Note

Extracellular Carbohydrate Esterase from the Basidiomycete

Coprinopsis cinerea Released Ferulic and Acetic Acids from Xylan

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eDNA encoding an extracellular carbohydrate esterase (CcEst1) was cloned from the basidiomycete Coprinopsis cinerea. The recombinant CcEst1 expressed in Pichia pastoris acted on p-nitrophenyl acetate, α-naphthyl acetate, and methyl hydroxycinnamates, except for methyl sinapic acid. The enzyme released ferulic and acetic acids from wheat arabinoxylan and acetylated xylan respectively. Activity increased on the addition of endo-β-1,4-xylanase.

Key words: basidiomycete; carbohydrate esterase; Coprinopsis cinerea

Plants are the most abundant renewable resources on earth, and their cell walls consist mainly of cellulose, hemicellulose, and lignin. A group of basidiomycetes known as white-rot fungi are rare microorganisms that can completely degrade lignin through an oxidative reaction involving lignin peroxidases, manganese peroxidases, versatile peroxidases, and laccases. On the other hand, they decompose cellulose and hemicellulose by various glycoside hydrolases such as cellulases and hemicellulases.

In addition to glycoside hydrolases and phenol oxidases, carbohydrate esterases are required for the complete degradation of cell walls, which contain various types of ester bonds. Acetyl xylan esterase (EC 3.1.1.72) and feruloyl esterase (EC 3.1.1.73) are major extracellular carbohydrate esterases produced by cellulolytic microorganisms. Acetyl xylan esterase hydrolyzes the ester linkages of the acetyl groups at positions 2 and/or 3 of the xylose moieties of natural acetylated xylan.1) Complete biodegradation of natural acetylated xylan requires the co-activity of xylanases and β-xylosidases together with acetyl xylan esterases. Feruloyl esterase catalyzes the hydrolysis of the ester bond between ferulic acid and the sugar present in plant cell walls, and acts synergistically with cellulases and xylosanes to decompose plant cell wall polysaccharides.2,3) To date, many acetyl xylan and feruloyl esterases have been isolated from cellulolytic ascomycetes and bacteria, but, limited information is available on these esterases from basidiomycetes.

The basidiomycete Coprinopsis cinerea produces multiple cellulases,4) extracellular phenol oxidases such as an extracellular peroxidase,5) and multiple laccases.6) Therefore, C. cinerea and white rot fungi have an efficient degradation system for plant cell wall components. In this study, we cloned a gene encoding a putative extracellular carbohydrate esterase, designated CcEst1, which is associated with C. cinerea hemicellulose degradation. We also characterized the recombinant enzyme produced by Pichia pastoris.

The publicly available genome database for C. cinerea (http://www.broadinstitute.org/annotation/genome/coprinus_ciner us/MultiHome.html) was searched with the amino acid sequences of known feruloyl esterases, and a gene encoding CcEst1 that had significant identity with these enzymes was found. To clone the gene, C. cinerea strain 53387) was grown at 25°C in 200 ml of Kremer and Wood medium,5) containing 2% w/v Avicel and 0.4% w/v birch wood xylan. The mycelia were harvested after 3 d and subjected to a cloning procedure, as described in a previous report.8) Primers (forward, 5′-AGTAA-CATGCCGCCTTA-CTG-3′, and reverse, 5′-GCAATTACCTAGTTGG-GTA-TGTATGAGAG-3′) were designed in the 5′- and 3′-untranslated regions to amplify the open reading frame of the target gene by polymerase chain reaction (PCR), followed by sequencing using a 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The cDNA revealed an open reading frame of 996 bp encoding a polypeptide of 332 amino acids (accession no. AB540992). The N-terminal 20-amino acid sequence was predicted to be a signal peptide by the SignalP program (http://www.cbs.dtu.dk/services/SignalP). The predicted molecular mass and pl of the mature protein were 33.5 kDa and 8.01 respectively. The serine esterase family motif (Gly-Xaa-Ser-Xaa-Gly)9) was conserved in CcEst1 (aa133–aa137). The amino acid sequence of this enzyme showed significant homology to NcFaeD of Neurospora crassa (46%, accession no. XP_330902), PfFaeA of Penicillium funiculosum (46%, accession no. AJ312296), and PfXYLD of Pseudomonas fluorescens (43%, accession no. AB540992).

Abbreviations: MSA, methyl sinapic acid; PCR, polymerase chain reaction; pNP, p-nitrophenyl; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography

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no. X58956), which belong to the type-D feruloyl esterase family. In addition to the catalytic domain, this enzyme contains a family 1 carbohydrate-binding module, previously called the cellulose-binding domain, at the C-terminal of the mature protein.

The cDNA encoding the mature protein was amplified by PCR with primers (forward, 5’-CCGGAATTCCGCT-TCCACCCCCCGCT-3’, and reverse, 5’-AAGGAAAAA-AGGCGGCCGCCAGGCACTGGTGGTACCACTG-3’). The amplified product was digested with EcoRI and NotI and ligated to the corresponding sites of the pPICZα vector (Invitrogen, Carlsbad, CA). P. pastoris KM71H was transformed, and the transformants were selected following the manufacturer’s instructions. The transformants were cultured in 900 ml of YPG medium (1% yeast extract, 2% peptone, and 1% glycerol) for 2 d. The cells were harvested, resuspended in 50 ml of YPM medium (1% yeast extract, 2% peptone, and 1% methanol), and incubated for 5 d at 26.5°C with shaking. Methanol (1% w/v) was added daily. The concentration of the recombinant CcEst1 (rCcEst1) reached 31.6 mg/l at day 5, when the amount of recombinant protein secreted into medium was estimated by the rate of hydrolysis against p-nitrophenyl (pNP) acetate, as described below. rCcEst1 was purified from the supernatant of the culture on day 2 using a Ni-NTA agarose column (GE Healthcare, Buckinghamshire, England). After purification, the concentrations of the protein were calculated from the molar extinction coefficient (73,350 M⁻¹ cm⁻¹). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band of approximately 46.4 kDa (Fig. 1). The molecular mass decreased after deglycosylation with endoglycosidase H (Endo-H) (45.1 kDa), indicating that the enzyme was N-glycosylated. However, the molecular mass of the deglycosylated protein was higher than that predicted using the amino acid sequence (33.5 kDa). Thus, the enzyme appears to undergo O-glycosylation.

The enzymatic properties of rCcEst1 were examined, as follows: because the enzyme was stable from pH 7.0 to 11.0 at 50°C, the enzyme assay was performed in Mcllvaine buffer (0.2 M Na₂HPO₄/0.1 M citric acid, pH 7.0) at 37°C. Substrate specificity was evaluated using pNP acetate, α-naphthyl acetate, methyl esters of ferulic acid, p-coumaric acid, caffeic acid, and methyl sinapic acid (MSA) (Apin Chemicals, Oxfordshire, UK) as substrates. An aliquot (900 μl) of the reaction mixture containing the enzyme (4.2 ng/μl) and the substrates (0.9 mM) was incubated for an appropriate length of time. The release of p-nitrophenol was monitored at 420 nm and quantified from the molar extinction coefficient (15,000 M⁻¹ cm⁻¹). The released α-naphthol was quantified from its standard curve (Sigma, Tokyo, Japan) obtained by high-performance liquid chromatography (HPLC). For the methyl esters of hydroxycinnamic acids, the reaction was terminated by adding 200 μl of glacial acetic acid, and the products were analyzed by HPLC with a CrestPack C18S column (Jasco, Tokyo, Japan), as previously described.¹⁰ The free acids of the substrates (Apin Chemicals Oxfordshire, UK) were used to prepare standard curves. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP, α-naphthol, or free acid per min from the substrates at 37°C, pH 7.0. As shown in Table 1, the enzyme showed activities against all substrates except MSA, and the highest activity was observed against pNP acetate. These results indicate that CcEst1 has broad specificities for synthetic substrates and has strong acetyl esterase activity. Similar specificities have been reported in research on PfXYLD and PfFaeA. That is, these enzymes also show higher activity against pNP acetate than the methyl esters of cinnamic acid.¹¹,¹² The release of acetic acid and ferulic acid from xylan was examined as follows: acetylated xylan was prepared from oat-spelt xylan as described previously.¹³ The reaction was performed with purified rCcEst1 (8 ng/μl) in 200 μl of Mcllvaine buffer (pH 7.0) containing acetylated xylan (10 mg/ml) in the absence and the presence of endo-β-1,4-xylanase from Thermomyces lanuginosus (Sigma, 2.9 ng/μl) at 37°C for 1 h. The reaction was terminated by adding 300 μl each of 0.3 M zinc sulfate and 0.3 M barium hydroxide. After 20 min of centrifugation, the supernatant was transferred to a new tube. The amount of acetic acid released was estimated using an F-kit (Roche, Basel, Switzerland), following manufacturer’s instructions. The release of ferulic acid was examined using wheat arabinoxylan (10 mg/ml). The reaction was performed with purified rCcEst1 (8 ng/μl) in 600 μl of Mcllvaine buffer (pH 7.0) in the absence and the presence of endo-β-1,4-xylanase from T. lanuginosus (2.9 ng/μl) at 37°C for 1 h. It was terminated by adding 200 μl of glacial acetic acid. The samples were filtered through a 0.45 μm filter, and 100 μl of filtrate was analyzed by HPLC, as described above. Ferulic acid was used to prepare standard curves.

Table 1. Substrate Specificity of rCcEst1 against Synthetic Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>U/mg*</th>
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<tbody>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>66.20</td>
</tr>
<tr>
<td>α-Naphthyl acetate</td>
<td>2.67</td>
</tr>
<tr>
<td>Methyl ferulate</td>
<td>1.26</td>
</tr>
<tr>
<td>Methyl p-coumarate</td>
<td>0.78</td>
</tr>
<tr>
<td>Methyl caffeate</td>
<td>0.26</td>
</tr>
<tr>
<td>Methyl sinapate</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*The reactions were performed with rCcEst1 (4.2 ng/μl) at a 0.9 μM substrate concentration in Mcllvaine buffer (pH 7.0) at 37°C, as described in the text.
As shown in Table 2, acetic acid was detected when rCcEst1 was mixed with acetylated xylan. The amount of acetic acid increased significantly (approximately 8-fold) following the addition of endo-β-1,4-xylanase to the reaction mixture. Generally, acetylated xylan esterases show no feruloyl esterase activity. In contrast, several feruloyl esterases can release acetic acid from xylan, but the amount released is remarkably lower than that of ferulic acid released from xylan.14,15 When the ferulic acid-releasing activity of rCcEst1 was assayed, a significant amount of ferulic acid was released from wheat arabinoxylan, and the amount released increased dramatically (approximately 13-fold) in the presence of endo-β-1,4-xylanase. However, no release was observed when only endo-β-1,4-xylanase was used. These results clearly indicate that rCcEst1 displayed not only acetic acid-releasing activity against xylan but also ferulic acid-releasing activity, which were promoted by the addition of endo-β-1,4-xylanase.

To date, few acetyl xylan esterases and no feruloyl esterase has been isolated from basidiomycetes, although the latter was roughly purified from Schizophyllum commune.16 In the present study, we cloned the gene encoding CcEst1 from C. cinerea. The substrate specificities of the recombinant enzyme suggest that the enzyme released acetic and ferulic acids when mixed with native acetylated and ferulated xylan respectively. The crystal structure of the enzyme should offer insight into its catalytic mechanism.

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