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The ferulic acid esterases of *Chrysosporium lucknowense* C1: Purification, characterization and their potential application in biorefinery

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ABSTRACT

Three ferulic acid esterases from the filamentous fungus *Chrysosporium lucknowense* C1 were purified and characterized. The enzymes were most active at neutral pH and temperatures up to 45 °C. All enzymes released ferulic acid and *p*-coumaric acid from a soluble corn fibre fraction. Ferulic acid esterases FaeA1 and FaeA2 could also release complex dehydrodiferulic acids and dehydrotriferulic acids from corn fibre oligomers, but released only 20% of all ferulic acid present in sugar beet pectin oligomers. Ferulic acid esterase FaeB2 released almost no complex ferulic acid oligomers from corn fibre oligomers, but 60% of all ferulic acid from sugar beet pectin oligomers. The ferulic acid esterases were classified based on both, sequence similarity and their activities toward synthetic substrates. The type A ferulic acid esterases FaeA1 and FaeA2 are the first members of the phylogenetic subfamily 5 to be biochemically characterized. Type B ferulic acid esterase FaeB2 is a member of subfamily 6.

1. Introduction

The use of plant biomass for second generation biofuel production requires the efficient and controlled degradation of their carbohydrate-rich cell walls. Straw and bran from grasses (Poaceae) or sugar beet pulp are two potential feedstocks for second generation biofuels. The cell walls of grasses contain arabinoxylans that are substituted with hydroxycinnamic acid constituents, such as ferulic acid and coumaric acid [1]. Also, the neutral sugar side chains of pectins in the cell walls of sugar beet (arabinan and galactan) are feruloylated [2]. Such phenolic substituents have been shown to hinder the enzymatic degradation of sugar beet pectin [3] and corn arabinoxylan [4]. The enzymatic removal of the hydroxycinnamoyl esters (e.g. by ferulic acid esterases) could greatly facilitate cell wall degradation.

Ferulic acid esterases (FAEs, EC 3.1.1.77) are structurally diverse and share sequence similarities with lipases, carbohydrate esterase (CE) family 1 acetyl xylan esterases, chlorogenate esterases and xylanases [5]. FAEs are classified into four subgroups (A–D), according to their activities toward synthetic substrates (Fig. 1) and dehydrodiferulic acids [5,6]. Type A FAEs prefer methyl ferulate and methyl sinapinate over methyl *p*-coumarate and methyl caffeate [5] (Fig. 1). They hydrolyze the O-6 ester linkage with galactose in pectic galactan and the O-5 linkage with arabinose in arabinoxylan from gramineae [7]. They also release dehydrodiferulic acids from arabinoxylans when co-incubated with xylanase [8].

Type B esterases prefer hydroxycinnamic acid esters with free hydroxyl substituents over methyl ferulate and methyl sinapinate [5] (Fig. 1). They release ferulic acid that is ester-linked to arabinose of arabinoxylans or pectic arabinans. They also release ferulic acid that is ester-linked to O-6-galactose in pectic galactans [9], but they are not able to release dehydrodiferulic acids [10].

Type C esterases hydrolyze all hydroxycinnamic acid methyl esters, but do not release dehydrodiferulic acids [5]. They are active on arabinoxylans and pectins.

Type D esterases are rather unspecific esterases that hydrolyze all hydroxycinnamic acid methyl esters. They can also release dehydrodiferulic acids and acetic acid from arabinoxylan oligomers [11].

Only very few ferulic acid esterases are found in the CAZy database in CE family 1 and GH family 10 (bifunctional esterase/xylanase [12]). A phylogenetic classification of putative FAEs into subfamilies (SFs) 1–7 was done by Benoit and co-workers [13]. Only SFs 1, 6 and 7 contain biochemically characterized FAEs. Fungal FAEs of the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Talaromyces* have been characterized, but sequence data is not always available to assign them to a single SF. In turn, a multitude of putative FAEs have been identified in genome sequencing projects, but they are rarely biochemically characterized. Type A feruloyl

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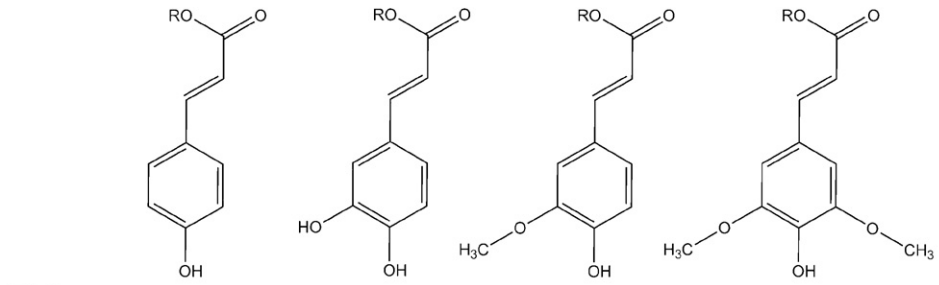

FAE type	p-coumaric acid	caffeic acid	ferulic acid	sinapinic acid	diFerA
A	+	no	++/+++	+++	yes
B	++/+++	++/+++	+	no	no
C	yes	yes	yes	yes	no
D	yes	yes	yes	yes	yes

Fig. 1. Chemical structures of hydroxycinnamic acids: R = H – free acid form, R = CH_3 – (synthetic) methyl-esterified form. Insert table: Classification of ferulic acid esterases based on their activities toward synthetic substrates and their ability to release diferulic acids (diFerA) [5].

esterase Fae-III and type B feruloyl esterase Fae-I from *Aspergillus niger* have been described in most detail [13].

The genome of the ascomycete *Chrysosporium lucknowense* C1 (C1) comprises six genes that putatively encode for ferulic acid esterases [14]. Of these six genes, four were successfully brought to expression and the enzymes were active toward methyl ferulate [14]. One enzyme was unstable and was not included in further experiments. In this research we purified and biochemically characterized three ferulic acid esterases from C1, aiming the removal of hydroxycinnamic acid esters from cell wall polysaccharides that frequently hinder their complete degradation to monomer constituents. The biochemical characterization of ferulic acid esterases toward natural substrates could allow a better fundamental understanding of the structure function relationship in ferulic acid esterases.

2. Methods

2.1. Materials

Methyl caffeate, methyl coumarate, methyl sinapinate and methyl ferulate were obtained from Apin Chemicals Ltd. (Abingdon, Oxon, UK). Alcohol insoluble solids of Brewers spent grain (BSG), corn cobs (CC) and wheat bran (WB) were present in the laboratory [15]. The following commercial enzyme preparations were used: Rapidase® Liq+ (Liq+; DSM, Delft, the Netherlands), Driselase® (Dri; Sigma–Aldrich, St. Louis, MO, USA), Pectinex® Ultra SP (USP; Novozymes, Bagsværd, Denmark) and Rocksoft™ MPL (MPL; Dyadic® Netherlands, Wageningen, The Netherlands).

Cell free fermentation supernatants of ferulic acid esterase overexpression lines were obtained from Dyadic®. Overexpression of the genes was performed using the C1 production platform [16,17]. The ferulic acid esterase candidate genes were identified based on a sequence homology screen with already identified ferulic acid esterases and they were named FaeA1, FaeA2 and FaeB2 (Table 1). Due to a lack of biochemical data for the best hits, the second best hit was used for annotation.

2.2. Preparation and isolation of feruloylated oligomers

A fraction of corn fibre arabinoxylan oligosaccharides, that contains monomeric, dimeric and trimeric ferulic acid constituents linked to the oligosaccharides was used ([4], subfraction M-5). Feruloylated oligosaccharides from sugar beet pulp were obtained from 5 g pretreated sugar beet pulp ([18], 140-0 supernatant fraction) digested with the C1 enzymes Abn1, Abn4 and Gal1 [14,19]. The feruloylated oligomers were isolated and fractionated by reverse phase solid phase extraction [4]. Samples were eluted with 10 v/v%, 60 v/v% and 100% methanol. Methanol was removed from the fractions by evaporation in a vacuum rotation evaporator and the fractions were subsequently freeze-dried. The 60 v/v% methanol fraction contained all the ferulic acid, as confirmed by UV spectroscopy at 325 nm (see Section 2.4.2).

2.3. Enzyme purification

The purification of the enzymes required a single chromatographic step for FaeA1 and two chromatographic steps for FaeA2 and FaeB2. Ferulic acid esterase activity in the purified fractions was monitored by measuring the activity toward methyl ferulate.

2.3.1. FaeA1

FaeA1 was purified by size exclusion chromatography using an ÄKTApurifier 10 analytical system (GE Healthcare, Uppsala, Sweden) equipped with a Superdex™ 75 analytical column (10/300 GL, 1 column volume (CV) = 23.56 ml; GE Healthcare) at a flow rate of 0.5 ml/min at 20 °C. The sample was manually injected via a 200 μl sample loop and eluted with 50 mM NaCl in 50 mM sodium acetate buffer (pH 5.0). Fractions of 200 μl were collected in microtiter plates using an ÄKTA Frac-950 fraction collector (GE Healthcare).

2.3.2. FaeA2 and FeaB2

FaeA2 and FaeB2 were purified by hydrophobic interaction chromatography followed by anion exchange chromatography using an ÄKTAexplorer 100 preparative system (GE Healthcare). The system was equipped with a self-packed Phenyl Sepharose™ HP column (XK16, 58 ml column volume; GE Healthcare) that was equilibrated with 5 CV of 10 mM sodium acetate buffer (pH 5.0) containing 1.5 M ammonium sulfate (buffer A). The samples (17.5 ml) were mixed 1:1 with 20 mM sodium acetate buffer (pH 5.0) containing 3 M ammonium sulfate. They were cooled on ice for 30 min and subsequently centrifuged (5 min, 5000 × *g*, 4 °C). The supernatants were injected onto the column via a sample pump at a flow rate of 2 ml/min.

Table 1
Sequence similarity based preliminary annotation of C1 ferulic acid esterases.

C1 FAE	First hit	Similarity	Second hit	Similarity
FaeA1 (JF826027[a])	*Chaetomium globosum* gene CHGG_06075 (EAQ89456[a])	84%	*Penicillium funicolosum* FAE A (AJ312296[a])	52%
FaeA2 (JF826028[a])	*Chaetomium globosum* gene CHGG_06916 (EAQ85663[a])	71%	*Penicillium funicolosum* FAE A (AJ312296[a])	45%
FaeB2 (JF826029[a])	*Chaetomium globosum* gene CHGG_10485 (EAQ84081[a])	77%	*Neurospora crassa* FAE B (AJ293029[a])	68%

[a] GENBANK accession numbers.