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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Thermostable feruloyl esterase for the bioproduction of ferulic acid from triticale bran

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Abstract A putative α/β hydrolase fold-encoding gene (locus tag TTE1809) from the genome of *Thermoanaerobacter tengcongensis* was cloned and expressed in *Escherichia coli* as a possible source of thermostable feruloyl esterase (FAE) for the production of antioxidant phenolic acids from biomass. Designated as TtFAE, the 33-kDa protein was purified to apparent homogeneity. The lipase-like sequence characteristics of TtFAE and its substrate specificity towards methyl ferulate, methyl sinapate, and methyl *p*-coumarate classify it as a new member of the type A FAEs. At 75°C, the enzyme retained at least 95% of its original activity for over 80 min; at 80°C, its half-life was found to be 50 min, rendering TtFAE a highly thermostable protein. Under different hydrolytic conditions, ferulic acid (FA) was shown to be released from feruloylated oligosaccharides prepared from triticale bran. An estimated recovery of 68 mg FA/100 g triticale bran was demonstrated by a 30% release of the total FA from triticale bran within a 5-h incubation period. Both the oxygen radical absorbing capacity values of the feruloylated oligosaccharides and free FA were also determined. Overall, this work introduces a new bacterial member to the growing family of plant cell wall degrading FAEs that at present is largely of fungal origin, and it benchmarks the bioproduction of FA from triticale bran.

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Introduction

In a biorefinery setting, the production of value-added chemicals besides biofuels and bioenergy derived from a generic feedstock represents an important component of the overall value chain and economics (Caesar 2008; Bozell 2008). Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid), a representative hydroxycinnamic acid, has been identified as one of the two top aromatic acid building blocks from lignocellulosic materials and biomass (Werpy and Peterson 2004; Dodd and Gross 2007). FA is best known for its antioxidant properties due to its radical scavenging ability, is widely used as a food preservative, and is an active ingredient in many skin lotions and sunscreens (Graf 1992; Ou and Kwok 2004). Besides, FA can be converted enzymatically to various value-added products, e.g., vinyl guaiacol and vanillin that are known flavoring agents in foods, beverages, or perfumes (Rosazza et al. 1995; Mathew and Abraham 2006).

Present in both monocots and dicots of the plant kingdom, FA is generally not found in free form but instead esterified to arabinose in various polysaccharides, e.g., arabinoxylans and pectins (Carnachan and Harris 2000; Bunzel 2009). Ferulic acid esterases (FAEs, also known as feruloyl esterases, cinnamic acid, or cinnamoyl esterases; EC 3.1.1.73) represent a diverse group of hydrolases that can release FA from its bound constituents in the plant cell wall. Interest in FAEs as key enzymes for cell wall hydrolysis and the extraction of phenolic acids from agricultural crops in particular is increasing (Benoit et al. 2006; Wong 2006; Faulds et al. 2006; Fazary and Ju 2007; Topakas et al. 2007; Koseki et al. 2009a).

To date, four types of microbial FAEs (A, B, C, and D) have been classified based on substrate preference, e.g., specificity for hydroxycinnamic acid methyl esters and release of diferulic acid from model and complex substrates (de Vries et al. 1997; Crepin et al. 2004). Additional subtypes or subfamilies (SF1-7) of the fungal FAEs have also been proposed according to phylogenetic clustering of the sequences (Benoit et al. 2008). Although many hypothetical FAE candidates exist in the microbial genome database, only over a dozen fungal FAE-encoding genes have been cloned and characterized so far (Benoit et al. 2008; Moukouli et al. 2008; Koseki et al. 2009b). On the bacterial side, even fewer clones are available. These are limited to *Cellvibrio japonicum* (DeBoy et al. 2008; formerly *Pseudomonas fluorescens*; Ferreira et al. 1993), *Burkholderia multivorans* (Rashamuse et al. 2007), *Butyrivibrio fibrisolvens* (Dalrymple et al. 1996; Dalrymple and Swadling 1997), *Pseudoalteromonas halosplanktis* (Aurilia et al. 2008), *Clostridium thermocellum* cellulosome (Blum et al. 2000), and *Prevotella ruminicola* (Dodd et al. 2009).

This study was conducted to characterize a genome-mined α/β-fold family hydrolase-encoding gene from a thermophilic organism, *Thermoanaerobacter tengcongensis* MB4^T (Bao et al. 2002), also known as *Caldanaerobacter subterraneus* subsp. *tengcongensis* (Fardeau et al. 2004). The resulting biocatalyst was characterized and applied to a laboratory-scale preparation of FA from triticale bran. Triticale is a relatively new, manmade hybrid crop of durum wheat (*Triticum* ssp.) and rye (*Secale* ssp.) (Oettler 2005). Due to its hardiness, tolerance to marginal growing conditions, disease resistance, draught tolerance, and largely unexplored potential, triticale is being developed as a bioindustrial cereal in Canada (http://www.ctbi.ca).

Materials and methods

Chemicals

trans-FA (99%) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Aldrich Chemical, Canada. Methyl esters of ferulate (MFA), *p*-coumaric acid (MpCA), caffeic acid, sinapinic acid (MSA), vanillic acid, and syringic acid were obtained from Extrasynthèse (France) and Bosche Scientific, New Brunswick (Canada). *p*-Nitrophenyl esters of various chain lengths, β-phycoerythrin, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma. High-performance liquid chromatography (HPLC)-grade methanol and other chemicals of analytical grade were purchased from J.T. Baker, USA, and were used without further purification.

Genome mining and genetic manipulations

The 246-residue amino acid sequence of a biochemically characterized cinnamoyl esterase (AAC44493) from *B. fibrisolvens* (Dalrymple et al. 1996) was used to perform a BLAST search of the National Center for Biotechnology Information (NCBI) database. We were interested in a potential thermophilic candidate. As a result, a putative α/β-fold family hydrolase-encoding gene in the thermophilic *T. tengcongensis* strain MB4^T (locus tag TTE1809; NP_623397; Bao et al. 2002) showing 26% sequence identity was chosen for this study.

The *T. tengcongensis* strain MB4^T (DSM number 15242) was obtained from DSMZ, Braunschweig, Germany, and grown as previously described (Grosse et al. 2010). Genomic DNA was prepared by conventional protocol (Sambrook et al. 1989). The target esterase gene was amplified by polymerase chain reaction (PCR) using Titanium Taq DNA polymerase (Clontech, Takara BIO Company) and the specific primers: FAE1, 5′-GCTCGGAATTCATGCAG AAGGCTGTAGAG-3′ and FAE2, 5′-CGACCTGCAGCTATC CCTTTAATTCTC-3′ that contain *Eco*RI and *Pst*I restriction recognition sites, respectively. The PCR cycle parameters were 94°C 3 min, 30 cycles of 94°C for 45 s, Tm-3°C for 30 s, 68°C for 1 min, followed by a final extension at 68°C for 10 min. The resulting 777-bp DNA fragment, following gel purification, was digested with *Eco*RI and *Pst*I and ligated into the corresponding sites of the IPTG-inducible plasmid pKK223-3 (Pharmacia). The transformants in *Escherichia coli* strain JM109 (Yanisch-Perron et al. 1985) were selected for ampicillin (100 μg/ml) resistance on Luria-Bertani (LB) medium agar plates at 37°C using standard protocols (Sambrook et al. 1989). The designated strain is *E. coli* [pKTtFAE]. The gene sequence of the cloned insert was verified by DNA sequencing using the Big Dye DNA sequencing kit (Applied Biosystems) and automated DNA sequencer (Model 377, ABI prism).

Protein purification and electrophoresis

Expression of TtFAE in *E. coli* [pKTtFAE] and processing of cells are described in Supplementary materials – Protein expression. TtFAE was purified to electrophoretic homogeneity in four steps using the ÄKTAexplorer™ 100 Air chromatography system (GE Healthcare). Crude enzyme extract was obtained by cell disruption using a French Press operating at 20,000 psi and subsequent centrifugation at 20,000×*g* for 30 min. In the first purification step, the crude extract was incubated at 60°C for 30 min, and precipitated protein was removed by centrifugation (20,000×*g*). Solid ammonium sulfate (AS) was slowly added to bring the solution to 40% AS, and the precipitated protein was removed by centrifugation (20,000×*g*). The enzyme con-

taining solution was then applied to a Butyl-S Sepharose FF (16/10) previously equilibrated with 40% AS in 20 mM sodium phosphate buffer (pH 7.0), and bound protein was eluted using a linear gradient of AS ranging from 40% to 0% in phosphate buffer. Active fractions were collected, pooled, and concentrated by ultrafiltration (YM 10 membrane, 50 mL stirring cell, Amicon, USA). TtFAE was further purified on a HiLoad Superdex 75 pg (10/60) gel filtration column, previously equilibrated with 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. Protein was eluted with the same buffer, and active enzyme was collected, pooled, and concentrated by ultrafiltration.

Protein concentration was determined by the BCA method (Smith et al. 1985). Denaturing polyacrylamide gel electrophoresis (PAGE) was carried out using 12% polyacrylamide and the gel stained with silver (Sambrook et al. 1989).

Analytical methods

Quantitative determination of phenolic compounds was conducted by HPLC (Agilent, serie 1100) using a Gemini-NX 3 µm C18 column (150×4.6 mm) (Phenomenex). The HPLC system was equipped with a diode-array detector, and samples were monitored at 280 nm. Sample elution was performed using a gradient of methanol [10% to 20% (0–35 min) and from 20% to 60% (35–60 min)] in a solution of 0.027 N phosphoric acid. The volume of sample injected was 10 µl. Phenolic acids were quantified according to corresponding standards.

Thermostability and pH optimum

Enzyme stability was studied over the temperature range of 65–85°C using purified TtFAE (0.7 mg/ml). Aliquots were withdrawn at defined times (2, 5, 10, 20, 40, and 80 min), chilled on ice, and assayed for activity against *p*-NP caprylate at room temperature. The experimental values were used to calculate the half-life time of the enzyme. The pH optimum was determined over a pH range of 5–11 in 50 mM Britton and Robinson's universal buffer (Surinenaite et al. 2002).

Enzyme assays, substrate specificity, and kinetics

Esterase activity was measured using a variety of *p*-NP esters (1 mM final concentration) of various chain lengths and on methyl esters of phenolic compounds (5 mM final concentration). The substrates were dissolved in isopropanol or DMSO and added to 50 mM sodium phosphate buffer pH 8.0 in a 1-ml reaction volume. The reaction was started by adding a defined amount of enzyme. The release of *p*-nitrophenol (ε=15 $mM^{-1}cm^{-1}$) was monitored at 410 nm using a Beckman UV-visual recording spectrophotometer (model DU 640). One unit of enzyme is defined as 1 µmol of product formed per minute. All assays were conducted at 25°C in triplicate with appropriate controls.

The enzyme assays for the methyl esters of phenolic acids were carried out at 65°C, and the reaction was terminated by acidification with HCl after 30 min reaction time. The products were quantified by HPLC.

Kinetic parameters of the esterase (K_m and k_{cat}) were determined by using the double-reciprocal transformation (Lineweaver–Burk plot) of the Michaelis–Menten equation under steady-state conditions. Initial reaction rates for the methyl esters were measured as described above by using substrate concentrations between 0.03 and 5 mM.

Xylanase activity was determined by measuring the release of reducing sugars (Chaplin 1986) from 1% (*w*/*v*) Birchwood xylan (Sigma) and expressed as xylose equivalents.

Preparation of plant materials

Triticale bran (Ultima variety) and straw were provided by Centre de recherche sur les grains Inc. (CEROM, Saint-Mathieu-de-Beloeil, Quebec, Canada). Freeze-dried triticale bran at −80°C was ground in a Cyclone sample Mill (UDY Corporation, Fort Collins, CO, USA). Preparation of alcohol insoluble residue (AIR) sample (extraction of unbounded low-molecular weight sugars, amino acids, organic acids, and various inorganic salts leaving behind the plant polymer) was carried out using the protocol of Waldron and Selvendran (1990). Destarched triticale bran (DSTB) was prepared according to Mukherjee et al. (2007). Briefly, triticale bran was treated with 0.3% (*w*/*v*) potassium acetate at 95°C for 30 min, followed by extensive washing with deionized water to remove starch. The AIR or DSTB material (1 g for a routine experiment) was suspended in deionized water and heated to 121°C in an autoclave for 1 h. The volume of the suspension was then adjusted to obtain a 5% aqueous suspension for further treatment.

Preparation and purification of soluble feruloylated oligosaccharides

To obtain soluble feruloylated oligosaccharides, enzymatic hydrolysis was carried out using 10 U *Trichoderma viride* xylanase (GH11 superfamily; Sigma) on 1 g AIR-treated triticale bran for 16 h. The samples were centrifuged, and the supernatant was analyzed at 375 nm in a spectrophotometer for the presence of feruloylated oligosaccharides (Micard et al. 1994; Saulnier et al. 1995; Barberousse et al. 2008). For purification, the supernatant was loaded onto an XAD-2 column (10×1.6 cm) to bind free phenolics. The column was washed with water, and bound chemicals were

eluted successively by three column volumes of 15% (*v*/*v*), 50% (*v*/*v*), and 100% (*v*/*v*) methanol, at a rate of 0.7 ml/min. Fractions were collected. Methanol was removed using an air stream, and the dried residue was dissolved in deionized water.

Chemical hydrolysis of triticale bran and straw

The total contents of phenolic compounds in either triticale bran AIR-sample or triticale straw were determined by 2 M NaOH hydrolysis at 55°C for 5 h. The soluble fractions, after centrifugation (3,600×*g*, 15 min) were acidified to pH 2 with HCl and analyzed by HPLC.

Extraction of FA from triticale bran by TtFAE

Three different hydrolysis conditions were used.

Scheme A An autoclaved triticale suspension (5% *w*/*v*) was incubated with 10 U of *T. viride* xylanase at 37°C with gentle shaking for 16 h. The supernatant containing soluble esters of phenolic acids was incubated with partially purified TtFAE (40 mU/ml) in 50 mM sodium phosphate buffer pH 8.0, at 55°C, with gentle shaking (100×g). Samples were taken at various intervals, and the released phenolic compounds were analyzed by HPLC.

Scheme B Feruloylated oligosaccharides purified from 1 g AIR-treated triticale bran were incubated with the partially purified TtFAE and processed as described above.

Scheme C The thermostable xylanase, Pentopan, from *Thermomyces lanuginosus* (125 U; Sigma) was used in combination with 100 mU/ml of TtFAE. The hydrolysis of 1 g AIR-treated triticale bran was carried out in a water bath shaker at 65°C in the same buffer as in Scheme A. At each hour interval, samples were taken, and the residues were separated by centrifugation (3 min, 13,000 g). Alternatively, *T. viride* xylanase was also tested; the incubation was carried out at 37°C since it is unstable at higher temperature. The amount of phenolic acids released was determined by HPLC. All assays were prepared in triplicates.

Oxygen radical absorbing capacity assay

The oxygen radical absorbing capacity (ORAC) assays for the free phenolic compounds and feruloylated oligosaccharides were carried out using a fluorescence method with β-phycoerythrin as fluorescent probe and AAPH as peroxyl radical generator as previously described (Cao et al. 1995). Specific details are described in Supplementary materials – ORAC assay.

Results

Sequence characteristics

The 258-codon sequence of the cloned TtFAE in pKTtFAE was verified by DNA sequencing to be identical to the reported TTE1809 in *T. tengcongensis* strain MB4^T (Bao et al. 2002) except for the expected G to A change in the initiator codon (from GTG to ATG) in the clone. In the NCBI BLAST search, the most identical homolog (99%) is one derived from the genome sequence of *Carboxydibrachium pacificum* DSM12653 and numerous hypothetical candidates (63–83% identity) from *Thermoanaerobacter* spp. (not shown). The NCBI Conserved Domain Search of TtFAE predicted an α/β fold-hydrolase enzyme of the esterase_lipase superfamily with the characteristic catalytic triad consisting of S113-D203-H233. The sequence LLGLSMGGA containing the S113 has the lipase-serine pattern PS00120 (PROSITE website). Sequence homologs of TtFAE in the Protein Data Bank are 2FUK, a *Xanthomonas campestris* α/β serine hydrolase (19% identity); 2HDW, a *Pseudomonas aeruginosa* PAO1 hypothetical carboxyl esterase (15% identity); and 1QZ3, *Alicyclobacillus acidocaldarius* EST2 (15% identity).

TtFAE expression and purification

TtFAE was found to be expressed constitutively even though *E. coli* [pKTtFAE] was an IPTG-inducible system (not shown). The purification scheme of TtFAE is shown in Table 1. The Butyl-S Sepharose FF (16/10) step afforded the most purification prior to Superdex 75 pg that resulted in a purification of 30.5-fold and 9% protein yield. The estimated molecular mass (M_r) of purified TtFAE was

Table 1 Purification of TtFAE

Purification step	Total prot. (mg)	Total act.[a] (U)	Spec. act.[a] (U/mg)	Yield/Purif. (%/fold)
Crude extract	510	196	0.38	100/1
Heat step (60°C, 30 min)	195	164	0.84	84/2.2
AS precipitation (40%)	114	110	0.97	56/2.5
Butyl-S Sepharose FF	7.7	40	5.2	20/13.5
Superdex 75 pg	1.5	18	11.7	9/30.5

[a] Towards *p*-NP-caprylate

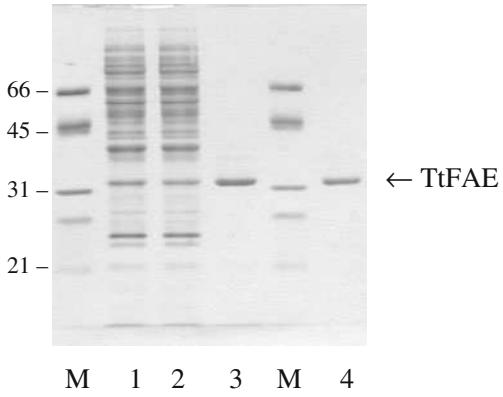

Fig. 1 Purification of TtFAE analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Lane 1*, crude cell extract of *Escherichia coli* (pKTtFAE); *lane 2*, heat-treated cell extract; *lane 3*, fraction after Butyl-S Sepharose; *lane 4*, fraction after Superdex 75 pg; *M*: molecular size standards in kilodaltons (trypsin inhibitor, 21.5; carbonic anhydrase, 31; ovalbumin, 45; bovine serum albumin, 66)

33 kDa by sodium dodecyl sulfate-PAGE (Fig. 1), compared to the theoretical value of 29,096. Native M_r was found to be about 36 kDa suggesting TtFAE is a monomeric protein.

Substrate specificity of TtFAE and enzyme kinetics

The purified TtFAE was active towards various esters of *p*-NP, with decreasing activity depending on the chain length of the alcohol portion of the ester (C_4–C_{16}) especially C_{14} and C_{16} (Table 2). More importantly, TtFAE cleaved the ester bonds in MFA, MSA, and MpCA at approximately the same rate, although considerably lower than the preferred *p*-NP esters. Methyl caffeate, vanillate, and especially syringate appeared as relatively poor substrates. On the other hand, the feruloylated oligosaccharides purified from triticale bran were converted at a comparable rate to those of MFA, MSA, and MpCA.

The K_m for MFA and MSA were determined to be very similar at about 0.3 mM (Table 2). The enzyme appeared to have a higher affinity for MpCA at a K_m of 0.16 mM. The V_{max} in all cases was very close.

Thermostability and pH profile

Initial experiment using partially purified TtFAE showed that the enzyme had a remarkable stability over a wide temperature range of 25–80°C (not shown). The purified enzyme was used to investigate its thermal stability between 65°C and 85°C (Fig. 2). The results indicated minimal loss of activity at 75°C after 80 min. At 80°C in the same timeframe, the enzyme retained 30% of its original activity. The half-life ($t_{1/2}$) of the enzyme was calculated to be >600 min at 75°C and 50 min at 80°C. The activation energy of denaturation (E_a) was estimated to be 162±10 kcal mol^{-1} using the Arrhenius plot ($\ln[k_d]$ vs. $1/T$).

The TtFAE enzyme showed maximal activity at pH 8.0. This dropped drastically at pH's above 10 and below 7 (Supplementary Fig. 1).

FA in triticale bran and straw by chemical extraction

In order to establish the maximum yield, total alkali-extractable FA content of triticale bran was determined to be 227.8±8.9 mg/100 g, representing 0.23% (*w*/*w*). The triticale straw was found to contain more *p*-coumaric acid (403.7±19.3 mg/100 g) than FA (303.7±12.7 mg/100 g).

Effect of pretreatment of triticale bran on its solubilization by *T. viride* xylanase

Both destarched and AIR-treated triticale bran could be solubilized by *T. viride* xylanase. Based on 1 g material, a slightly higher amount of feruloylated oligosaccharides was

Table 2 Substrate specificity and selected kinetic parameters of TtFAE

Substrate	Spec. activity (U/mg)	K_m (mM)	k_{cat} (s^{-1})
Methyl ferulate	0.456±0.001	0.32±0.05	0.28
Methyl sinapate	0.511±0.040	0.29±0.04	0.28
Methyl *p*-coumarate	0.426±0.005	0.16±0.02	0.22
Methyl caffeate	0.050±0.001	ND	ND
Methyl vanillate	0.030±0.001	ND	ND
Methyl syringate	Trace	ND	ND
Feruloylated oligosaccharides	0.462±0.004	ND	ND
p-Nitrophenyl acetate (C2)	18.5±1.5	ND	ND
p-Nitrophenyl butyrate (C4)	36.0±0.6	ND	ND
p-Nitrophenyl caprylate (C8)	10.5±1.0	ND	ND
p-Nitrophenyl myristate (C14)	1.7±0.1	ND	ND
p-Nitrophenyl palmitate (C16)	0.004±0.001	ND	ND

ND not determined

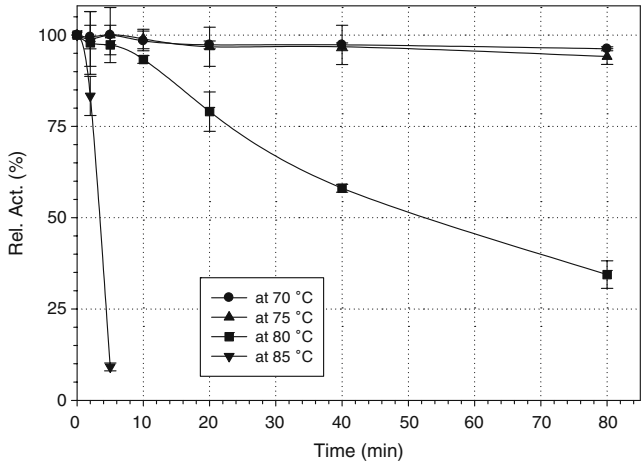

Fig. 2 Thermostability of TtFAE. Purified enzyme was incubated at temperatures between 65°C and 85°C at 5° intervals. Samples were taken at indicated times and assayed for activity against *p*-NP caprylate. Residual esterase activity was reported as a function of time at the indicated temperatures

obtained for the DSTB, 6.4±0.4 vs 5.0±0.1 mg for AIR. A higher dose of xylanase had no apparent effect on the extent of solubilization (not shown).

Bioproduction of FA from triticale bran

TtFAE proved to be an active biocatalyst for the conversion of feruloylated sugars as shown by the time course of FA recovery under three hydrolytic conditions (Fig. 3). The best yield of FA was 68.2±5.8 mg/100 g after 5 h incubation when the unpurified FA-sugar fraction was used in scheme A. This represented 30% of the total FA present in this biomass. The XAD-resin purified FA-sugar fraction gave a similar degree of bioconversion although slightly lower in scheme B. The possible synergistic action of xylanase and TtFAE was examined under the hydrolysis scheme C. *T. viride* xylanase combined with TtFAE could release 29.2±4.1 mg/100 g material which is lower than the recovery of 47.7±2.9 mg/100 g by the use of the thermostable *T. lanuginosus* xylanase at 65°C (scheme C). Increasing the concentration of TtFAE and xylanase did not affect the total yield of FA. In scheme C, prolonged incubation up to 24 h led to a slight improvement of FA recovery (data not shown).

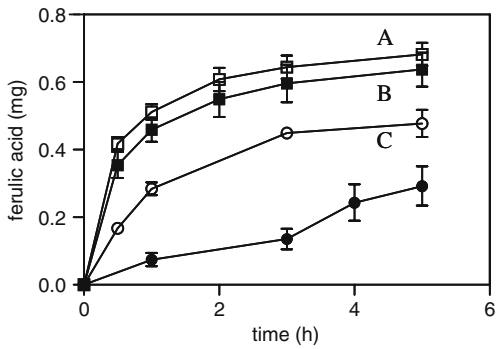

Fig. 3 Time course of ferulic acid recovery from triticale bran. *Open squares* scheme A: soluble phenolic acid esters treated with TtFAE; *closed squares* scheme B: purified esters of phenolic acid (XAD 2 column) treated with TtFAE; *open circles* scheme C: joint action of *Thermomyces lanuginosus* xylanase (thermostable) and TtFAE; *closed circles* scheme C: joint action of *Trichoderma viride* xylanase and TtFAE

Without TtFAE, xylanase alone was unable to release FA from triticale bran. However, xylanase led to the release of feruloylated sugar, which could be detected by measuring the absorbance of the soluble fraction at 375 nm. Treatment of this fraction with TtFAE produced a shift in the absorbance from 375 to 345 nm, the latter peak being characteristic for FA. In HPLC analysis, the appearance of a major FA peak at a retention time of 50.66 min (chromatogram not shown) confirmed the spectrophotometric result.

Oxygen radical absorbing capacity values of feruloylated oligosaccharides and free FA

The β-phycoerythrin fluorescence decay curves are illustrations of the ability of the purified feruloylated oligosaccharides to provide protection against oxidative damage by AAPH. Minimum loss of fluorescence of β-phycoerythrin was observed at 153 ng of feruloylated oligosaccharides under the experimental conditions. The ORAC values indicated that the antioxidant capacity of free FA extracted by alkaline hydrolysis was ten times higher than that of the feruloylated oligosaccharides. These values are 67.9±0.3 and 6±0.1, μmole Trolox equivalents/milligram extracted compound, respectively. The enzymatically recovered FA showed some six times higher value (37.8±0.1) than the feruloylated oligosaccharides. A higher ORAC value of the FA fraction extracted from alkaline hydrolysis is expected since it breaks both the ester and ether linkages compared to an esterase action that is specific for ester-linkages.

Discussion

Otherwise annotated as a hypothetical α/β hydrolase, the TTE1809 gene locus in the *T. tengcongensis* genome was characterized to encode a new alkaline and thermostable feruloyl esterase that we named TtFAE.

TtFAE is a new member of the type A FAE that is characterized by its lipase-like sequence and activity towards model substrates such as MFA, MSA, and MpCA (Crepin et al. 2004). Like the prototype type A FAE, represented by *Aspergillus niger* FAEA, TtFAE exhibited weak or insignificant lipase activity (Aliwan et al. 1999). In

a phylogenetic analysis that consisted of FAEs of the known types A to D or subfamilies SF1-7 (Crepin et al. 2004; Benoit et al. 2008), TtFAE, together with the cinnamoyl esterases CinI and CinII from *B. fibrisolvens* and numerous hypothetical FAEs of bacterial origin, were found in a cluster that is adjacent to those of the type A FAEs (not shown). It is noteworthy that there is no strict clustering of type A FAEs according to sequence-function relationship. For a discussion of the evolutionary relationships, the notion of neofunctionalization, between fungal lipases and type A FAE family, readers are referred to Levasseur et al. (2006) and Vieites et al. (2010).

Triticale bran and straw were recently analyzed for their phenolic content, proanthocyanidins, and lignans by alkaline (2 M NaOH) extraction (Hosseinian and Mazza 2009). Among several phenolic acids, FA was most abundant and estimated at 97.1 mg/100 g in triticale bran and twice as much in the straw. In our study, enzymatic treatment (TtFAE together with xylanase) produced a comparable value of 68 mg FA/100 g from triticale bran. This represented 30% of the total FA extractable by NaOH. This sub-optimal hydrolysis has been encountered in other agricultural feedstocks, e.g., a combination of *Fusarium proliferatum* FAE and *T. lanuginosus* xylanase provided only about 35% recovery of FA from corn bran (Shin and Chen 2006). Among various agricultural crops, corn residues (bran and fibers) appear to be the best possible source of FA (up to 3% by weight), and some recent efforts using either chemical or enzymatic methods to extract FA have been described (Akin and Rigsby 2008; Tilay et al. 2008; Knoshaug et al. 2008; Torre et al. 2008; Buranov and Mazza 2009).

Factors limiting efficient extraction of FA by enzymatic action on various biomasses have been discussed (Faulds et al. 1997, 2006; Shin et al. 2006; Panagiotou et al. 2007; Barberousse et al. 2009; Xiros et al. 2009). These include the complexity of the plant materials—compositional and linkage differences in the arabinoxylans—so that the right choice of enzymes is important. Faulds et al. (2006) reported that the source of xylanase is important as well as the type of FAE used in conjunction with the xylanase. For example, family 10 xylanases (specificity towards the substituted or decorated region of the arabinoxylan backbone) in conjunction with *A. niger* FAEA were more effective in releasing the 5,5′ form of diferulic acid from arabinoxylan derived from brewers' spent grain, whereas family 11 xylanases (cleaving the unsubstituted regions of the arabionoxylan backbone) preferentially released FA. In other studies, a multi-enzyme approach has proven successful by employing the full enzyme complement of the whole fungal system, e.g., the filamentous fungus *Neosartorya spinosa* NRRL185 was able to release 99.5% FA from corn fibers (Shin et al. 2006); the crude enzyme extract from *Penicillium brasilianum* released over 70% of the available phenolic acids in a number of agricultural substrates (Panagiotou et al. 2007); and a 2.5-fold increase of FA hydrolysis from Brewer's spent grain using *Fusarium oxysporum* instead of a two-enzyme cocktail (a recombinant type C-FAE and a *Trichoderma* xylanase) was also demonstrated (Xiros et al. 2009).

Antioxidant activity in cereal grains and other plant products is an important biological property that has health benefits upon consumption by protection from various oxidative damages (Balasundram et al. 2006; Yu 2008). The antioxidant potential of the feruloylated oligosaccharides prepared from triticale bran was evaluated for the first time. The resulting Trolox equivalents, together with the study carried out by Hosseinian and Mazza (2009) on alkaline-extracted FA, demonstrated the potential of triticale bran (and straw) as a valuable bioresource for antioxidant.

Following the completion of this work, Royter et al. (2009) reported the cloning of two thermostable lipases, one, designated as LipCst, originating from the same source as TtFAE. Our studies differ in terms of assay conditions and the type of substrates examined, particularly, the methyl-substituted phenolic esters and feruloylated oligosaccharides were not examined by Royter et al. (2009). Otherwise, the physiochemical properties of the enzyme in terms of thermostability and pH profile are very similar. An added property of LipCst, assayed with *p*-NP-palmitate, was its solvent tolerance to *tert*-butanol, ethanol, acetonitrile, isopropanol, pyridine, and acetone, up to a concentration of 99% (*v*/*v*) and also 50% (*v*/*v*) DMSO, making it a solvent-stable biocatalyst.

In summary, a new alkaline and thermostable FAE (TtFAE/LipCst) of the α/β hydrolase fold-superfamily is now available for optimization and further exploration. The FAE family of enzymes holds promise in biomass deconstruction and application in both the agri-food and pharmaceutical industries (Benoit et al. 2006, 2008; Wong 2006; Fazary and Ju 2007; Topakas et al. 2007; Koseki et al. 2009a).

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