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BIOCATALYSIS

Cloning of a novel feruloyl esterase gene from rumen microbial metagenome and enzyme characterization in synergism with endoxylanases

Dominic W. S. Wong · Victor J. Chan · Hans Liao · Mary J. Zidwick

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Abstract A feruloyl esterase (FAE) gene was isolated from a rumen microbial metagenome, cloned into *E. coli*, and expressed in active form. The enzyme (RuFae2) was identified as a type C feruloyl esterase. The RuFae2 alone released ferulic acid from rice bran, wheat bran, wheat-insoluble arabinoxylan, corn fiber, switchgrass, and corn bran in the order of decreasing activity. Using a saturating amount of RuFae2 for 100 mg substrate, a maximum of 18.7 and 80.0 μg FA was released from 100 mg corn fiber and wheat-insoluble arabinoxylan, respectively. Addition of GH10 endoxylanase (EX) synergistically increased the release of FA with the highest level of 6.7-fold for wheat bran. The synergistic effect of adding GH11 EX was significantly smaller with all the substrates tested. The difference in the effect of the two EXs was further analyzed by comparing the rate in the release of FA with increasing EX concentration using wheat-insoluble arabinoxylan as the substrate.

Keywords Feruloyl esterase · Ferulic acid esterase · Ferulic acid · Metagenomics · Corn fiber · Arabinoxylan

D. W. S. Wong (✉) · V. J. Chan
Western Regional Research Center, USDA-ARS, Albany, CA, USA
e-mail: dominic.wong@ars.usda.gov

H. Liao
OPX Biotechnologies, Boulder, CO, USA

M. J. Zidwick
Cargill Biotechnology Development Center, Excelsior, MN, USA

Introduction

Polysaccharide-bound hydroxycinnamic acids are known to be widely present in the cell walls of many plant species. The lignocelluloses of monocotyledons such as grasses (Poaceae) are particularly rich in ferulic acid (FA, 3-methoxy-4-hydroxy-cinnamic acid) ester-linked to glucuronoarabinoxylans in the hemicellulose fraction [8, 18, 30]. In cereals such as corn, wheat, and barley, FA is ester-linked via its carboxylic acid group to the C(O)5 position of the arabinofuranosyl side group attached at the C(O)2 to the xylan chain. In dicotyledons, such as spinach and sugar beet, FAs are found ester-linked to pectic polysaccharides at the C(O)6 of galactosyl and C(O)2 or C(O)3 position of the arabinosyl substituent. Ferulic acid also forms cross-links via oxidative coupling through either its aromatic ring or aliphatic side group, resulting in polysaccharide–polysaccharide, polysaccharide–protein, and polysaccharide–lignin crosslinks. The covalent crosslinks mediated by FA cause the cell wall polymers to assemble in extended networks, contributing to a major barrier in the conversion of plant biomass for bioenergy production [2, 19].

Bacteria and fungi produce feruloyl esterases (EC 3.1.1.73) that are able to cleave the ester bond between FA and polysaccharide. Enzymatic hydrolysis of FA ester linkages has been shown to improve biodegradation of corn stover and grass lignocellulose materials to fermentable sugars [1,3–17]. The action of feruloyl esterase is enhanced in synergism with endo-xylanases, because short-chain feruloylated xylooligosaccharides are more accessible as FAE substrates compared to the xylan polymer [11, 27, 30]. It has been suggested that hemicellulases and accessory enzymes may be used to enable less severe pretreatment conditions in biomass conversion and thus enhance the efficiency of the process [23, 24]. The products

generated from the degradation of feruloyl xylan, including ferulic acids, ferulate oligomers, and feruloyl xylooligosaccharides, are known to have potential applications for pharmaceutical and food uses [5]. Ferulic acid can be a precursor of vanillin via microbial biotransformation. The use of enzyme technology in their production and manufacturing represents a unique aspect of value-added utilization of agricultural waste and biomass materials.

Direct cloning of metagenomes of uncultured microflora has proven to be a powerful tool for exploring the sequence space of a microbial community and for discovering new genes and biocatalysts with potential industrial and biotechnological applications [6,27–31]. The objective of this paper is to report the screening for a FAE gene from rumen microbial metagenome, cloning and expression of the gene in *E. coli*, purification and characterization of the recombinant enzyme, demonstration of continuous release of FA from natural substrates, and its synergistic action with two families (GH10, GH11) of EXs.

Materials and methods

Genomic DNA library construction and screening

Genomic DNA was extracted and purified collectively from the microflora of a cow's rumen using a genomic DNA extraction kit (Q-biogene, Irvine, CA, USA) according to the supplier's protocol. The genomic DNA was partially digested by *Eco*RI, sized for ~8 kb fragments by agarose gel electrophoresis, ligated into λZAPII vector, and packaged into Gigapack III (Stratagene, La Jolla, CA, USA). The resulting genomic DNA library was amplified and screened for enzyme activity using *p*-nitrophenyl ferulate as the substrate following procedures described previously [33–36].

Subcloning, expression, and purification

The identified gene was amplified by PCR and cloned into the pET29b vector, which contains a C-terminal His-tag (Novagen, Madison, WI, USA). Transformants in *E. coli* BL21(DE3) were screened by halo formation using LB agar plates supplemented with kanamycin and 0.1 % ethyl ferulate. Positive clones were further confirmed by liquid assay of FAE activity using *p*-nitrophenyl ferulate as the substrate. A single positive transformant was used to inoculate 2 ml of LB and incubated overnight at 37 °C at 225 rpm. A 2-ml overnight culture was transferred to 200 ml of fresh LB, and incubated for 3 h to an OD_{600nm} of 0.7. Induction of protein expression was initiated by the addition of 0.1 mM of IPTG, and incubation was allowed to proceed for 4 more hours at 30 °C. Thereafter, the cells were pelleted and lyzed using 8 ml of primary amine-free Bug-Buster reagent (Novagen, Madison, WI, USA). The extracted protein was purified by Ni Sepharose affinity chromatography, using 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4 binding buffer, and the same buffer containing 500 mM imidazole as the elution buffer. Collected 1-ml fractions were analyzed for purity by SDS-PAGE. The buffer of the combined active fractions was exchanged into 20 mM sodium phosphate buffer, pH 7.4 with 10 % glycerol, and concentrated by ultrafiltration [33, 34].

Electrophoresis

The purified and concentrated enzyme was run on a 4–12 % bis–Tris NuPAGE gel using 50 mM MOPS, 50 mM Tris, and 0.1 % SDS, at 200 V constant for 1 h, and stained with SimplyBlue SafeStain (Invitrogen). For molecular weight determination, the protein marker bands were analyzed by image analysis software (Alpha Innotech, San Leandro, CA). For p*I* determination, the sample was run on an electrofocusing gel (pH 3–10, Invitrogen) according to the supplier's procedure. Serva IEF markers 3–10 mix was used (Heidelberg, Germany).

Enzyme activity

Typically, the enzyme reaction mixture consisted of 100 mg of various natural substrates, 3–9 μg of FAE in 0.5 ml 100 mM phosphate, pH 7.0, incubated at 37 °C for 2 h. Enzyme activity unit was expressed as μg FA released from 100 mg substrate per min measured by HPLC analysis. The system consists of a Gilson 307 pump equipped with a Brownlee analytical C18 5 μ ODS column (260 × 4.6 mm). The mobile phase consisted of water/formic acid/acetonitrile (7:1:2 v/v) at a flow rate of 0.2 ml/min and at ambient temperature. The absorbance detector was set at a wavelength of 310 nm. Ferulic acid standards were prepared by saponification of corn bran, followed by TLC separation and HPLC purification [26].

Optimum pH and temperature

For the measurement of pH optimum, the reaction mixture was incubated for 2 min at 37 °C, with various substrates in pH of 5–9 buffers (sodium citric pH 5, sodium phosphate pH 6–8, glycine pH 9). For determining pH stability, the enzyme was incubated at various pH at 20 °C for 16 h, reconstituted to pH 7.0 with 100 mM phosphate buffer, and the residual activity was measured by adding the substrate. To determine the temperature optimum, the reaction mixture was incubated for 2 h in 100 mM phosphate buffer, pH 7.0. Temperature stability was measured by incubating the enzyme at various temperatures from 20 to 60 °C for 15 min, after which the substrate was

added, and the residual activity was determined. For qualitative analysis in some cases, the released of FA was analyzed by TLC (10 × 10 cm HPTLC silica gel 60 w/uv254), developed in benzene/dioxane/acetic acid (90:25:4).

Activity comparison on different natural substrates

The release of FA from corn fiber (Cargill, Wayzata, MN, USA), corn bran, wheat bran, rice bran (Bob's Red Mill, Milwaukie, OR, USA), wheat-insoluble arabinoxylan (Megazyme, Wicklow, Iceland), and switchgrass (ARS Grain, Forage & Bioenergy Research Unit, Lincoln, NE, USA) was compared using the protocol outlined below, all performed under the same reaction conditions.

Alkali hydrolysis of the substrates

To determine the total alkali-extractable FA of the substrate, 1.5 ml of 1 M NaOH was added to 100 mg substrate, followed by incubation at 37 °C for 24 h. After centrifugation, the supernatant was neutralized using 6 N HCl, and the FA content was measured by HPLC. For TLC analysis, the supernatant was adjusted to pH < 2, extracted with ethyl acetate, dried over Na_2SO_4, evaporated under N_2, and reconstituted in 1:1 MeOH/H_2O.

Synergistic activity with addition of other xylanolytic accessory enzymes

For 100 mg of various natural substrates, 0.2 nmol of RuFae2 was added, supplemented with pure EX (from *Cellvibrio mixtus* or *Trichoderma longibrachiatum*) (Megazyme, Wicklow, Ireland). The mixture was incubated for 2 h in a 37 °C shaker bath.

Preincubation with endoxylanase followed by addition of RuFae2

Endoxylanase was added at 0.15 or 0.40 nmol to 20 mg of wheat-insoluble arabinoxylan in 0.7 ml final volume of 100 mM K_2HPO_4 buffer, pH 7.0. The mixture was preincubated for 30 min in a 37 °C shaker, followed by addition of 0.2 nmol of RuFae2. The reaction mixture was further incubated for 30 min. For control samples, both EX and RuFae2 were added in the initial reaction under the same incubation conditions. The amount of FA released with and without preincubation with EX was determined by HPLC.

Bioinformatics

Geneious and Vector NTI were used for sequence analysis. Homology modeling was accomplished by the use of Swiss Model [4].

Results

Isolation and cloning of RuFae2 gene

The genomic fragment isolated from the metagenomic clone is 3,466 bp long containing a 792-bp open reading frame, with an upstream transcription element TACAAT Pribnow box located in the promoter region. The structural gene encodes a protein of 263 amino acids, with a predicted molecular mass of 28.92 kD and a p*I* of 7.70. The N-terminus is devoid of a signal peptide, suggesting the gene product was not secreted. BLAST search reveals that the amino acid sequence is closely related to the primary structures of FAE from *Prevotella oris* C735 (41.9 % identities, 42.3 % positives) and *Lactobacillus johnsonii* (47.0 % identities and 42.2 % positives) (Fig. 1). The rumen enzyme (RuFae2) also aligns with a hydrolase of α/β family recently isolated from *Prevotella bryantii* B14 (45.4 % identities and 48.8 % positives). The structure revealed by homology modeling [4] shows a typical α/β hydrolase fold as observed in various esterases and lipases. RuFae2 is structurally closely related to the *L. johnsonii* enzyme (3pf9A), which consists of a central β sheet of eight parallel strands flanked by α-helices with a small α/β lid domain above the active site pocket [22]. Similar architecture has also been characterized for the feruloyl esterase (Est1E) from the rumen bacterium *Butyrivibrio proteoclasticus* [16] and AnFae enzyme from *Aspergillus niger* [20].

Enzyme activity on synthetic substrates

The purified protein of RuFae2 was estimated by SDS-PAGE, a molecular mass of 29 kD, corresponding to the predicted value. Isoelectrofocusing gel showed a basic protein with a p*I* of 8.5 (data not shown). RuFae2 acted on all four hydroxycinnamic acid methyl esters, methyl ferulate (MFA), methyl *p*-coumarate (MpCA), methyl sinapinate (MSA), and methyl caffeate (MCA), liberating the corresponding acids (Fig. 2). It did not release diferulic acid from the natural substrate corn fiber as also confirmed by HPLC analysis. According to the classification system described by Crepin et al. [10], RuFae2 is a type C feruloyl esterase.

Enzyme activity on natural substrates

The purified enzyme was characterized using corn fiber as the substrate. It exhibited a pH optimum at pH 7, and was relatively stable from pH 5–8, retaining more than 90 % of the activity (37 °C, 2 h) using corn fiber as the substrate (Fig. 3a). The temperature optimum occurred at 50 °C, and the enzyme was stable at ≤50 °C. The enzyme retained 26 % activity after incubation at 60 °C at pH 7 for

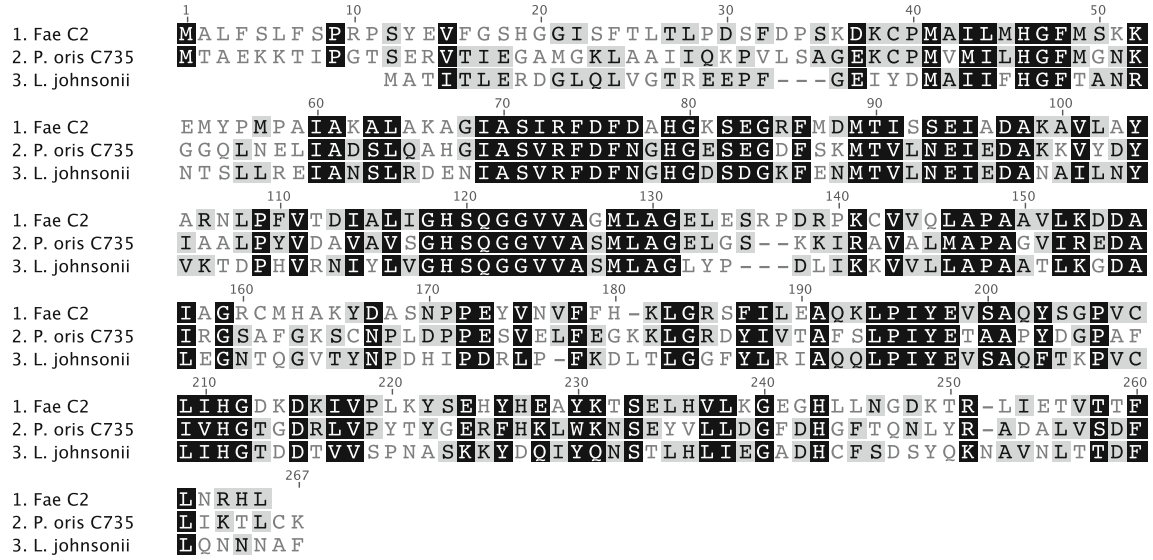

Fig. 1 Multiple sequence alignment of RuFae2 with *Prevotella oris* C735 (ZP_07034946), *Lactobacillus johnsonii* (ADD11991) feruloyl esterases using Geneious software

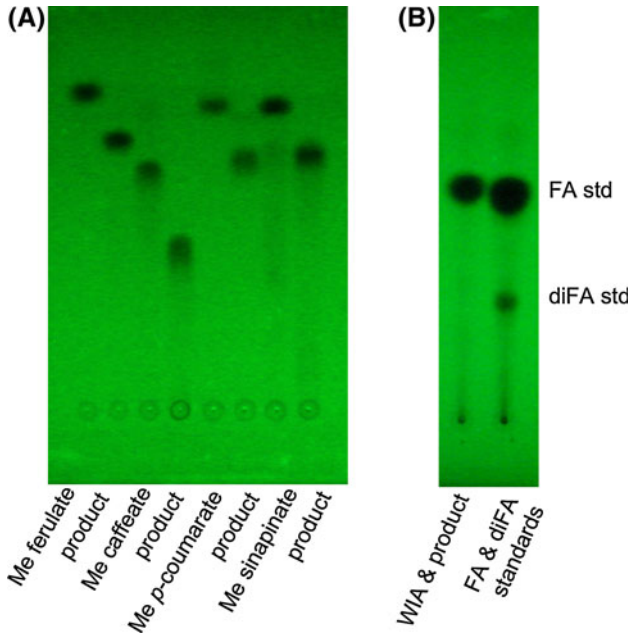

Fig. 2 TLC separation of substrate and product components: **a** *Lanes 1, 2* methyl ferulate (MFA) ester substrate and acid product, 3, 4 methyl caffeate (MCA) ester substrate and product), 5, 6 methyl *p*-coumarate (MpCA) ester substrate and product), 7, 8 methyl sinapinate (MSA) ester substrate and product) in RuFae2-catalyzed reactions; **b** *Lane 1*: wheat-insoluble arabinoxylan and FA products using RuFae2 and endoxylanases: *Lane 2*: FA and diFA as standards. Reaction conditions: 20 mM substrate in 50 mM sodium phosphate buffer, pH 6.0, 1 μg RuFae2, 30 °C, 30 min (see details in the "Materials and methods" section)

15 min (Fig. 3b). The enzyme was most active on wheat bran, followed by rice bran, corn fiber, wheat-insoluble arabinoxylan, corn bran, and switchgrass, with enzyme activity of 10.3, 8.6, 7.8, 7.4, 2.2, and 0.5 U/mg, respectively (Fig. 4).

A comparison of alkali extraction and RuFae2 hydrolysis on several natural substrates using a constant amount of RuFae2 and substrate is presented in Table 1. The highest recovery was observed in rice bran followed by wheat bran, wheat-insoluble arabinoxylan, corn fiber, switchgrass, and corn bran, with 5.46 and 5.33, 1.82, 0.57, 0.15, and 0.14 % of the total ferulic acids released, respectively. The concentration effect was studied by increasing the amount of the enzyme using the same amount of corn fiber and wheat-insoluble arabinoxylan at constant reaction conditions. A rapid release of FA from corn fiber was observed proportional to the enzyme added to the corn fiber at concentrations up to 45 μg/100 mg CF (Fig. 5a). A steeper and more linear slope of 0.19 + 0.01 was observed for the rate versus enzyme concentration up to 450 μg when wheat-insoluble arabinoxylan was the substrate (Fig. 5b). Using a saturating amount of 45 and 450 μg RuFae2, a maximum of 18.7 and 80.0 μg FA (1.3 and 18.2 % of total FA) was released from 100 mg of corn fiber and wheat-insoluble arabinoxylan, respectively.

Synergistic action with endoxylanases

To investigate the action of RuFae2 in synergism with endo-xylanases, GH10 and GH11 EXs were individually added to the reaction of various substrates with a constant concentration of RuFae2. Using reaction mixtures of 0.6 nmol EX, 0.2 nmol RuFae2, and 100 mg substrate, addition of the GH10 EX (from *Cellvibrio mixtus*) enhanced the release of FA from corn bran, corn fiber,

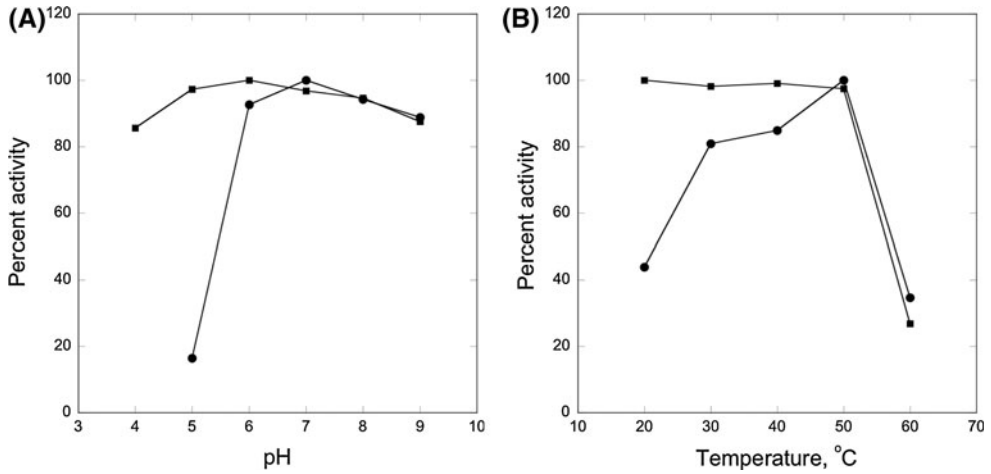

Fig. 3 **a** Effect of pH on optimum activity and stability. Reactions were conducted for 2 h in a 37 °C shaker bath using corn fiber as the substrate in variable pH buffers. For pH stability measurements, the enzyme was preincubated at various pH buffers at 20 °C for 16 h, reconstituted to pH 7.0, and assayed for residual activity of FA release. **b** Effect of temperature on optimum activity and stability. For temperature optimum, the reaction was incubated in 100 mM K_2HPO_4 buffer, pH 7.0 for 2 h at variable incubation temperature in shaker baths. For temperature stability, the enzyme was incubated in the buffer at a specific temperature for 15 min, before assay for residual activity of FA release. Activity of RuFae2 was equal to 6.38 ± 0.15 μg FA per 100 mg CF. *Circles*: pH and T optima. *Squares*: pH and T stability

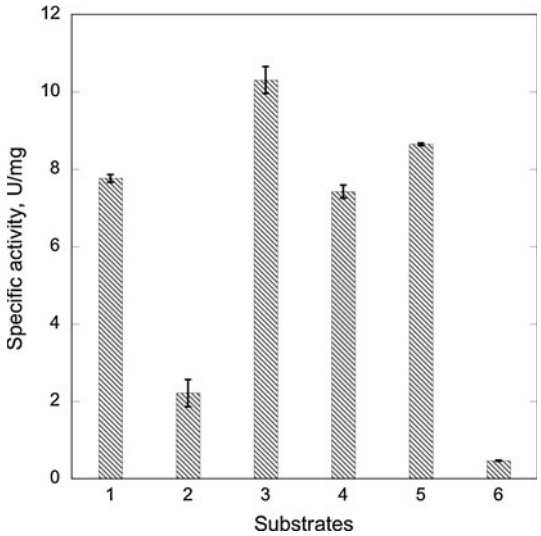

Fig. 4 Specific activity of RuFae2 on natural substrates: 1. corn fiber, 2. corn bran, 3. wheat bran, 4. wheat-insoluble arabinoxylan, 5. rice bran, 6. switchgrass. Reaction conditions: 100 mg substrate, 0.3 nmol RuFae2, 0.5 ml 100 mM K_2HPO_4, pH 7.0, incubated at 37 °C for 2 h

Table 1 Alkaline versus enzymatic hydrolysis of ferulic acid from natural substrates

Substrates	μg ferulic acid per 100 mg of substrate		
	Alkaline extraction	RuFae2 hydrolysis	%
Corn fiber	1,475.5 ± 86.0	8.4 ± 0.1	0.57
Corn bran	1,751.3 ± 100.8	2.4 ± 0.4	0.14
Wheat bran	208.1 ± 13.2	11.1 ± 0.4	5.33
Wheat-insoluble arabinoxylan	440.0 ± 8.0	8.0 ± 0.2	1.82
Rice bran	170.3 ± 7.4	9.3 ± 0.1	5.46
Switchgrass	335.3 ± 42.2	0.5 ± 0.1	0.15

Alkaline extraction: 100 mg substrate, 1.5 ml 0.1 N NaOH, incubated at 37 °C for 24 h. Enzyme reaction conditions: 100 mg substrate, 0.3 nmol RuFae2, 0.5 ml 100 mM K_2HPO_4, pH 7.0, incubated at 37 °C for 2 h. Right column % = (FA released by RuFae2 hydrolysis / FA measured by alkaline extraction) × 100

switchgrass, wheat-insoluble arabinoxylan, and wheat bran in increasing order with the highest 6.73-fold increase for wheat bran, followed by 2.76 for wheat-insoluble arabinoxylan (Table 2). Using the same molar concentration of the GH11 EX (from *Trichoderma longibrachiatum*), the enhancement of FA release showed a similar pattern, but the magnitude was significantly less than that of the GH10 enzyme in all cases. The synergistic effect was not observed for rice bran with the addition of either enzyme.

The concentration effect of the two EXs on the synergistic action was further studied using wheat-insoluble arabinoxylan (WIA) as the substrate. The synergistic effect of adding varying concentration of 0.06—1.92 nmol the GH10 EX to a constant amount of RuFae2 (0.2 nmol) and WIA (100 mg) showed a linear correlation to FA released (Fig. 6a). The slope of the curve showed an increase of 13.7 μg of FA (released from 100 mg substrate) per nmol EX added. In the case of the GH11 EX, a minimal increase (~1 μg) in FA was observed initially but the curve remained flat with further addition of the enzyme. The extent of hydrolysis of WIA xylan chain was analyzed for

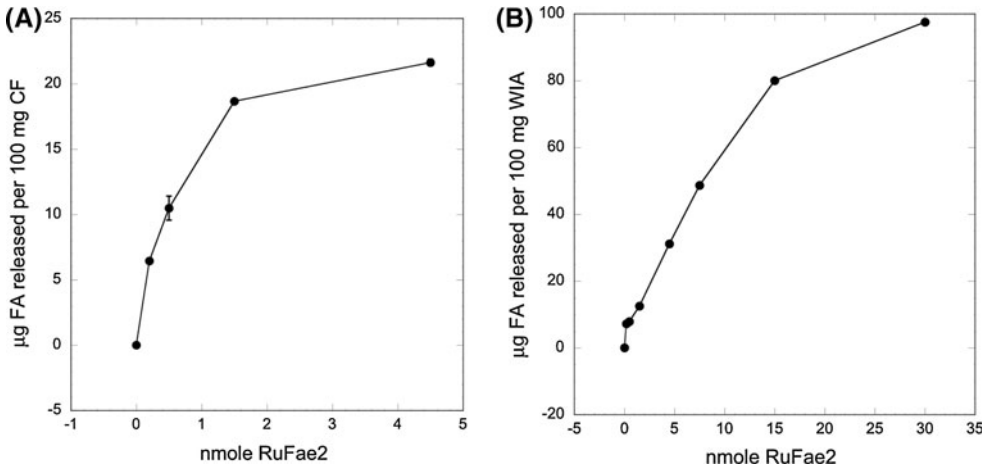

Fig. 5 Effect of enzyme concentration on FA release from **a** corn fiber **b** wheat-insoluble arabinoxylan. Reaction conditions: 100 mg substrate, 0.2—30 nmol RuFae2, 0.5 ml 100 mM K_2HPO_4, pH 7.0, incubated for 2 h in a 37 °C shaker bath

Table 2 Release of FA from natural substrates by RuFae2 in synergism with endoxylanases

Substrate	GH10 EX (*C. mixtus*)	GH11 EX (*T. longibrachiatum*)
Corn fiber	1.21 ± 0.08	1.08 ± 0.02
Corn bran	1.19 ± 0.03	1.08 ± 0.20
Wheat bran	6.72 ± 0.23	1.41 ± 0.10
Wheat-insoluble arabinoxylan	2.72 ± 0.01	1.14 ± 0.04
Switchgrass	1.38 ± 0.05	1.11 ± 0.04

Reaction conditions: 0.2 nmol RuFae2, 0.6 nmol endoxylanase, and 100 mg substrate in 0.7 ml 0.1 M K_2HPO_4, pH 7.0 buffer, incubation at 37 °C for 2 h. Fold increase based on comparing with the hydrolysis by RuFae2 alone

the same reactions. Both the GH10 and GH11 EXs hydrolyzed the xylan chain with similar activity producing comparative amount of reducing xylose equivalents (Fig. 6b).

Effect of preincubation with EX on RuFae2-catalyzed release of FA

The effect of preincubation with EX (GH10 or GH11) endoxylanase on the release of FA by RuFae2 is presented in Fig. 7. Preincubation with EX increased the amount of FA released by RuFae2. The increase was 2.43 ± 0.11 and 1.72 ± 0.25-fold for the GH10 and GH11 enzymes, respectively. The result also confirms that the GH10 enzyme showed a synergistic effect (with RuFae2 in the release of FA) significantly greater than that of the GH11 EX, with or without preincubation.

Discussion

The characterization of RuFae2 was conducted using natural substrates, which are all insoluble. The use of synthetic substrates in one experiment was primarily for the purpose of identifying the enzyme as a type C feruloyl esterase according to the classification scheme of FAE enzymes [10]. Our previous study on *Aspergillus niger* AnFae has shown the importance of using natural substrates for studying enzyme properties [32]. The use of synthetic substrate such as methyl ferulate would not provide results representative of the conditions in the natural and physiological environment relevant to bioprocessing conditions. Synthetic substrates differ from naturally occurring substrates in terms of the type and accessibility of the ester bonds being hydrolyzed, and their reactions are unlikely to reveal the true enzyme action on natural substrates.

The present study suggests that a small fraction of the total ferulate content in the substrate could be recovered by using the esterase enzyme alone. The % recovery of FA in rice bran and wheat bran falls within the range reported in literature. It has been shown that *Aspergillus niger* AnFae alone releases a maximum of 4 % of the FA present in destarched wheat bran [13]. The types of FA bonds could explain to some extent the limitation in the use of FAE alone to recover FA from biomass materials. Alkali extraction releases both ester-linked and ether-linked ferulates. FAE-catalyzed hydrolysis, however, acts only on the ferulic acid ester-linked to arabinosyl residues in the xylan, but not on the ether linkages between ferulic acid and lignin. Furthermore, ferulic acids are also known to link to pectins and proteins, which could not be hydrolyzed by FAE enzymes [18, 30]. An additional restriction is due to the fact that cell wall-bound ferulic acids are embedded in the lignocellulosic matrix and therefore largely inaccessible and resistant to enzymatic hydrolysis. In fact, the variations observed in the activity on the different substrates as presented in Fig. 5 are mostly indicative of compositional and structural differences among the plant materials. The local environment and arrangement of the ferulic acids as well as other side groups along the xylan chain may influence the hydrolytic action of the enzyme

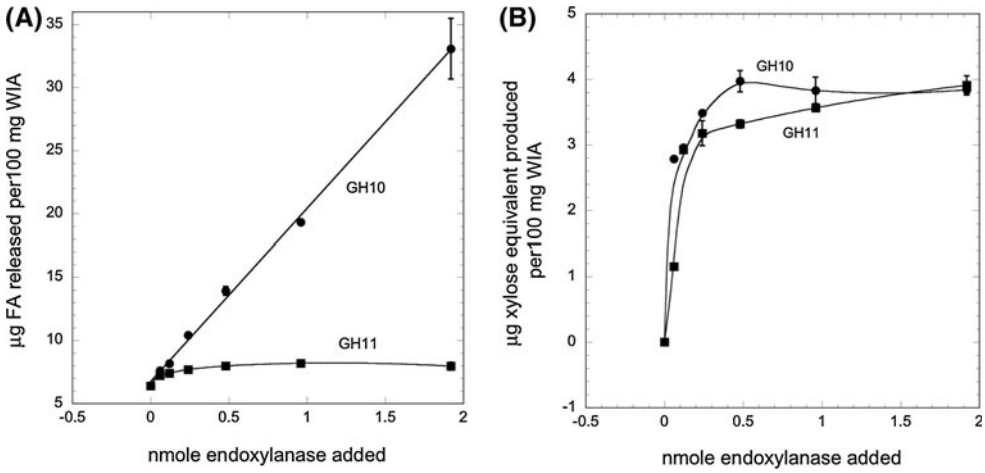

Fig. 6 Concentration effect of endo-xylanases on **a** RuFae2-catalyzed release of FA from WIA analyzed by HPLC **b** hydrolysis of WIA analyzed by DNSA. Reaction conditions: 20 mg wheat-insoluble arabinoxylan, 0.2 nmol RuFae2, 0.06—1.92 nmol endo-xylanase, 0.7 ml 100 mM K_2HPO_4, pH 7.0, incubated at 37 °C for 2 h. The zero point on the x-axis: RuFae2 alone was used (with no addition of endoxylanase)

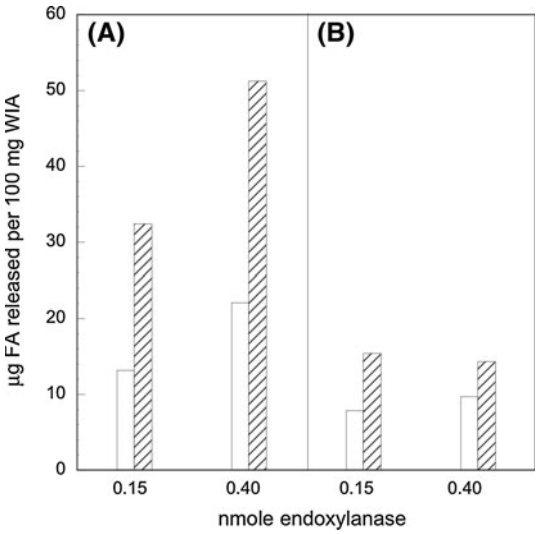

Fig. 7 Effect of preincubation with **a** GH10 and **b** CH11 endoxylanase on RuFae2-catalyzed release of FA. Reaction conditions: 20 mg wheat-insoluble arabinoxylan preincubate with 0.15 or 0.40 nmol endoxylanase at 37 °C for 30 min, followed by 0.2 nmol RuFae2 for another 30 min. Total reaction volume was 0.7 ml. Buffer used was 100 mM K_2HPO_4, pH 7.0. Control: RuFae2 and endoxylanase were added together in the initial reaction. Symbols: *line-shaded* preincubation, *no shade* no preincubation

[14]. The insolubility nature of the substrate also imposes limitation on accessibility for the enzyme to initiate hydrolysis.

Various studies have shown that FAE activity can be enhanced by the synergistic action of endoxylanases because shorter-chain feruloylated xylooligosaccharides are hydrolyzed at faster rates than the polymeric substrates [12, 13]. The addition of other xylanolytic (accessory) enzymes to further remove the various side groups from the xylan main chain may also synergistically increase the hydrolysis of the xylan chain and in turn the breakdown of ferulic acid linkages [11,21–29]. The synergistic effect of adding EXs depends on (1) the type and the degree of side group decoration on the xylan chain of the substrate (2) physical properties of the substrate, including solubility/insolubility, particle size, contaminating components, and (3) the ability of the enzyme to accommodate the various aspects of variability and catalyze degradation of the heteroxylan substrate.

The present study on adding two different families of EXs to RuFae2 yielded a wide variation in FA release among various substrates. These results (as presented in Table 2) underscore the importance of consideration of the variability of the nature of the substrates in assessing the synergistic interactions of FAE with EXs and other accessory enzymes. The complication entailed from the obvious structural differences of the xylan chain, variations in side-group substitutions, and heterogeneous nature in the substrates has prompted us to use WIA for further study of the concentration effect of adding EX enzymes. WIA is a xylan preparation purified to remove starch, β-glucan and protein from wheat flour, and would provide a more suited substrate, allowing better interpretation in the study of synergism between RuFae2 and EXs.

Comparative study of the two EXs using WIA as the substrate unequivocally showed the overwhelming advantage of the GH10 enzyme. The release of FA increased continuously with increased concentration of the GH10 EX, whereas for the GH11 EX, the curve reached a plateau at a very lower concentration (Fig. 7a). There have been reports that the two major families of EXs, GH10 and GH11, differ considerably in their action on heteroxylan. GH10 EXs have the ability to cleave glycosidic linkages in the xylan chain closer to the substitutions, forming smaller oligosaccharide products [7, 9]. This observation is ascribed to the substrate binding site of GH10 EXs, which can accommodate binding of xylose residues decorated with various side chains, including arabinose (Araf), methylglucuronic acid (MeGlcA), and feruloyl-arabinose (Araf-FA) [25]. In contrast, GH11 EXs are not able to

accommodate side chains of the heteroxylan backbone, and exhibit a higher substrate specificity requiring unsubstituted regions for cleavage [28]. The inability to accommodate decorated xylan backbone would result in a smaller number of cleavages, and therefore adding GH11 EX in increasing amount would not increase the production of FA in the reaction. On the other hand, the GH10 EX can cleave glycosidic bonds close to side group substitutions on the xylan chain, producing smaller fragments with a greater number of cleavages, and generating increasingly shorter feruloyl oligosaccharides that are more susceptible to the action of the FAE enzyme. The formation of reducing xylose equivalents as measured by DNSA suggests that there was indeed a difference in the total *amount* of oligosaccharide fragments formed by the two enzymes, but insufficient to explain the large difference observed in the synergistic effect of the two EXs on the release of FA (Fig. 7a, b).

One likely explanation could be that the hydrolysis product feruloyl xylooligosaccharide formed by the two EX enzymes might have different structural arrangement and thus affect the action of RuFae2 in the cleavage of the FA ester bond. It has been reported that in the reaction with GH10 EXs, the major decorated xylooligosaccharide product from arabinoxylan consists of Araf (or Araf-FA) group linked at the non-reducing end [7, 38]. In contrast, GH11 EXs produce xylooligosaccharides with substitutions at internal positions due to inability of the enzyme to cleave the xylan chain at glycosidic bonds close to the side group. The mode of action by these two families of EXs producing products of varying architecture have been observed in the hydrolysis of glucuronoxylan, and recent structural analysis shows that α-glucuronidases can only remove the uronic acid when it is linked to the non-reducing end of the glucuronyl xylooligosaccharide [7, 15, 25]. An analogy may be drawn for xylooligosaccharide product with the Araf-FA substitution linked at the non-reducing end. It is likely that RuFae2 could hydrolyze only or cleave at a higher rate end-linked FA groups from xylooligosaccharides, whereas FA linked to the xylose sugar other than the end position may produce a dead-end reaction product [25]. Further investigations will be required to confirm the enzyme's preference on end-linked Araf-FA substitution, and also to answer the question whether this phenomenon generally holds for all FAE enzymes.

Acknowledgments Reference to a company and/or products is only for purposes of information and does not imply approval of recommendation of the product to the exclusion of others that may also be suitable. All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

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