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A Protease-Insensitive Feruloyl Esterase from China Holstein Cow Rumen Metagenomic Library: Expression, Characterization, and Utilization in Ferulic Acid Release from Wheat Straw

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ABSTRACT: A metagenomic library of China Holstein cow rumen microbes was constructed and screened for novel gene cluster. A novel feruloyl esterase (FAE) gene was identified with a length of 789 bp and encoded a protein displaying 56% identity to known esterase sequences. The gene was functionally expressed in *Escherichia coli* BL21 (DE3), and the total molecular weight of the recombined protein was 32.4 kDa. The purified enzyme showed a broad specificity against the four methyl esters of hydroxycinnamic acids and high activity (259.5 U/mg) to methyl ferulate at optimum conditions (pH 8.0, 40 °C). High thermal and pH stability were also observed. Moreover, the enzyme showed broad resistance to proteases. FAE-SH1 can enhance the release of ferulic acid from wheat straw with cellulase, β-1,4-endoxylanase, β-1,3-glucanase, and pectase. These features suggest FAE-SH1 as a good candidate to enhance biomass degradation and improve the health effects of food and forage.

KEYWORDS: *feruloyl esterase, metagenomic library, ferulic acid, cow rumen microorganism, wheat straw, protease resistant*

■ INTRODUCTION

Agricultural wastes such as rice straw, wheat straw, corn stover, and sugar cane bagasse are abundant, renewable, and carbon-neutral resources, but they have not been utilized in environmentally friendly and efficient ways.[1] These biomaterials are mainly composed of cellulose, hemicelluloses, and lignin, which are intermeshed and chemically bonded through covalent cross-linkages. This cross-link has been demonstrated to be a natural barrier for biomass degradation and utilization.[2]

Ferulic acid (FA) attached to polysaccharides has been suggested as a linkage between polysaccharides and lignin in plant cell wall.[3−5] Feruloyl esterases (FAEs; EC 3.1.1.73) are a class of enzymes involved in catalyzing the ester bonds between polysaccharide and FA. This property makes the FAEs key tools to degrade, modify, and elucidate the structures in plant cell walls.[6,7] In addition, these enzymes are important to de-esterify dietary fibers, releasing hydroxycinnamates and derivatives, which may have positive health effects.[8] This property makes these enzymes potentially suitable for wide applications in biomass degradation related industries such as the animal feed, pulp, textile, fuel, food, and pharmaceutical industries. Several microbial FAEs have been discovered, characterized, and overexpressed in the past three decades.[5,9] Nevertheless, knowledge about these enzymes is limited, and few FAEs are used in industry. Identification, cloning, and utilization of novel FAEs in industry remain hot topics.[6]

The complex microbiome of the rumen functions as an effective system in degrading plant cell walls in nature, which makes it the ideal environment for microbes that possess FAE activities.[10] However, few FAEs have been reported in rumen.[11] Also, because no more than 1% of microbes present in environments can be cultured,[12] screening of novel FAEs via traditional cultivation techniques is technically limited.[13] This limitation may be conquered by metagenomics, a culture-independent strategy that provides access to valuable genetic resources of the uncultured microbes by directly extracting the total DNA from their natural environments.[10] Thus, the metagenomics become a powerful tool to discover novel biocatalysts in rumen.[14]

In this study, a fosmid metagenomic library of China Holstein cow rumen microbes was constructed. A new gene cluster was discovered, and a novel FAE gene (*FAE-SH1*) was subcloned and functionally expressed in *Escherichia coli* BL21 (DE3). Characterization studies of the purified FAE-SH1 were performed. Synergistic effects of cellulase, β-1,3-glucanase, β-1,4-endoxylanase, and pectase with FAE-SH1 were also observed.

■ MATERIALS AND METHODS

Strains, Vectors, and Enzymes. *E. coli* EPI300 and pcc2FOS (Epicenter, USA) were used as the host and vector for metagenomic library construction, respectively, whereas *E. coli* BL21 (DE3) and pET30a (+) (Novagen, USA) were used for protein production. Restriction enzymes, T4 DNA ligase, and DNA polymerase were purchased from Takara, Japan. All clones were replicated in Luria−Bertani (LB) medium and stored at −80 °C with 30% v/v glycerol.

Proteinase K, α-chymotrypsin, pepsin, trypsin, papain, and collagenase (pH 7.5, 37 °C) were purchased from Biodee (Beijing, China). Commercial enzymes including cellulase, β-1,3-glucanase, β-1,4-endoxylanase, and pectase were supplied by Ankesd (Shandong, China) for wheat straw enzymatic hydrolysis. Standard chemicals were obtained from J&K Chemical, China.

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Total DNA Purification, Library Construction, and FAE Screening. The methods of sampling and total environmental DNA extraction were followed as described.[15] *Hin*d III digested DNA fragments around 30−50 kb were electroeluted by pulse field gel electrophoresis (PFGE), linked to the pcc2FOS vector, and transformed to *E. coli* EPI300.

To screen the clones exhibiting FAEs, a function-driven screening method was used. Chloramphenicol (10 mg/L, dissolved in 70% v/v ethanol) and ethyl ferulate (10% w/v, dissolved in dimethyl formamide (DMF)) were filtration-sterilized and added to LB agar plates at the plate-pouring stage. A homogeneous haze formed when the agar plates cooled. The transformants were then inoculated on the LB−ethyl ferulate plates and cultured at 37 °C for 24 h. The formation of a clear zone around the inoculation point indicated FAE production.[16]

One positive clone was sequenced on the basis of shotgun sequencing technology by Beijing Genomic Institute. The putative FAE, named FAE-SH1 in this study, was deposited in the GenBank database (accession no. HQ338149).

Gene Annotation and Analysis. The open reading frame (ORF) was analyzed by using the online software ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf). Multiple alignments of catalytic domains of FAEs were performed using the ClustalX program. Sequence similarities were analyzed on the Basic Local Alignment Search Tool (BLAST) online server, and several programs were used accordingly (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenic tree was generated by MEGA4 software (http://www.megasoftware.net/). The amino acid sequences of esterases most similar to the cloned FAEs were retrieved from the GenBank database. Molecular weight (MW), isoelectric point (p*I*), and protein sequence translation were performed using ExPASy proteomics server tools (http://expasy.org/tools/). Protein conserved domain analyses were performed on the Conserved Domains database (CCD, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml); signal peptide analysis was conducted on the online SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). To classify the FAE-SH1, the putative protein sequence was submitted on the server (http://faeclassification.webs.com), which was based on the analysis of amino acid sequence descriptors of experimentally verified and putative FAEs.[17]

Recombined Expression and Purification. The putative FAE was amplified by PCR using the primers as follows: MFaeF/*Bam*HI, 5′-CGCGGATCCGCGATGGCAAGACTGCAGATTG-3′ and MFaeR/*Xho*I, 5′-CCGCTCGAGCGGGCCGAACATCCATTCCACGAC-3′ (the restriction sites are underlined). The purified PCR products were subcloned into pET30a(+) expression vector, which was double digested with *Bam*HI and *Xho*I. His-tag was conjugated for further enzyme purification.

Crude enzyme production and harvest were performed as described.[18] The recombined enzyme was then purified using Ni Sepharose 6 Fast Flow (GE Healthcare, USA) according to the manufacturer's instructions, and the proteins were eluted with a linear imidazole gradient concentration (30−100 mM). The purified enzyme samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). MW was determined on the basis of relative mobility in SDS-PAGE gels according to the standard curve generated from marker proteins.

Enzyme Activity Assay and Characterization of FAE-SH1. FAE activity was determined by analyzing the released FA from methyl ferulate (MFA). The assay was performed at 40 °C for 30 min in 3-(*N*-morpholino)propanesulfonic acid (MOPS)−NaOH buffer (pH 8.0) containing appropriately diluted enzyme and 0.2 mM MFA as the substrate. The liberated FA was analyzed by high-performance liquid chromatography (HPLC) using an Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA). Detection was carried out at 320 nm, and FA was quantified using calibration curves prepared using standard FA (Sigma, USA). A constant elution was used with 65% methanol and 35% water (containing 2.5% acetic acid) at a flow rate of 1.0 mL/min. One unit of FAE activity was defined as the amount of enzyme that liberated 1 μmol of FA per minute.[19] The protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) as standard. Hydrolysis specificities to methyl *p*-coumarate (M*p*CA), methyl caffeate (MCA), and methyl sinapinate (MSA) of FAE-SH1 were also determined. In addition, K_m and k_{cat} of the four individual substrates were tested.

The optimal pH was determined by measuring the enzyme activities in different buffers: pH 2.0−8.0, 50 mM Na_2HPO_4−citric acid buffer; pH 3.0−6.0, 50 mM sodium citrate buffer; pH 7.0−9.0, 50 mM Tris-HCl buffer; pH 9.0−10.0, 50 mM glycine−NaOH buffer. For pH stability determination, the enzyme was kept under different pH buffers at 4 °C for 3 h, and the residual enzyme activities were measured under optimum conditions (pH 8.0, 40 °C). The optimal temperature was determined by measuring the enzyme activity at different temperatures. For thermostability determination, the enzyme was incubated at 40−60 °C, and the residual enzyme activities were measured at the optimum conditions at 1 h intervals.

The purified enzyme was preincubated with different reagents (20 mM) for 30 min at 4 °C, and the residual enzyme activities were assayed according to the method above. Several kinds of protease (1 mg/mL), including proteinase K (pH 7.5, 37 °C), α-chymotrypsin (pH 8.0, 25 °C), pepsin (pH 2.0, 37 °C), trypsin (pH 8.0, 37 °C), papain (pH 7.0, 37 °C), and collagenase (pH 7.5, 37 °C), were used to determine the resistance to proteases. After reaction in respective temperature and pH conditions for 30 min, the residual activities of purified FAE-SH1 were determined under optimum conditions (pH 8.0, 40 °C). For the control sample, the purified FAE-SH1 was incubated in BSA solution.

Release of FA from Wheat Straw. Wheat straw, sampled immediately after harvest, was dried for 8 h in 50 °C and ground in a laboratory mill to fine powder by passing a 250 μm sieve. Total FA was prepared using an alkaline lysis method.[20] One thousand unit doses of each cellulase, β-1,3-glucanase, β-1,4-endoxylanase, and pectase, were inoculated to the 2.0 g wheat straw powder solely or combined with 30 U of FAE-SH1. The reaction was performed in Tris-HCl buffer (pH 8.0) at 40 °C for 3 h. FA in the aqueous assay was performed as above.

RESULTS

Metagenomic Library Evaluation and FAE Screening. A fosmid metagenomic library derived from total microorganisms of China Holstein cow rumen was constructed in this study. A *Not*I cut of the recombined plasmid showed that the average insert size was 32 kb. The library consisted of around 30000 clones and covered around 0.96 Gbp genomic DNA. All of the clones were screened on LB−ethyl ferulate agar. A total of 157 clones were screened in plates and all confirmed with FAE activity using activity assay. One clone exhibiting the highest activity was fully sequenced for further studies.

Analyses of FAE-SH1 DNA and Protein Sequence. A 789 bp DNA fragment, encoding 262 amino acids, was identified as a putative esterase gene (FAE-SH1) by ORF analysis. Its predicted MW and p*I* were 29.5 kDa and 5.76, respectively. A BLASTp analysis of the amino acid sequence against the nonredundant (nr) protein sequence database demonstrated that the putative esterase exhibited the highest identity (56%) to the predicted esterase from *Eubacterium siraeum* V10Sc8a (GenBank accession no. CBL34630) and 55% identity to the predicted esterase from *E. siraeum* (GenBank accession no. CBK96609).

The translated amino acid sequence was further analyzed using the CCD to verify the protein functions. CCD search showed that hydrolase superfamily, carboxylesterase superfamily, lipase superfamily, and esterase family domains were observed in FAE-SH1 primary amino acid sequence. SignalP program results showed that there was no predicted signal peptide available (data not shown).

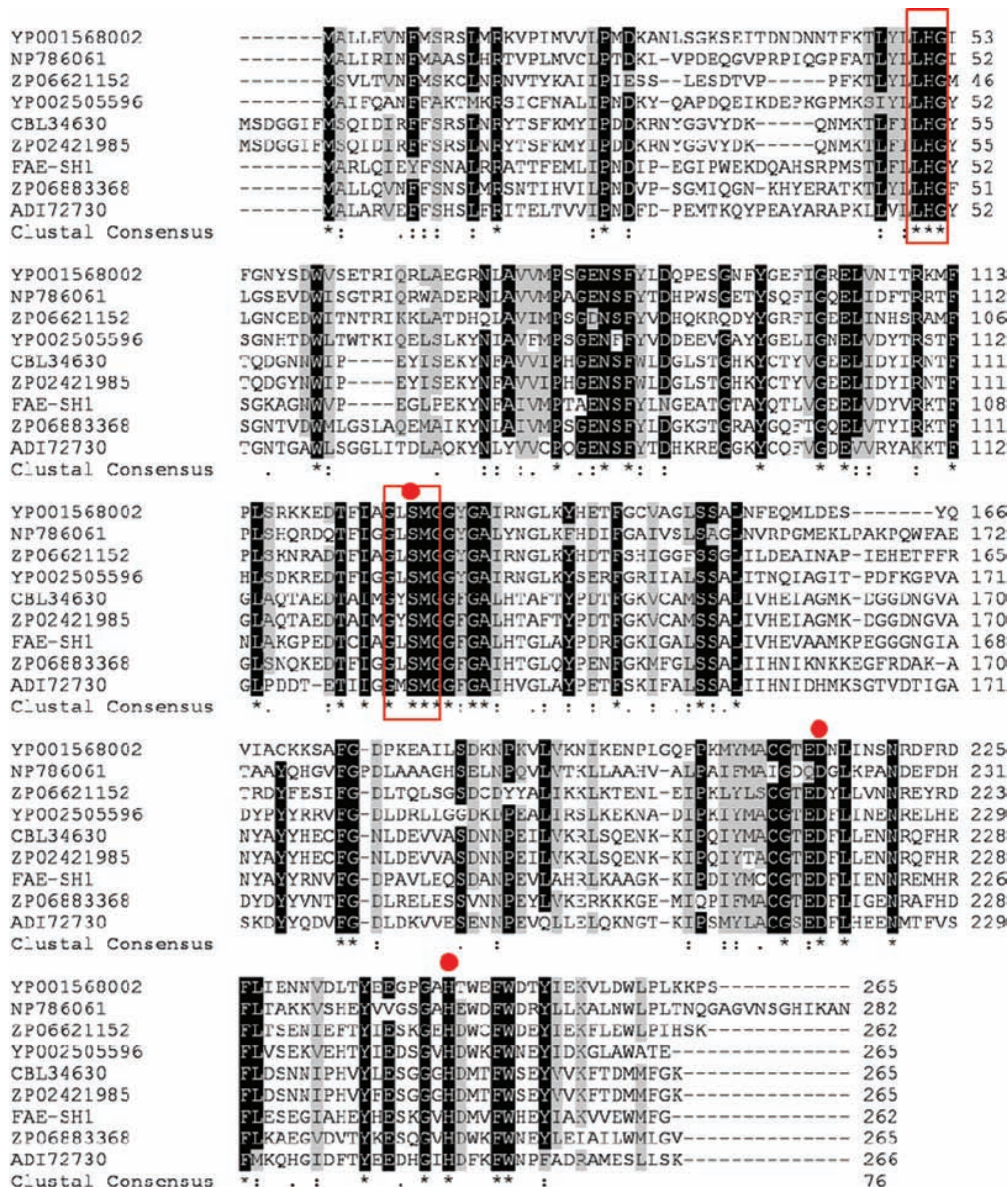

Figure 1. Multialignment of the FAE-SH1 with the most similar sequences. Identical residues are shaded, and the same amino acid residues are marked by an asterisk (*). Active sites of the FAE-SH1 are marked with dots and rectangles. ADI72730, *Lactobacillus buchneri* ferulic acid esterase; CBL34630, *E. siraeum* V10Sc8a predicted esterase; NP786061, *Lactobacillus plantarum* WCFS1 acetylesterase; YP001568002, *Petrotoga mobiliz* SJ95 putative esterase; YP002505596, *Clostridium cellulolyticum* H10 putative esterase; ZP02421985, *E. siraeum* DSM 15702 hypothetical protein; ZP06621152, *Turicibacter* sp. PC909 tributyrin esterase family protein; ZP06883368, *Clostridium lentocellum* DSM 5427 putative esterase.

The multiple alignments data of the translated FAE-SH1 amino acid sequence with the most similar sequence are shown in Figure 1, and the identical residues are highlighted. The results showed that the G-x-S-M-G sequence (amino acid positions 121−127) corresponded with a classical pentapeptide signature G-x-S-x-G motif. The serine (S, 123) residue in this motif may form the catalytic triad of typical esterase together with aspartate (D, 215) and histidine (H, 244) residues.[21] A putative oxyanion hole LHG (amino acid positions 49−51) was also discovered on the upstream sites, which was also conserved and might be involved in the stabilization of intermediates formed during the ester hydrolysis.[22−24]

Phylogenic Relationship Studies of the FAE-SH1. A phylogenetic tree was constructed using FAE-SH1 protein sequence (underlined in Figure 2) with 25 classified FAEs (indicated with an ellipse in Figure 2). The results indicated that FAE-SH1 was most similar to the type E FAEs.[25] Udatha et al.[16] classified FAEs into 12 proposed families. According to the online server analysis, the FAE-SH1 sequence showed features that were most similar to the FEF1 family and subfamily 1B.

Overexpression and Purification of FAE-SH1. To analyze the biochemical properties of FAE-SH1, the gene was subcloned in pET30a(+) vector and expressed in *E. coli* BL21 (DE3). The recombinant and empty host strains were separately inoculated on the LB−ethyl ferulate agar plates using isopropyl *β*-D-1-thiogalactopyranoside (IPTG) as the inducer. A clearer and larger halo was found around the recombinant colonies compared with the empty host strains. SDS-PAGE analysis also verified that

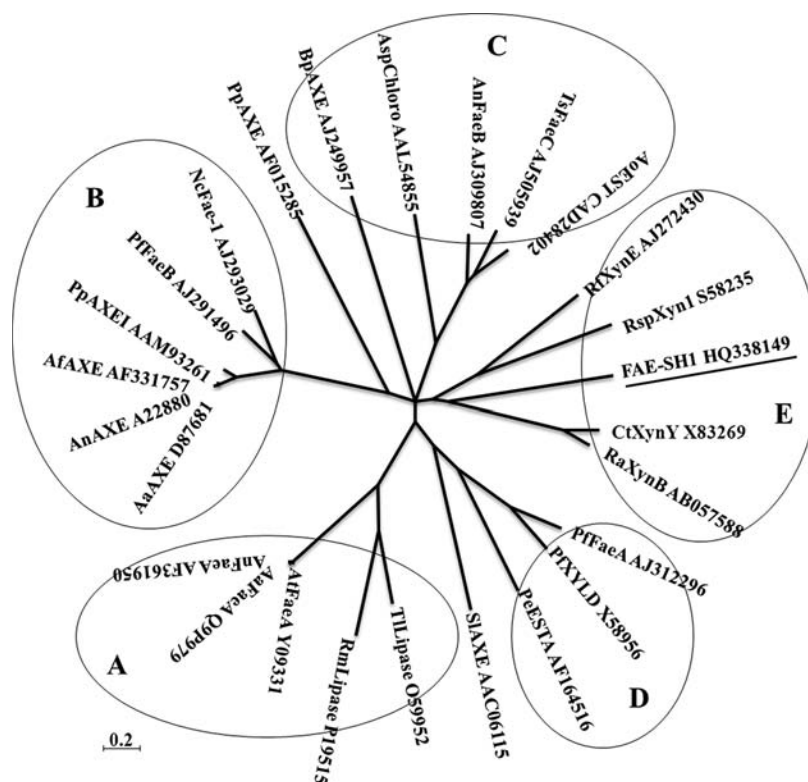

Figure 2. Phylogenetic tree of FAE-SH1 and other classified FAEs. AaAXE, *A. awamori* AXE (D87681); AaFaeA, *A. awamori* FaeA (Q9P979); AfAXE, *Aspergillus ficuum* AXE (AF331757); AnAXE, *A. niger* AXE (A22880); AnFaeA, *A. niger* FaeA (AF361950); AnFaeB, *A. niger* FaeB (AJ309807); AoEST, *A. oryzae* selective esterase (CAD28402); AspChloro, *Acinetobacter* sp. chlorogenate esterase (AAL54855); AtFaeA, *Aspergillus turbingensis* FaeA (Y09331); BpAXE, *Bacillus pumilus* AXE (AJ249957); CtXynY, *Clostridium thermocellum* XynY (X83269); NcFae1, *Neurospora crassa* Fae1 (AJ293029); PeESTA, *Piromyces equi* ESTA (AF164516); PfFaeA, *Penicillium funiculosum* FaeA (AJ312296); PfFaeB, *P. funiculosum* FaeB (AJ291496); PfXYLD, *P. fluorescens* XYLD (X58956); PpAXE, *Penicillium purporogenum* xylan esterase II (AF015285); PpAXEI, *P. purpurogenum* AXE I (AAM93261); RaXynB, *Ruminococcus albus* XynB (AB057588); RfXynE, *Ruminococcus flavefaciens* XynE (AJ272430); RmLipase, *Rhizomucor miehei* lipase (P19515); RspXyn1, *Ruminococcus* sp. Xyn1 (S58235); SlAXE, *Streptomyces lividans* AXE (AAC06115); TlLipase, *Thermomyces lanuginosus* lipase (O59952); TsFaeC, *Talaromyces stipitatus* FaeC (AJ505939).

the cytoplasmic extracts of the expression cells showed a distinctive band. After purification of the protein by immobilized Ni^{2+} affinity chromatography and SDS-PAGE analysis, a highly pure protein was obtained (Figure 3, lane B). The purified protein showed a MW of 32.4 kDa and was consistent with the predicted MW of the protein in the vector.

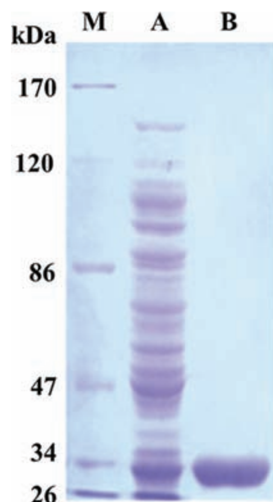

Figure 3. SDS-PAGE analysis of FAE-SH1. Lanes: M, standard protein marker (26−170 kDa); A, crude cytoplasmic protein extracts of recombinant cells; B, purified recombinant enzyme FAE-SH1.

Biochemical Characterization of FAE-SH1. Activities of the purified FAE to the four substrates including MFA, M*p*CA, MCA, and MSA were examined. As shown in Table 1, FAE-SH1 was capable of catalyzing the hydrolysis of all four substrates. The specificity activity to MFA was the highest (up to 259.5 U/mg protein) and followed by that to MCA. However, the enzyme activity to M*p*CA and MSA was about half that to MFA. From the values of K_m, MFA exhibited the highest affinity to the enzyme. In addition, comparison of k_{cat} and k_{cat}/K_m values

Table 1. Substrate Specificity and Selected Kinetic Parameters of Purified FAE-SH1[a]

substrate	specific activity (U/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
MFA	259.52 ± 6.07	1.63 ± 0.03	66.46 ± 0.28	40.84
MCA	202.09 ± 2.11	4.28 ± 0.21	111.42 ± 2.59	26.02
M*p*CA	121.89 ± 2.33	3.23 ± 0.8	47.51 ± 9.6	14.73
MSA	143.48 ± 9.19	9.48 ± 0.81	117.60 ± 8.21	12.41

[a]Substrate concentrations between 0.1 and 10 mM were employed to estimate the kinetic constants from the Michaelis−Menten equation. Values are given as the mean of three repeats ± standard deviation.

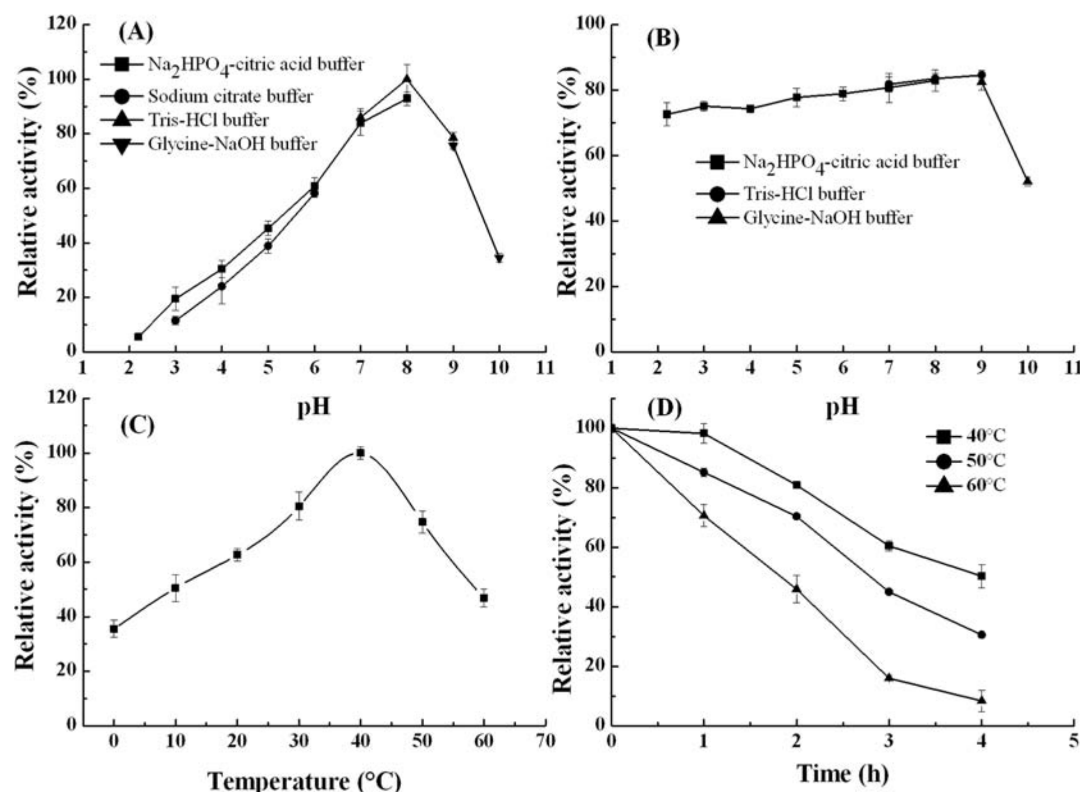

Figure 4. Characterization of the purified FAE-SH1. (A) Effect of pH on FAE-SH1 activity. The activity assay was performed at 37 °C in different buffers: Na_2HPO_4–citric acid buffer (squares); sodium citrate buffer (circles); Tris-HCl buffer (up triangles); and glycine–NaOH buffer (down triangles). (B) Effect of pH on FAE-SH1 stability. The enzyme was preincubated at 4 °C for 3 h in different buffers: Na_2HPO_4–citric acid buffer (squares), Tris-HCl buffer (circles), and glycine–NaOH buffer (triangles). The remaining activity was determined at pH 8.0 and 40 °C. (C) Effect of temperature on FAE-SH1 activity. Activity was determined under different temperatures in Tris-HCl buffers (pH 8.0). (D) Thermostability of purified FAE-SH1. The enzyme was preincubated at 40 °C (squares), 50 °C (circles), and 60 °C (triangles). Aliquots were removed at different time points for the measurement of residual activity in Tris-HCl buffer (pH 8.0) at 40 °C. Values are given as the mean of three experiments ± standard deviation.

indicated that the hydrolysis of MFA and MCA by the enzyme was faster and more efficient.

The effects of pH on the activity and stability of the purified FAE-SH1 in different buffer systems were determined. Figure 4A shows that the optimum FAE-SH1 activity was obtained around pH 8.0. Over 60% of its activity was retained at pH 6.0–9.5, whereas 11% of the highest activity was obtained at pH 3.0. Over 70% residual activities were retained after FAE-SH1 was incubated in different buffers from pH 2.0 to 9.0 for 3 h at 4 °C (Figure 4B). The optimum temperature of the purified FAE-SH1 toward MFA was around 40 °C, and it exhibited >60% of the maximum activity between 20 and 55 °C (Figure 4C). After 3 h of incubation at 40, 50, and 60 °C, 60.4, 44.9, 16.0% of initial activity can be retrieved, respectively (Figure 4D).

As demonstrated in Table 2, some chemicals such as EDTA, dimethyl sulfoxide (DMSO), acetone, isopropanol, Na^+, K^+, Ba^{2+}, Mg^{2+}, Ca^{2+}, and Zn^{2+} had little effect on FAE-SH1 activity. However, phenylmethanesulfonyl fluoride (PMSF), SDS, Fe^{3+}, Fe^{2+}, and Cu^{2+} had inhibitory effects on FAE-SH1 activity. Residual activities of FAE-SH1 were also tested after protease treatment. Compared with BSA-treated samples, >95% initial activities remained to α-chymotrypsin, pepsin, trypsin, papain, and collagenase, whereas about 90% was retained to proteinase K.

Release of FA from Wheat Straw. FA release from wheat with FAE-SH1 and lignocellulose destruction enzymes was studied. The total extractable FA was 21.55 μg/mL in this study. As illustrated in Figure 5, treatments with high doses of cellulase, β-1,3-glucanase, β-1,4-endoxylanase, and pectase were unable to produce FA from straw powder solely. FAE-SH1 could significantly ($p < 0.05$) release FA up to 9.57 μg/mL, representing 44.4% of total FA. Higher yields of FA were also obtained by joint action of FAE-SH1 with the other four lignocellulose destruction enzymes. Cellulase showed the highest synergistic effect on FA release, followed by β-1,4-endoxylanase, β-1,3-glucanase, and pectase. Cellulase could enhance the release of FA by 70% over FAE-SH1 single reaction with a yield of 16.7 ± 0.16 μg/mL, accounting for 77.5% of total FA.

Table 2. Effect of Metal Ions and Chemical Reagents on the Activity of Purified FAE-SH1[a]

reagent	relative activity (%)	reagent	relative activity (%)
control	100 ± 1.91	Na^+	98.77 ± 2.31
EDTA	102.15 ± 5.32	K^+	102.18 ± 4.01
SDS	37.26 ± 2.85	Ba^{2+}	101.46 ± 2.54
DMSO	102.11 ± 4.01	Zn^{2+}	95.432 ± 1.6
acetone	103.97 ± 2.98	Mg^{2+}	88.30 ± 7.1
isopropanol	98.76 ± 3.2	Ca^{2+}	94.69 ± 6.52
PMSF	12.45 ± 1.6	Fe^{2+}	62.50 ± 6.50
Cu^{2+}	24.99 ± 1.91	Fe^{3+}	43.75 ± 3.58

[a]Activities of FAE-SH1 were determined at optimum conditions (pH 8.0, 40°C). Double-distilled water was taken as control (100%). Values are given as the mean of three repeats ± standard deviations.

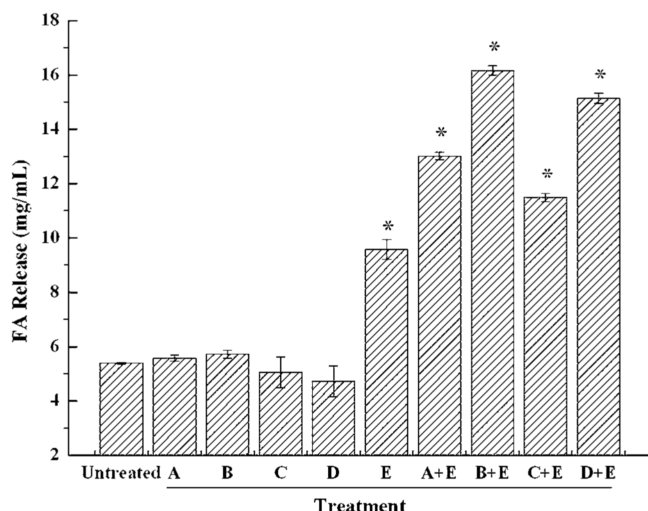

Figure 5. Release of FA from wheat straw powder. Data shown are the average of three independent experiments; error bars represent standard deviation. Statistical analysis was performed using analysis of variance (ANOVA), followed by least significant difference test (LSD); *, statistically significant difference from untreated ($p < 0.05$). Untreated, double-distilled water treated; A, β-1,3-glucanase treated; B, cellulase treated; C, pectase treated; D, β-1,4-endoxylanase; E, feruloyl esterase FAE-SH1 treated; A+E, β-1,3-glucanase and FAE-SH1 treated; B+E, cellulase and FAE-SH1 treated; C+E, pectase and FAE-SH1 treated; D+E, β-1,4-endoxylanase and FAE-SH1 treated.

■ DISCUSSION

Although many FAEs have been studied, identification and utilization of novel FAEs were profound in agricultural waste utilization and food industries.[8] Metagenomic technology, enabling harvesting and archiving of genetic resources from diverse environments directly, provides an ideal tool to new enzyme discovery.[13] Mining of FAEs with new characters and industrial prospects using this technology is in its initial stage. In this study, a fosmid library was constructed by extracting DNA of China Holstein cow rumen microbes by cell lysis in agarose without microorganism enrichment. The FAE-SH1 exhibited 56% identity to the predicted esterase sequence of *E. siraeum*, which was still not verified by experiment. The finding of FAE-SH1 would deepen the understanding of this family of enzymes.

Adjacent sequence analysis showed that a predicted endo-1,4-β-xylanase and IclR family protein were at the upstream of FAE-SH1. The specific functions regulated by members of the IclR family reported are diverse, including carbon metabolism in Enterobacteriaceae sp.[26,27] and degradation of aromatic compounds by soil bacteria.[28] This novel gene cluster may encode enzymes that function for hemicellulose or cellulose degradation in its original host.

Classification of feruloyl esterase has been controversial since its discovery, and classification schemes were developed with the increasing gene discoveries. According to the substrate utilization data and primary sequence identity, FAEs were previously classified into four subclasses and named as types A, B, C, and D.[25] Benoit et al.[5] introduced a different classification method named SF, which was based on phylogenetic analysis of fungal FAEs. Udatha et al. further classified FAEs into 12 proposed FEFs based on the descriptor of experimentally verified and putative FAEs, covering 365 protein sequences from fungus, bacterium, and plant kingdoms.[17] According to the Crepin classification scheme, FAE-SH1 should be classified as a type C FAE member, but phylogenetic tree results showed that FAE-SH1 was most similar to the type E FAEs. However, FAE-SH1 was distinct from the characterized family E member of CtXynY, which is a bifunctional enzyme with feruloyl esterase and xylanase activities and domains.[29] According to the Udatha classification scheme, FAE-SH1 can be categorized into the FEF1 family and subfamily 1B. This subfamily showed no distribution of Crepin classified type A, B, C, or D FAEs with the data available. Due to the low similarity to the known FAEs, the finding of FAE-SH1 would deepen the understanding of this family enzyme.

Increasing research has been conducted on the enzymes that are able to withstand the often relatively harsh conditions of industrial processing.[30] As summarized before, the optimum pH of FAE activity was mostly in acid conditions, the optimum temperature from mesophilic microorganisms ranged from 45 to 60 °C,[1,5] and the highest enzyme activity reported was 132 U/mg (FAE from *Aspergillus niger*).[29] In the present study, FAE-SH1 obtained highest enzyme activity under slightly basic conditions (pH 8.0). Acid-stable FAEs have been found in many species, such as FoFaeC from *Fusarium oxysporum*,[31] rAoFaeB from *Aspergillus oryzae*,[9] and AN1772.2 FAE from *Aspergillus nidulans*.[19] FAE-SH1 was stable over the range from pH 2.0 to 9.0. The pH stability profile of FAE-SH1 was similar to rAoFaeB. The highest activity of FAE-SH1 was 259.5 U/mg toward MFA, which was one of the highest enzyme activities ever reported. Currently, a few thermostable FAEs have been obtained using genome data from extreme microorganisms such as *Thermoanaerobacter tengcongensis*[20] and *Thermobacillus xylanilyticus*.[32] Site-directed mutagenesis technology was also used to improve the thermostability of feruloyl esterase A from *A. niger*.[33] As revealed in Figure 4D, the themostability of FAE-SH1 was not as high as theirs. Nevertheless, the optimum temperature of FAE-SH1 was around 40 °C, which was among the lowest ones and corresponded to the cow body temperature.[34] From the applied perspective, this property makes FAE-SH1 a good candidate to cut the energy cost during processing.

FAE-SH1 was inhibited by Fe^{2+} and Cu^{2+}, whereas EDTA, Ca^{2+}, and Zn^{2+} were not. This character was quite similar to that of FAE from *Aspergillus awamori* G-2[35] and *p*-coumaroyl esterase from *Neocallimastix* sp. MC-2.[36] In addition, FAE-SH1 was highly sensitive to PMSF, which specifically binds to the active site serine residue in serine enzymes.[37] This result agreed with the primary protein sequence analysis that FAE-SH1 was a serine-dependent esterase group enzyme.[38] Protease-treated experiment indicated that FAE-SH1 was resistant to a broad range of proteases, including pepsin. This property suggested its potential applications in broad industries such as textile and food with proteases.

FA, known for antioxidant and anti-inflammatory properties, is a high-value product widely distributed in straw. The enzyme compositions and relative ratios are vital to guide the design and optimization of enzymatic FA production. Utilizations of FAE with β-1,4-endoxylanase, cellulase, or crude enzyme have been conducted, and high yields of ferulic acid were achieved.[39,40] However, research on functions of β-1,3-glucanases and pectases in this process is limited up to now. As illustrated in this study, FAE-SH1 was capable of releasing FA from wheat straw powder individually. Moreover, FA release was significantly ($p < 0.05$) enhanced by the addition of these four lignocellulose-degrading enzymes without optimization, which could function as accessory

enzymes in this process. This result was informative for the biorefinery of FA from biomaterials.

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ABBREVIATIONS USED

BLAST, basic local alignment search tool; BSA, bovine serum albumin; CCD, conserved domains database; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FAEs, feruloyl esterases; FA, ferulic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria−Bertani; MCA, methyl caffeate; MFA, methyl ferulate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; M*p*CA, methyl *p*-coumarate; MSA, methyl sinapinate; MW, molecular weight; nr, nonredundant; ORF, the open reading frame; PFGE, pulse field gel electrophoresis; p*I*, isoelectric point; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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