reported from *Lactobacillus plantarum* (pH 7.0),[15] higher than that recorded from *Lactobacillus plantarum* CECT 748^{T} (pH 5.0)[29] and lower than that recorded from *Serratia ficaria* DTC (pH 8.9).[30] The optimum temperature of 30 °C was comparable to that recorded from *Bacillus cereus* KBR9, *Lactobacillus plantarum* CECT 748^{T} and *Serratia ficaria* DTC (30−40 °C).[10,29,30] Tannase from *Lactobacillus plantarum* CECT 748^{T} was reported to be stable below 37 °C and no activity was detected after incubation at 45 °C for 4 h.[29] It was reported that tannase from *Bacillus cereus* was stable below 30 °C and its activity was lost at 50 °C for 60 min.[10] Curiel et al. reported that recombinant *Lactobacillus plantarum* tannase showed less than 20% of its activity after incubation at 37 °C for 3 h.[15] However, recombinant Tan410 retained close to 50% of its activity after incubation at 50 °C for 12 h, and the thermostability of the enzyme was beneficial for application in the food industry.

The effects of some metal ions, chelators or enzyme inhibitors on the recombinant Tan410 activity were studied. Ca^{2+}, Mg^{2+} and Cd^{2+} had a stimulatory effect on tannase activity, indicating that these metals acted as cofactors to increase the catalytic activity of the enzyme.[29] The enzyme activity was completely inhibited by Hg^{2+}, suggesting that thiol may be involved in the active catalytic site.[29] Triton X-100 and urea just slightly affected Tan410 activity at the concentration tested. EDTA inhibited about 14% of Tan410 activity under the assay conditions. However, Kasieczka et al. reported that EDTA had a strong inhibitory effect on tannase from *Verticillium* sp. P9.[31] Tan410 activity was strongly inhibited by 1 mM PMSF, suggesting that Tan410 was a serine hydrolase which needed a serine residue for its catalytic function; this property of Tan410 was similar to that reported from *Arxula adeninivorans*.[24,32] In addition, Tan410 was stable in the presence of 4 M NaCl, whereas tannases from *Bacillus cereus* and *Aspergillus aculeatus* were only stable below 2 M NaCl and 3 M NaCl, respectively.[10,33] This halotolerant property made Tan410 useful for the treatment of tannery wastewater.

Tan410 not only released gallic acid from tannic acid, ECG and EGCG, but also released ferulic acid from ethyl ferulate and caffeic acid from chlorogenic acid and rosmarinic acid (Figure 8 and Figure S1in the Supporting Information). This property was different from that of tannase from *Lactobacillus plantarum*, which could not hydrolyze chlorogenic acid, rosmarinic acid and ethyl ferulate.[15] To our knowledge, this is the first report about tannase which could hydrolyze ethyl ferulate, rosmarinic acid and chlorogenic acid. Tannins, ferulic acid, rosmarinic acid and chlorogenic acid are widely spread in the plant kingdom. For example, green tea contains 1−2% ECG and 5−8% EGCG. EGCG is the major constituent of tea catechins accounting for approximately 40% of the total catechins content in green tea.[34] High concentrations of chlorogenic acid, rosmarinic acid and ferulic acid were also found in several agroindustrial byproducts.[35] The ability to hydrolyze tannins, rosmarinic acid, ethyl ferulate and chlorogenic acid is an important feature of Tan410 which can further be used in the industry of instant tea, degradation of agroindustrial wastes and production of bioactive compounds such as caffeic acid, ferulic acid and gallic acid which have various biotechnological applications.

In conclusion, a novel tannase gene was successfully isolated from a metagenomic library with the sample from cotton field by functional screening and expressed in *E. coli*. The recombinant Tan410 was purified and characterized. The enzyme exhibited halotolerance and moderate thermostability. It was able to hydrolyze chlorogenic acid, rosmarinic acid, ethyl ferulate, ECG, EGCG and tannin acid. All of these properties made Tan410 a potential candidate for biological applications. Further studies of directed evolution, three-dimensional structure and kinetics are expected to explain the uniqueness of Tan410, and the comparison of Tan410 with purified commercial fungal tannase (e.g., from *Aspergillus oryzae*) is necessary to evaluate the novel tannase's industrial application.

ASSOCIATED CONTENT

Supporting Information. Figure S1 depicting HPLC analysis of commercial GA, CA and FA. This material is available free of charge via the Internet at http://pubs.acs.org.

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