Hydrolysis of Ferulic Acids in Corn Fiber by a Metagenomic Feruloyl Esterase

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A feruloyl esterase (FAE) gene was isolated from rumen microbial metagenome that consisted of 774 bp encoding 258 amino acid residues. The gene was subcloned into pET 32b vector, expressed in *Escherichia*. *coli*, and the enzyme was purified in active form. Homology modeling showed that the FAE contained the catalytic triad composed of Ser_{80}-His_{236}-Asp_{177}, and a classical Gly-X-Ser-X-Gly nucleophile motif commonly found in esterases. Under optimum pH and temperature (pH 7.0, 40 °C), 1 nmole FAE catalyzed the release of 19.75 ± 0.24 µg ferulic acid (FA) from 100 mg corn fiber (CF) in 1 h, which represents 3.5% of FA present in saponified CF. Addition of GH10 endoxylanase (XYN) to 0.5 nmole FAE enhanced the yield by 1.1 fold, equivalent to a 5% increase in FA release. Using CF pretreated with hot water, the synergistic effect of adding XYN resulted in 59.0 ± 0.2 mg FA/100 mg CF (5 nmole XYN, 0.5 nmole FAE), equivalent to a 4-fold increase compared to using the untreated CF substrate under the same reaction conditions.

Keywords: Feruloyl esterase; Ferulic acid esterase; Ferulic acid; Metagenomics; Corn fiber

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INTRODUCTION

In the wet-milling process, the corn is steeped, coarsely ground, and processed with hydrocyclones to remove the germ (for corn oil), finely ground again to release the starch and gluten (zein and glutelin) from the fiber, and filtered over screens to catch the fiber, which is then pressed and dried for use in animal feed. Corn fiber (CF) is a mixture of coarse fiber from kernel pericarp or hull and fine fiber from endosperm cellular materials in the corn kernel. The major components consist of 50% hemicellulose, 20% cellulose, 11% to 23% starch, 15% protein, 13% lignin and ash, and 2% to 3% oil. In 2017 (NASS), 6.7×10^5 tons of corn was wet-milled. The corn fiber is produced in yields of 80 to 110 g/kg total corn kernel. The corn-ethanol industry produces approximately 6.4×10^4 tons of corn fiber yearly. The corn fiber is almost completely insoluble because of the hemicellulose fraction, which has a complex structure of heteroxylan, consisting of a β-(1,4)-xylopyranosyl (Xyl*p*) backbone attached with a variety of side groups (Biely *et al.* 2016). Over 70% of the Xyl*p* residues have arabinofuranosyl, acetyl, and glucuronyl side units. Oligosaccharide side chains containing galactose, xylose, and arabinose are also found. Some of the arabinofuranosyl side units are ester-linked with ferulic acid moieties. Due to the complexity of this structural architecture, CF is highly recalcitrant to enzymatic degradation. There have been efforts to characterize the remaining (non-degraded) oligosaccharides that are resistant to mild acid pretreatment and simultaneous saccharification and fermentation (as used in corn ethanol production). It has been shown

that resistant oligosaccharides represented 50% of the starting material, which survives pretreatment, enzymatic hydrolysis, and fermentation. All of these complex xylan oligomers were found to contain ferulic acid, diferulates, acetic acid, galactose, arabinose, and uronic acid groups (Appeldoorn *et al.* 2010). A common feature was (part of) an α-L-galactopyranosyl-(1,2)-β-D-xylopyranosyl-(1,2)-5-*O*-*trans*-feruloyl-L-arabinofuranosyl side chain attached to the *O*-3 position of the β-1,4 linked xylose residue (Agger *et al.* 2010; Appeldoorn *et al.* 2013). The recalcitrance of CF hemicellulose can be ascribed to feruloylation of the side units and/or backbone of the xylan structure, which have not received adequate attention in enzymatic hydrolysis in biomass conversion.

A variety of bacteria and fungi are known to produce feruloyl esterases (FAE, EC 3.1.1.73) that catalyze the cleavage of feruloyl-arabinose (Araf-FA) ester bonds linking ferulic acids to arabino-furanosyl side groups of the xylan main chain. It has been proposed that accessory enzymes, including FAE, be used to augment the hydrolytic efficiency of cellulases and hemicellulases in biomass conversion, thus requiring less severe pretreatment conditions (Mosier *et al.* 2005; Akin 2007). Furthermore, FAE are known to act synergistically with endo-xylanases, because short-chain feruloylated xylo-oligosaccharides are better substrates with a higher rate of Araf-FA cleavage (Wong *et al.* 2013). Other xylanolytic accessory enzymes that act on various side chains may also enhance the hydrolysis of xylan main chain and in turn the ferulic acid linkage (Vardakou *et al.* 2004; Wong 2006). Direct cloning of metagenomes provides a powerful tool to explore the sequence space of unculturable microbial communities for novel gene discovery and biocatalyst development. The objective of this paper is to report the application of a metagenomic feruloyl esterase (FAE-C3) for the hydrolysis of CF, and characterize its synergistic action with endo-xylanases.

EXPERIMENTAL

Cloning of Feruloyl Esterase

Metagenomic DNA was isolated from microflora of a cow's rumen, and it was used to construct a λZAP library and screened for FAE enzyme activity. The identified gene was subcloned in pET32b and transformed in BL21. The protein was purified to homogeneity, using previously reported procedures (Wong *et al.* 2013, 2015; Shang *et al.* 2018).

Bioinformatics

Vector NTI (Informax, Bethesda, MD, USA) and Geneious (Biomatters Ltd., Auckland, New Zealand) were used for sequence analysis. The gene sequence was submitted to GenBank with the following accession number: MH785296.

Electrophoresis

The purified enzyme was run on a 4% to 12% Bis-Tris NuPAGE gel using a 50 mM buffer solution of 3-morpholinopropane-1-sulfonic acid (MOPS) at constant 100 V for 2 h and stained with SimplyBlue Safe stain (Invitrogen, Carlsbad, CA, USA). The protein and marker bands were analyzed by image analysis software (Alpha Inotech, AlphaImager, San Jose, CA, USA). For pI determination, the enzyme protein was run on an electrofocusing gel (pH 3 to 10, Invitrogen, Carlsbad, CA, USA) using Serva IEF markers 3−10 mix (Biophoretics, Reno, NV, USA, Heidelberg, Germany).

Enzyme Activity Measurement

A typical enzyme reaction mixture contained 100 mg CF, various nmole concentrations of FAE-C3 in 2 mL 50 mM K_2HPO_4, pH 7.0 buffer, and was incubated for 2 h in a 40 °C shaker bath. For all reactions, the CF was milled for three times for 10 s each (Micro-Mill, Technilab Instruments, Vineland, NJ, USA), washed 5 times with water, and oven-dried at 50 °C. Before using in enzyme reaction, the CF was exhaustively washed 4 times with water. Activity was expressed as μg FA released from 100 mg substrate per hour measured by high performance liquid chromatography analysis. The HPLC system (Gilson 307 HPLC, Middleton, WI, USA) consisted of a Gilson 307 pump equipped with a Brownlee analytical C18 m ODS column (260 × 4.5 mm), using a mobile phase of water/formic acid/acetonitrile (7/1/2 v/v) at a flow rate of 0.2 mL/min at ambient temperature. The UV-absorbance detector was set at 315 nm.

Optimum pH and Temperature

For pH optimum, the reaction mixture of 100 mg CF and 0.4 nmole FAE-C3 in 1 mL universal buffer of varying pH was incubated for 2 h in a 37 °C shaker bath. To determine temperature optimum, the enzyme reaction mixture was incubated in 100 mM K_2HPO_4 buffer, at pH 7.0 at temperatures from 20 °C to 70 °C for 2 h.

Hot Water Pretreatment of Corn Fiber

The pretreatment reactor tube with a working volume of 30 mL was made out of stainless steel with dimensions of 1" OD × 4.5" L × 0.065" thickness, capped with 1" swagelock end fittings. The CF (1.5 g) was soaked overnight in 28.5 mL H_2O, then autoclaved for 20 min at 121°C at 20 to 21 psi. The pretreated CF was washed 4 times with H_2O before using for enzyme reaction.

Alkali Hydrolysis of Corn Fiber

To 200 mg CF, 6 mL 1 N NaOH was added, and the mixture was incubated for 16 h in a 37 °C shaker bath (Bartolome *et al.* 1997). Following centrifugation, the supernatant was recovered, and adjusted to a pH ≤ 2 with HCl. Ferulic acid products were then extracted with ethyl acetate (3 × 3 mL), dried under N_2, and dissolved in CH_3OH:H_2O (1:1 v/v) for HPLC analysis.

Synergistic Activity with Added Accessory Enzymes

To 100 mg of CF, 0.5 nmole FAE-C3 was added, supplemented with endo-xylanase (XYN GH10 from *Cellvibrio mixtus*), α-L-arabinofuranosidase (ABF from *Bifidobacterium* sp.), and acetylxylan esterase (AXE from *Orpinomyces* sp.) (Megazyme, Bray, Ireland) in various nmole concentrations. The XYN, ABF, and AXE were added individually and in combinations to the reaction. The mixture was incubated for 2 h in a 40 °C shaker bath.

RESULTS AND DISCUSSION

Isolation, Cloning, and Bioinformatics of the FAE Gene

The genomic insert isolated from the metagenomic library contained (1) a TlpA-like sequence of 1,323 bp (136 amino acid residues) at the 5' end, and (2) an esterase

domain (FAE-C3) of 774 bp (258 residues). The FAE-C3 gene was subcloned into pET 32b vector as a fusion protein containing a thioredoxin (Trx) tag, a Met start codon and a 6xHis tag. A BLASTP search reveals the amino acid sequence related to the primary structures of *Prevotella oris* C735 feruloyl esterase (EF148533), *Lactobacillus johnsonii* cinnamoyl esterase (ADD11991), and *Bacteroides vulgatus* α/β fold hydrolase (WP_117885235) with identity percentages of 65.2%, 42.1%, and 59.2%, respectively (Fig. 1) (Chenna *et al.* 2003). Homology modeling showed an α/β hydrolase fold commonly observed in various esterases (Fig. 2) (Guex *et al.* 2009). The FAE-C3 is closely related to the *Lactobacillus johnsonii* feruloyl esterase (3S2Z) structure. The *L. johnsonii* enzyme has a 3D structure consisting of a central β sheet of eight parallel strands flanked by α-helices (Lei *et al.* 2011). Based on sequence and structural comparison, the FAE-C3 contains the catalytic triad composed of Ser_{80} $His_{205}$$Asp_{177}$, and a classical Gly_{39}-X-Ser_{41}-X-Gly_{43} nucleophile motif commonly found in esterases (Brenner 1988).

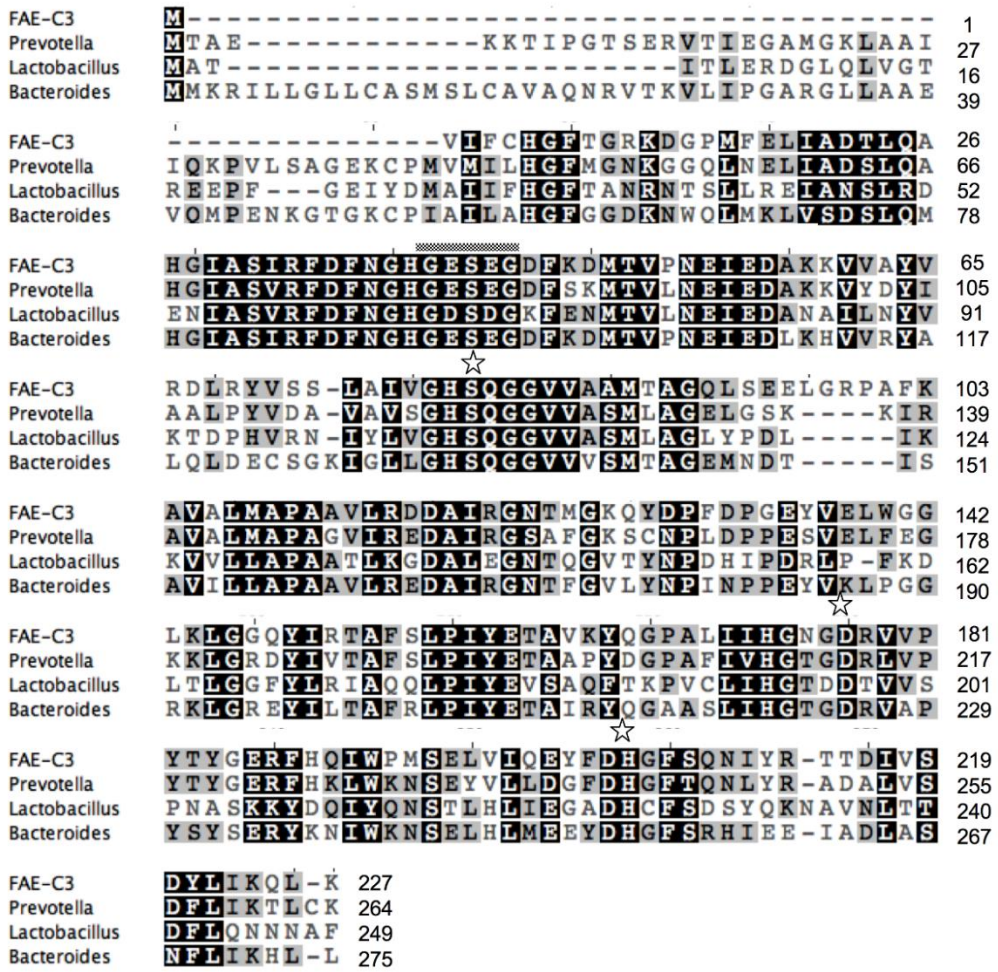

Fig. 1. Multiple sequence alignment of FAE-C3 with *Prevotella oris* C735 feruloyl esterase (EF148533), *Lactobacillus johnsonii* cinnamoyl esterase (ADD11991), and *Bacteroides vulgatus* α/β fold hydrolase (WP_117885235), using the Geneious software. The Gly_{39}-X-Ser_{41}-X-Gly_{43} motif sequence is highlighted with a striated bar above the residues. The catalytic triad Ser_{106} $His_{205}$$Asp_{177}$ is indicated with stars above the residues.

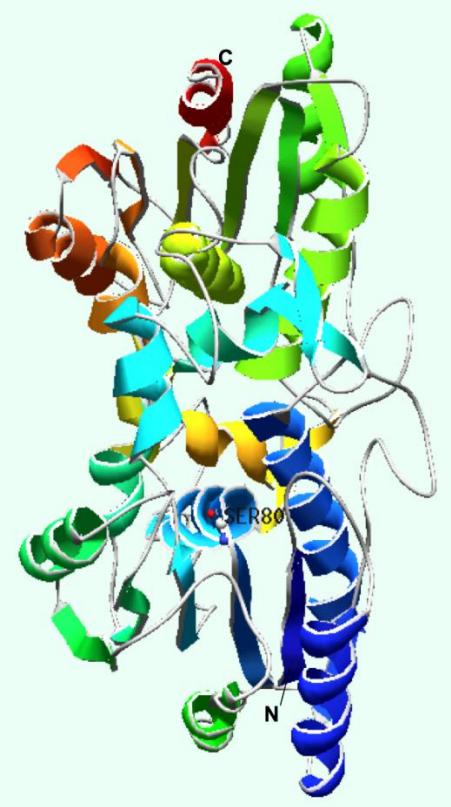

Fig. 2. SWISS-MODEL homology modeling of FAE-C3 based on comparison with the crystal structure of *Lactobacillus johnsonii* enzyme (3s2z.1), showing the α/β-fold architecture with the essential Ser_{80} labeled (at the α3 helix). Elements of secondary structure are colored from the N- to the C-terminal ends in the order of violet, blue, green, yellow, orange, and red

Enzyme Activity on Corn Fiber

The enzyme FAE-C3 was characterized using CF as the substrate. An enzyme unit was defined as the amount of enzyme that catalyzes the formation of 1 μg of ferulic acid product per 100 mg of substrate per hour reaction. The FAE showed a pH optimum at pH ≥ 7, and a temperature optimum of 40 °C (Figs. 3A and 3B). Under the described reaction conditions (100 mg CF, in 1 mL pH 7.0 buffer, incubated for 2 h at 40 °C), 1 nmole FAE catalyzed the release of 19.75 ± 0.24 μg FA from 100 mg CF/h equivalent to 3.5% of total FA content in the 100 mg CF (as determined by alkaline hydrolysis) used in the reaction mixture. Using optimum temperature and pH, the effect of enzyme concentration was determined. The sharpest increase in FA release occurred in the first nmole FAE range (Fig. 3C). Thereafter, the slope started to level progressively with increasing enzyme concentration. This may suggest that some FA residues were relatively less accessible and more resistant, requiring higher enzyme concentration for hydrolytic cleavage. Two possible factors may be involved: (1) The FA residues could be crosslinked, resulting in resistance to enzymatic hydrolysis; and (2) The FA may have been located at interior positions of the polymer, rendering it inaccessible to the FAE enzyme. Recent studies show that GH67 α-glucuronidases remove only the uronic acid linked to the non-reducing end of the glucuronyl xylooligosaccharides (Bielty *et al.* 1997; Pell *et al.* 2004). An analogy may be drawn that the FAE-C3 hydrolyzes only or cleaves at a higher rate the Araf-FA substitution linked at the non-reducing end of the feruloyl oligosaccharide.

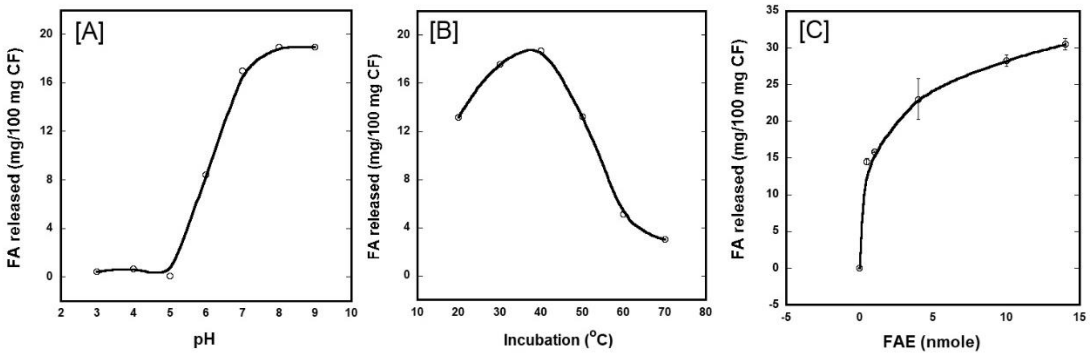

Fig. 3. FAE-catalyzed release of ferulic acid from CF. (A) Effect of pH (reaction conditions: 100 mg CF, 1 mL universal buffer of varying pH, 0.4 nmole FAE-C3, 37 °C for 2 h); (B) Effect of incubation temperature (reaction conditions: 100 mg CF, 1 mL 0.1 M K_2HPO_4 pH 7.0 buffer, 0.4 nmole FAE, varying incubation temperature for 2 h); and (C) Effect of enzyme concentration (reaction conditions: 100 mg CF, 1 mL K_2HPO_4 pH 7.0 buffer, 0.5 to 14 nmole FA-C3E, 40 °C for 2 h). Enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 µg of ferulic acid product per 100 mg CF/h at 40 °C

Effect of Endo-xylanase on FAE Reaction

The synergistic action of FAE-C3 with endo-xylanase was investigated by adding increasing concentrations of XYN (1.0 nmole to 20 nmole) at a constant concentration (0.5 nmole) of FAE. An average increase of 1.11 ± 0.14 µg of FA was observed for each doubling of the concentration of XYN (Figs. 4A and 4B).

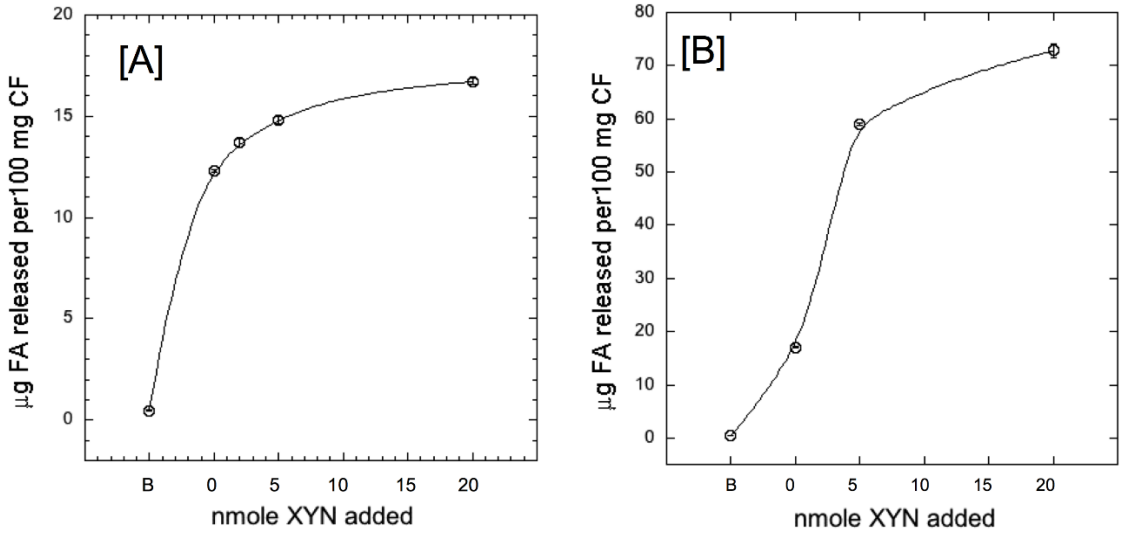

Fig. 4. Effect of endo-xylanase on FAE-catalyzed release of FA analyzed by HPLC: (A) CF and (B) pre-treated CF (reaction conditions: 100 mg CF, 1 mL 0.1 M K_2HPO_4 pH 7.0 buffer, 0.5 nmole FAE, varying concentrations (0.5 to 40 nmoles) XYN, 40 °C for 2 h). The "B" label on the *x*-axis stands for "Blank", which contained only 100 mg CF (without enzymes).

The synergistic effect of adding XYN was equivalent to a 5% or 1.23 fold increase in FA release over the use of FAE-C3 alone. The release of FA from CF and corn bran by FAE in synergism has been reported to be in a similar range, 1.21 and 1.19 fold, respectively (Wong *et al.* 2013). These amounts are less than using wheat bran or wheat

insoluble arabinoxylan as substrates (6.72 and 2.72 fold increase) (Wong *et al.* 2013). Various investigations have shown that FAE activity may be enhanced by the synergistic action of endo-xylanase because shorter chain xylooligosaccharides are more susceptible (hydrolyzed at faster rates by FAE) than long chain substrates (Faulds and Williamson 1993, 1995; Vardakou *et al.* 2004; Wong 2006; Wong *et al.* 2013). The other two accessory enzymes, ABF and AXE, did not show synergistic effect on the CF substrate under similar experimental conditions.

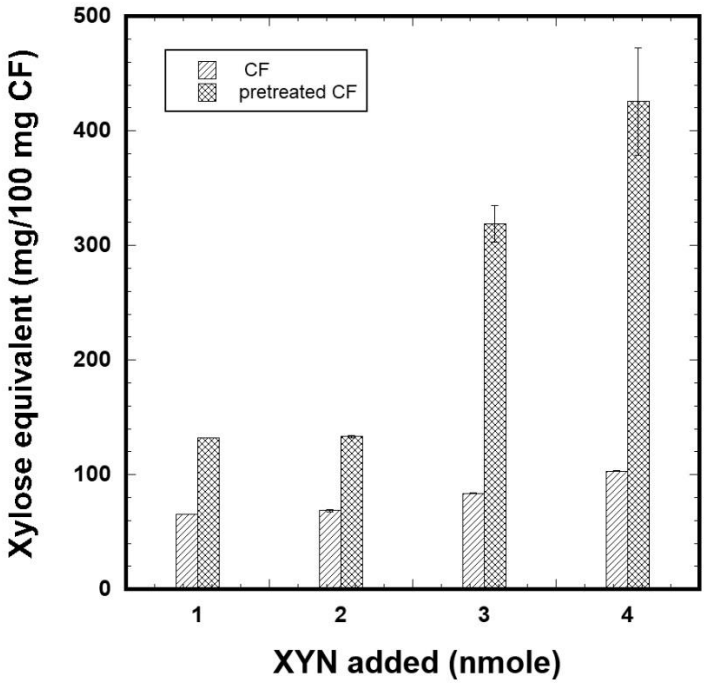

Fig. 5. Mixed endo-xylanase and FAE-C3 on hydrolysis of the xylan main chain assayed by DNSA (3,5-dinitrosalicyclic acid) (reaction conditions: 100 mg CF compared to pre-treated CF, 1 mL 0.1 M K_2HPO_4 pH 7.0 buffer, 0.5 nmole FAE-C3, varying concentrations (0.5 to 40 nmoles) XYN, 40 °C for 2 h)

Effect of Hot Water Pretreatment of Corn Fiber on FA Hydrolysis

Pretreatment has been a key step of cellulosic ethanol production for improving hydrolysis efficiency. The pretreatment loosened the substrate structure, increased porosity of the materials, and enhanced the accessibility of the hydrolytic enzymes. Hot water pretreatment has been shown to produce dissolution of the carbohydrates with minimum degradation (Kim *et al.* 2009). With pretreated CF, the synergistic effect of adding XYN resulted in *ca.* 60 mg FA/100 mg CF (5 nmole XYN, 0.5 nmole FAE), a 4-fold increase compared with the use of the untreated CF under similar reaction conditions.

Synergistic Action of Endo-xylanase and FAE on Main Chain Hydrolysis

The addition of FAE resulted in improving the endo-xylanase action on the hydrolysis of the xylan main chain. The synergistic effect was shown by the increase in the amount of reducing sugar (measured as xylose equivalent) in the reactions. The effect was more noticeable when pretreated CF was used. With 0.5 nmole FAE-C3 added, 5 nmole of XYN was produced, 319 μg xylose equivalent per 100 mg pretreated CF, compared to 84 μg for untreated CF.

CONCLUSIONS

1. A feruloyl esterase (FAE) gene was isolated from rumen microbial metagenome, expressed in *E. coli*, and the enzyme protein (fae-C3) was purified in active form. It consists of an esterase domain with an α/β hydrolase fold, composed of a catalytic triad $Ser_{80}His_{205}Asp_{177}$.

2. The FAE-C3 was characterized using corn fiber as substrate. Its synergistic action with endo-xylanase was studied. Under optimum pH and temperature (pH 7.0, 40 °C), 0.5 nmole FAE-C3 with 5 nmoles XYN were able to catalyze the release of 59.0 ± 0.2 mg ferulic acid and 319 μg xylose equivalent from 100 mg of pretreated CF.

3. It was determined that a combination of selected feruloyl esterase and endo-xylanase can lead to simultaneous increase in the hydrolysis of ferulic acid and the xylan main chain. It consists of an esterase domain with an α/β hydrolase fold, composed of a catalytic triad $Ser_{80}His_{205}Asp_{177}$.

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Reference to a company and/or product 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. The authors declare that there is no conflict of interest regarding the publication of this paper.

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