Molecular cloning, expression and characterization of a novel feruloyl esterase enzyme from the symbionts of termite (Coptotermes formosanus) gut

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Termites play an important role in the degradation of dead plant materials and have acquired endogenous and symbiotic cellulose digestion capabilities. The feruloyl esterase enzyme (FAE) gene amplified from the metagenomic DNA of Coptotermes formosanus gut was cloned in the TA cloning vector and subcloned into a pET32a expression vector. The Ft3-7 gene has 84% sequence identity with Clostridium saccharolyticum and shows amino acid sequence identity with predicted xylanase/chitin deacetylase and endo-1,4-beta-xylanase. The sequence analysis reveals that probably Ft3-7 could be a new gene and that its molecular mass was 18.5 kDa. The activity of the recombinant enzyme (Ft3-7) produced in Escherichia coli (E.coli) was 21.4 U with substrate ethyl ferulate and its specific activity was 24.6 U/mg protein. The optimum pH and temperature for enzyme activity were 7.0 and 37°C, respectively. The substrate utilization preferences and sequence similarity of the Ft3-7 place it in the type-D sub-class of FAE.

INTRODUCTION

Ferulic acid (FA) is the most abundant hydroxycinnamic acid present in the cell walls of several plants. It is to be found covalently linked to polysaccharides by ester bonds and to components of lignin through ether linkages (1). It plays an important role in maintaining the structural integrity of the plant cell wall matrix, which partially limits the biodegradability of non-lignified plant cell wall polysaccharides and regulates the growth by the cross-linking of cell wall polymers.

FAs are esterified to arabinose in the arabinoxylans of wheat bran, barley straw, maize and sugar-cane bagasse and to arabinose in the pectins of sugar beet and spinach. FA also mediates the polysaccharide-protein crosslink via tyrosine or cysteine residues. Feruloyl esterase (FAE) is an enzyme capable of hydrolyzing sugar-phenolic acid ester linkages. FAE forms a part of the enzyme complex that acts collectively and synergistically to completely hydrolyze xylan to its monomers. There has recently been considerable interest in a large number of potential applications of these enzymes due to their roles in many biotechnological processes, in various industries (chemicals, fuel, animal feed, textile and laundry, pulp and paper (2), food and agriculture and the pharmaceutical industry. FA is a precursor for vanillin and its access through biotechnological methods was crucial in the quest for natural vanillin (3, 4). As well as being exploited as a hydrolyase, FAE was shown to be a good catalyst in synthesizing sugar-phenolic esters (5, 6). Some of the ester-linked substituents on plant cell wall polysaccharides retard or inhibit microbial infection (7). Phenolic components of the plant cell wall, especially p-coumaric acid, FA and p-hydroxybenzaldehyde, inhibit the growth of rumen microorganisms (8) and phenolic acids derived from plant cell walls have long been used as food preservatives (9) to inhibit microbial growth. FAEs, also known as cinnamoyl esterases (E.C. 3.1.1.73), represent a subclass of carboxylic acid esterase. FAEs have been classified into four subclasses: types A, B, C and D based on their specificity for aromatic substrates and their varying ability to release diferulic acid (diFA) from esterified substrates. Type A is active on methyl ferulate (MFA), methyl p-coumarate (MPC) and methyl sinapate (MSA). These FAEs have sequences related to those of lipases and are able to hydrolyze synthetic ferulate dehydrodimers. Type B FAEs are specific against MFA, MPC, methyl caffeate (MCA), but not MSA. These enzymes do not release diFA and show sequence similarities to carboxylic esterase family 1 - acetyl xylan esterase. Type C and D act on all four hydroxycinnamic acid methyl esters. Type C enzymes do not release diFAs from model and complex substrates, whereas type D enzymes are able to hydrolyze dimers. Type D is active on the substrates MFA, MSA, MPC and MCA. These FAEs have sequence sim-
Termites are considered to be one of the most important bioreactors on the planet, converting cellulose into the ubiquitous energy source, glucose (12). The main role of these gut symbionts was thought to relate more to anaerobic respiration and nitrogen fixation than cellulose digestion (13). Now it has been observed that endosymbiotic and termite-derived cellulases are both present in termites (14, 15) supporting the connection that the cellulases of both endosymbiont and termite origin are important to termite cellulose digestion. There have also been reports of FAEs that are part of large multidomain structures, such as cellulases. The metagenomic approach, a culture independent method, has been used to clone a number of genes, including FAE genes. Therefore, the objective of the present study was to amplify FAE enzyme encoding sequences from the microorganisms present in termite gut by metagenomic approach. The amplified FAE gene was further expressed and characterized by sequence and activity based studied. Hence, we have isolated DNA from symbionts of Coptotermes formosanus gut, amplified, cloned and over-expressed the recombinant protein in E.coli. The Purified recombinant enzyme was biochemically characterized.

RESULTS AND DISCUSSION

Nucleotide sequence and expression of the FAE gene

The FAE gene was amplified from the symbionts of Coptotermes formosanus gut metagenomic DNA using enzyme-specific primers. The obtained PCR product of 1.3 kb was cloned into the pGEMT-Easy TA cloning vector for DNA sequence analysis and the FAE gene was subcloned into the Eco RI digested pET32a expression vector (Fig. 1). The resulting clone was named pET32a Ft3-7 recombinant plasmid. The positive clone plasmids carrying our gene of interest were sequenced. The 1.3 kb nucleotide sequence of the Ft3-7 positive clone was compared with the genome database, which shows 84% primary sequence identity with Clostridium saccharolyticum and a query coverage of 52%. On translation of the above nucleotide sequence using the EXPasy proteomics server, the predicted protein sequence matched with polysaccharide deacetylase of Clostridium saccharolyticum (ADL05911) with 87% identity. This Ft3-7 sequence shows a high homology to the Endoxylanase, xylan/chitin deacetylase, putative carbohydrate esterase family 4 protein, endo-1,4-beta-xylanase (EC 3.2.1.8), indicating an evolutionary relationship among FAEs, acetyl xylan esters and certain lipases. Ft3-7 gave 61% identity to the predicted xylan/chitin deacetylase enzyme of the butyrate-producing bacterium accession no CBL40699 and 59% to the polysaccharide deacetylase family of Clostridiales bacterium ZP_04667470, 59% to the hypothetical protein of Clostridium bolteae ZP_02083187 and 46% to the Clostridium asparagiforme strain no. ZP_03762399 (Fig. 2A). The other domain of Ft3-7 has a sequence identity of 90% with the ABC transporter related protein of Clostridium saccharolyticum WM1 (ADL05909), 68% with ATPase component of butyrate-producing bacterium SSC2 (CBL37620), 62% with ABC-type multidrug transport system, ATPase component of Faecalibacterium prausnitzii L2-6 (CBK99948) (Fig. 2B).

Ft3-7 has polysaccharide deacetylase/xylanases domains from residues 1 to 143 amino acids (AAs), which correspond to FAE type-D. This FAE module hydrolyzes the ester linkage between arabinofuranoside side chains and FA. There are reports that FAE can also hydrolyze xylan/ acetyl or 4-O-methylglucuronyl moieties (16). One of these ORFs present in Ft3-7 encodes a putative protein that features an ABC transporter/ ATP-binding domain from AA residues of 352-436. ABC transporters are a large family of proteins involved in the transport of a wide variety of different compounds like sugars, ions, peptides and more complex organic molecules. Thus, all available evidence indicates that Ft3-7 is a component of the FAE
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Fig. 3. Analysis of recombinant protein Ft3-7 expression on PAGE. (A) SDS-PAGE of the fractions obtained during purification of recombinant Ft3-7 from E.coli BL21 cell extract. Lane 1, molecular mass markers; lane 2, cell lysate of uninduced E.coli BL21 cells; lane 3, cell lysate of induced E.coli BL21 cells; lane 4 flow-through of Ni-NTA Biorad column and lane 5, Ft3-7 (FAE) purified protein; (B) Native-PAGE of purified Ft3-7 protein compared with native PAGE molecular markers as follows. Lane 1, bovine serum albumin (BSA) 67 kDa; lane 2, Ovalbumin 43 kDa; lane 3, Trypsine soyabean inhibitor 20.1 kDa and lane 4, purified Ft3-7 protein.

Fig. 4. Effects of pH and temperature on the activity and stability of the enzyme. (A) Influence of pH on the activity of Ft3-7 from C. formosanus gut. Enzyme activity was measured at pH 4-9. The highest level of activity, 22 U/mg, was obtained at pH 7.75 and was set at 100%. (B) Influence of temperature on the activity of Ft3-7 from C. formosanus gut. Enzyme assay was measured according to the slandered assay from 10-60°C. The highest level of activity, 24.6 U/mg, was obtained at 40°C and was set at 100%.
Release of phenolics from crude plant cell walls
The ability of the purified Ft3-7 protein to release phenolic compounds from crude plant cell wall materials in the form of crop residues was tested. The enzyme was found to be able to release FA and difA from wheat bran and spent grain in combination with xylanase (17). When the 20 μl Ft3-7 protein was incubated with wheat bran in the presence of 2 U xylanase at 37°C for 6 hr, 72% of FA and 28% of difA was released. On the other hand, with spent grain 46% of FA and 23% of difA was released. However, these measurements have only been carried out on phenolic acid-substituted xylan polysaccharide isolated from wheat straw and rarely on untreated crude cell wall materials (18). It was found that phenolic acids were released from the same substrates of wheat bran and spent grain in the presence of enzymes xylanase and FAE (19). Our results are agreeing the Crepin et al. 2004 findings (20). The synergetic nature of the association between xylanases and FAE activity has now been accepted and reports of the purification of FAE have either concluded that FAE activity could only be detected in the presence of xylanases or that in the presence of xylanase activity FAE activity increased. One explanation why FAE activity has been detected in only a few strains is the lack of a complete set of xylanase enzymes, resulting in low and perhaps undetectable FAE activity (21). The results suggest that Ft3-7 is able to release phenolic residues from wheat bran and spent grain.

The FAE from the symbionts of termites (Coptotermes formosanus) gut was cloned and an expressed protein of Ft3-7 was purified and characterized in this work. Ft3-7 is distinct from the other subclasses of type B and C FAE which does not release 5-5' difA (20). However, Ft3-7 releases 5-5' difA from plant cell wall materials. The enhancement of FAE activity by xylanase has also been elucidated. Therefore, based on these sequence similarity with xylanase and substrate utilization properties of Ft3-7, we propose that it is indeed a novel type-D FAE as predicted from a comparative sequence analysis. These findings enable us to develop superior fibrolytic enzymes that are impractical for commercial enzyme production. Thus, cloning of the desired genes is essential for the commercial production of plant cell wall polysaccharide-degrading enzymes.

MATERIALS AND METHODS

Bacterial strains, vectors and media
For cloning the FAE gene from the metagenomic DNA isolated from endosymbionts of termites, E.coli DH5α was employed as a host cell, with the pGEMT-Easy (Promega) TA cloning vector being used as the host-vector system. The pET32a (Novagen) vector and an E.coli BL 21 strain were used for protein expression. E.coli was grown at 37°C in Luria-Bertani (LB) agar plates containing 60 μg ampicillin/ml, X-Gal and IPTG for the selection of positive clones.

Collection of termites and DNA isolation
Worker termites were collected from small breaches in nests, wood or foraging trails. 50-60 worker termites were randomly chosen from each colony. Immediately after collection, the termites were transported to the laboratory. The exterior surfaces had been washed with distilled water and then the entire gut was collected from these individuals by using a pair of sterile forceps. The genomic DNA from the symbionts of termite’s gut was isolated by the CTAB (Cetyl Trimethyl Ammonium Bromide) method (22) with the some modifications. The Purity of the isolated genomic DNA was checked using a spectrophotometer. The plasmid DNA from the E.coli strains was isolated and purified using a Gene Jet plasmid Miniprep Kit from Fermentas and a Gene Jet gel extraction kit (Fermentas) was used for extracting DNA from agarose gels.

PCR amplification and cloning of the FAE gene
PCR was performed with purified genomic DNA as a template, using primers 5'-CTC GCT GAC GAC AAA TTT CCT GTA GCA G-3' and 5'-CAC CTC GAG TTC ATG GAA GAA ATA TGG-3' (16) The amplified gene was sent for the sequencing and based on the sequence obtained the following enzyme specific primers have been designed: 23F (5'-AAA ATA CGT TTG CCG GCA GGT AAG AGA T) and 31R (5'-ACA GCG TAC TGC AAT CGC CGG CCG CCA GGT AAC AGA T) to amplify the near full-length FAE gene. A high fidelity Novagen KOD XL DNA polymerase was used for DNA amplification which was carried out with 35 cycles of denaturation (45s at 95°C), annealing (1 min at 63°C) and extension (2 min at 72°C), followed by 10 min of further extension at 72°C. The PCR product was purified by the StrataPrep PCR cleanup kit (Stratagene) and ligated into the pGEMT-Easy TA cloning vector according to the manufacturer’s instructions. The pGEMT easy vector carrying the FAE gene was digested with the Eco R1 enzyme and the produced fragment was gel-purified before its subcloning into an expression vector. The Gel-purified FAE gene was ligated with the EcoR1 digested pET32a expression vector and transformed by electroporation into E.coli BL 21.

High-level expression and purification of Ft3-7 recombinant protein
E.coli cells containing the recombinant plasmid pET32a-Ft3-7 were cultured overnight on a LB agar plate containing ampicillin (100 μg/ml). The culture obtained was then inoculated into 500 ml of LB broth containing ampicillin (100 μg/ml) and allowed to grow at 37°C until it reached an optical density of 0.6 at 600 nm. At this point, 150 μM of IPTG (isopropyl-β-thio-galactopyranoside) was added and the cells were re-incubated for 6 hrs at 37°C. The bacterial culture was centrifuged at 6,000 × g for 10 min to separate the cells from the media. The induced bacterial cell pellets were resuspended in 10 ml of buffer A (10 mM TRIS, 100 mM NaCl, pH 8.0) and lysed by ultra sonication on ice. After centrifugation at 15,000 ×
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For 10 min at 4°C, a supernatant fraction containing a recombinant fusion protein was loaded on pre-equilibrated columns containing Ni-NTA agarose affinity resin and incubated for binding. The Ni-NTA resin was washed with 50 ml of buffer B (10 mM TRIS-Cl pH 8.0, 100 mM NaCl and 5 mM imidazole) to remove non-specific proteins from the column. The affinity-bound recombinant protein was eluted in buffer C consisting of 10 mM TRIS-Cl pH 8.0, 200 mM NaCl and 500 mM imidazole. The purity of the recombinant fusion protein was determined by SDS-PAGE and the protein concentration was determined by the dye-binding procedure of Bradford et al. 1976 (23) using bovine serum albumin as a standard.

**SDS-PAGE analysis**

The purified recombinant protein was analyzed on SDS-PAGE according to the method of Laemmli (24). The stacking gel was 4% polyacrylamide and the running gel was 12.5% polyacrylamide, used for the separation of polypeptides. Samples containing 1 mg/ml protein were solubilized by boiling for 2 min in a sample buffer containing 5% glycerol, 0.1 M Tris-HCl (pH 6.8), 2% SDS and 100 μM mercaptoethanol along with 0.1% of bromophenol blue. The molecular mass of the resolved proteins was calculated by using Novagen markers containing the 9 precise recombinant proteins of 225, 150, 100, 75, 50, 35, 25, 15 and 10 kDa.

**Enzyme assay**

The recombinant protein was subjected to dialysis and concentrated by ultrafiltration using Sartorius concentrators with a 10-kDa cutoff. The protein so obtained was used for FAE activity studies. FAE activity was assayed according to the method of Latha et al. (25). FAE activity was assayed by the analysis of free FA released from ethyl ferulate (substrate). The assay was carried out in 50 mM of TRIS buffer, pH 7 (5-8) at 37°C (18-40). The Ferulate release was analyzed by HPLC using a Nucleosil C18 column. One unit (U) of activity was defined as the amount of enzyme that catalyses the release of 1 cm FA per minute. The substrate is prepared by suspending 2 mM of ethyl ferulate in 10 ml of 50 mM TRIS buffer pH 7. One volume of substrate solution and three volumes of enzyme solution were incubated at 37°C for different time intervals. Adding three volumes of methanol or ethyl acetate to the reaction mixture immediately after the incubation period terminates the enzyme reaction. The released FA was collected by phase separation from the reaction mixture by centrifugation (15,000 g for 5 min, 15°C). Samples were filtered through a 0.45 μm syringe filter and analyzed by HPLC. The specific activity was defined as number of enzyme units per ml divided by the concentration of protein in mg/ml. Therefore the specific activity is quoted as units/mg.

The temperature optimum was determined by running the standard assay at 15-60°C. Thermostability was measured by incubating the enzyme (15 mg/ml in phosphate buffer, pH 7.0) at 30°C for 1 h. Samples were taken at different intervals and the residual activity was measured by the standard assay. All the assays were done in triplicate. The optimum pH was measured by running the standard assay at different pH values. The buffers used were a citrate/phosphate buffer (0.2 M) for pH 4-5 and a sodium phosphate buffer (0.2 M) for pH 6-9. The pH was monitored before and after the reaction. Significant changes in pH due to the reaction were not observed.

**Sequence alignments**

AA sequence alignments were performed by using the BLAST programs (26) at the server of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and ClustalW (www.ebi.ac.uk).

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