Molecular cloning, and characterization of a modular acetyl xylan esterase from the edible straw mushroom

Volvariella volvacea

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Introduction

Xylan is the most abundant hemicellulosic polysaccharide in plants and generally contains heterogeneous substituents such as arabinose, O-acetyl, and ferulic (4-hydroxy-3-methoxycinnamic) acid, p-coumaric (4-hydroxycinnamic) acid and 4-O-methylglucuronic acid (Coughlan & Hazlewood, 1993). Complete biodegradation of this structurally complex polymer requires the cooperation of xylanases and β-xylosidases along with several accessory enzymes. These include acetyl xylan esterases (EC 3.1.1.72) (AXEs) that hydrolyze the ester linkages of the acetyl groups in position 2 and/or 3 of the xylose moieties of natural acetylated xylan present in the plant cell wall (Biely et al., 1985). Several genes encoding for AXEs have been isolated and characterized from bacteria, including Fibrobacter succinogenes (Kam et al., 2005), Bacillus pumilus (Degrassi et al., 2000), Streptomyces lividans (Puchart et al., 2006), and Thermoanaerobacterium sp. (Lorenz & Wiegel, 1997), and ascomycetous fungi such as Aspergillus oryzae (Koseki et al., 2006), Trichoderma reesei (Margolles-Clark et al., 1996), and Penicillium purpurogenum (Gutierrez et al., 1998). However, there are no reports of corresponding genes from basidiomycetous fungi.

Volvariella volvacea, the edible straw mushroom, is grown on an industrial scale in many tropical and subtropical regions of Southeast Asia (Chang, 1999). Volvariella volvacea is a highly nutritious food source. Furthermore, earlier empirical observations on the health-promoting properties of V. volvacea are now being reinforced by an increasing number of reports ascribing immunomodulating, antitumor, and hypcholesterolemic activity to various components isolated from mushroom fruit bodies and mycelium (Buswell & Chen, 2005). Volvariella volvacea grows naturally on rice straw, a prominent component of which is arabinoxylan. Genes encoding for cellobiohydrolases and endoglucanases were differentially expressed during substrate colonization and fruiting (Ding et al., 2006). Because 22–50% of the xylose residues in this hemicellulose are substituted with acetyl groups in the O-2 or O-3 position (Chesson et al., 1983), the release of acetic residues is a crucial step in the breakdown of the polymer and in colonization of the rice straw substrate by the mushroom.

In this paper, the cloning and characterization of a gene encoding a modular VvAXE1 from the edible mushroom V. volvacea are reported. To the authors’ knowledge, this is also the first reported AXE from a basidiomycetous fungus.
Materials and methods

Fungal and bacterial strains, culture conditions, and vectors

Volvariella volvacea V14 was obtained from the culture collection of the Centre for International Services to Mushroom Biotechnology located at The Chinese University of Hong Kong (accession no. CMBO02). Escherichia coli XL-1 Blue MRF was used as the host for recombinant plasmids. PGEM-T vector (Promega) was used to subclone DNA fragments for sequencing.

For extraction of RNA, the fungus was grown in 250 mL flasks containing 50 mL basal medium with 1% Avicel (Sigmacell type 20) as a carbon source as described previously (Ding et al., 2001). The cultures were incubated at 32 °C in an orbital incubator shaker operated at 150 r.p.m.

Cloning of AXE gene (Vvaxe1)

Mycelium from 4-day-old *V. volvacea* cultures was harvested, frozen with liquid nitrogen, and ground to a fine powder with a mortar and pestle. Total RNA was isolated from this material using a TRizol reagent (Invitrogen) according to the manufacturer’s instructions and used to synthesize cDNA. The degenerate primer [5'-GG(AGCT) CAG TGC GG(AGCT) GGA GGT TGG C-3'] was designed to anneal to the DNA sequence encoding a conserved region of the cellulose-binding domain (CBD) sequence (GQQCGGIG) present in many fungal cellulases and hemicellulases, and used for the RACE-PCR using the SMART RACE cDNA Amplification Kit (Clontech). PCR amplification of the 3'-cDNA end fragment encoding the CBD region was carried out as described by Ding et al. (2006). The fragment was subcloned into a pGEM-T vector and sequenced.

The gene-specific primer 5'-GTG TAT TGG CAA GTG AAG GGA GGT TGG C-3' based on the sequence obtained above was designed to generate the 5'-end DNA fragment coding for acetyl xylan esterase1 (*Vvaxe1*). The full-length cDNA of *Vvaxe1* was then generated by 3'-RACE using the gene-specific primer 5'-GGA GAA CCC ACC ACC CGC ATA TTT TC-3' designed from the sequence of the extreme 5'-end of *Vvaxe1*, and was cloned and sequenced as above.

Expression of *Vvaxe1* in *Pichia pastoris*

In order to achieve expression in *Pichia pastoris*, the codon was optimized according to the codon preference of *Pichia pastoris* using the Backtranslation program (Entelechon GmbH, Regensburg, Germany). The gene was synthesized by the primer extension PCR procedure using a Pfu polymerase. The synthetic gene was fused with a 6-histidine tag (to aid subsequent purification by affinity chromatography) attached at the C-terminal and ligated at the EcoRI/NotI sites of the *Pichia pastoris* expression vector pPICZαA to yield the construct pPICZαA-AXE1. The gene insert was confirmed by dideoxy chain-termination DNA sequencing. Transformation and expression of *Vvaxe1* in *Pichia pastoris* was carried out according to the manufacturer’s protocol (Invitrogen).

Purification and characterization of recombinant acetyl xylan esterase (reVvAXE1)

Supernatants from 100 mL cultures were collected by centrifugation (5000 g for 15 min) and reVvAXE1 with the 6-histidine tag was purified by affinity chromatography using Ni-NTA Agarose gel (Qiagen) according to the manufacturer's manual. Enzyme homogeneity and the molecular weight of purified reVvAXE1 were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [10% (w/v)] and SDS-PAGE LMW protein markers (Pharmacia) under reducing conditions as described by Laemmli (1970).

Optimal pH and temperature values were determined over the ranges pH 6.5–10.0 (universal buffer: 50 mM H3PO4, 50 mM CH3COOH, 50 mM H3BO3, pH adjusted by 0.2 M NaOH at 25 °C) and 30 and 80 °C, respectively. To determine thermal stability, reAXE1 was incubated at temperatures between 30 and 60 °C for 0–60 min. Residual activity towards 4-methylumbelliferyl acetate was compared with control samples. The kinetic constants (*V*max and *K*m) for reVvAXE1 were determined from Lineweaver–Burk plots of data obtained by measuring the rate of 4-methylumbelliferyl acetate hydrolysis under the standard assay conditions using a substrate concentration range of 0.33–1.33 μM.

Deglycosylation of reVvAXE1

Purified reVvAXE1 was treated with Endoglycosidase H (endo-N-acetylglucosaminidase H of *Streptomyces plicatus*; NEB) according to the manufacturer’s protocol. Enzyme samples (containing 2 μg of the enzyme) in 1 × glycoprotein denaturing buffer were boiled for 10 min. After cooling, 1 μL of Endoglycosidase H was added and the samples were incubated at 37 °C overnight. The products of Endoglycosidase H treatment were analyzed by SDS–PAGE.

Substrate binding

Reaction mixtures (0.5 mL) containing 1 mg of substrate and 0.064 mg of purified reVvAXE1 in 100 mM potassium phosphate buffer (pH 7.5) were incubated on a rotating shaker (200 r.p.m.) at 4 °C for 60 min. After incubation, samples were centrifuged (10 000 g, 4 °C, 10 min) and the protein in the filtrate was quantified from the A280 nm, and
the residual activity in the supernatant was assayed with 4-methylumbelliferyl acetate.

**Enzyme assays**

ReVvAXE1 activity was determined spectrophotometrically at 50 °C by measuring the increasing in A254 nm during the initial 2 min of the assay resulting from the release of 4-methylumbelliferone from 4-methylumbelliferyl acetate (Shao & Wiegel, 1995). ReVvAXE1 activity towards acetylated xylan and xylose tetraacetate (Sigma) was assayed by the method of Degrassi et al. (2000). Reaction mixtures consisted of: 250 μL acetylated xylan [5% (w/v) suspension in 50 mM potassium phosphate buffer, pH 7.5] or xylose tetraacetate (1.25 mM) with 10 μL of purified enzyme in a total volume of 500 μL. Mixtures were incubated at 50 °C for 10 min with orbital shaking (150 r.p.m.). The acetic acid released was quantified using an enzymatic analysis kit (Boehringer Mannheim, catalog no. 148 261) according to the manufacturer’s instructions. Acetylated oat spelt xylan was prepared in accordance with the method of Mitchell et al. (1990). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of product per minute under the assay conditions. Specific activity is defined as the number of units per micromole of protein calculated from the A230nm using molar extinction coefficients (εVvAXE1 = 88 320 M⁻¹ cm⁻¹). Activity towards p-nitrophenyl acetate (pNA, Sigma), α-naphthyl acetate (Sigma), was assayed as described previously (Blum et al., 1999).

**Nucleotide sequence accession number**

The nucleotide sequence of *V. volvacea* VvAXE1 has been assigned the GenBank accession no. DQ888226.

**Results**

**Cloning and sequence analysis of axe1**

The full-length cDNA of VvAXE1 (Genbank no. DQ888226) consisted of 1363 bp encoding 349 amino acids with a putative signal peptide of 20-amino acids and a mature protein of 329-amino acids containing a family I CBD at the C-terminus and family I AXE catalytic domain at the N-terminus. Alignment of the deduced amino acid sequence of VvAXE1 with esterase genes of other organisms showed the highest sequence similarities to cinnamoyl esterase B from *Penicillium janiculans* (49%, accession no. AJ291496), and AXEs from *A. oryzae AoaxeA* (52%, accession no. AB167976), *Penicillium purpurogenum* axe1 (45%, accession no. AF529173), *Aspergillus ficuum* (48%, accession no. AF331757), and *Aspergillus fumigatus* AF293 (46%, accession no. XM 742365) (Fig. 1).

**Purification and characterization of reVvAXE1**

The synthetic gene encoding VvAXE1 from *V. volvacea* was expressed in *Pichia pastoris*. The recombinant protein was purified in a one-step procedure by affinity chromatography using Ni-NTA Agarose gel, but SDS-PAGE analysis revealed that the purified reVvAXE1 migrated as two bands with molecular masses of 48 and 66 kDa, respectively. After endoglucosidase H (endo H) treatment, only one protein band with an estimated molecular mass of 45 kDa was observed (Fig. 2).

**pH and temperature optima**

pH and temperature optima for reAXE1 are pH 8.0 and 60 °C, respectively (Fig. 3a and b). However, reVvAXE1 was not stable at temperatures above 60 °C and, after a 10-min incubation at 60 °C, over 80% of the activity was lost (Fig. 4). Hence, other characteristics of reVvAXE1 were determined in potassium phosphate buffer (pH 8.0) at 50 °C.

**Kinetic analysis and substrate specificity**

ReVvAXE1 had $K_m$ and $V_{max}$ values of 307.7 μm and 24 733 IU μmol⁻¹ protein, respectively, when 4-methylumbelliferyl acetate was used as the substrate. ReVvAXE1 released acetate from several substrates (Table 1), with the highest specific activity recorded for xylose tetraacetate (52 450 IU μmol⁻¹). α-Naphthyl acetate was a poor substrate for the enzyme and was hydrolyzed at only 1.8% of the rate observed with xylose tetraacetate. No hydrolytic activity was detected towards pNA.

**Substrate binding**

Binding experiments revealed that reVvAXE1 bound to Avicel and acid-swollen cellulose. Residual reVvAXE1 protein concentrations in reaction mixture supernatants following incubation with these two substrates decreased to 84.3% and 22.9%, respectively, of the control levels. Corresponding residual reVvAXE1 activities also decreased to 77.3% and 12%, respectively. However, no significant adsorption of reVvAXE1 to insoluble oat spelt xylan was observed (Table 2).

**Discussion**

AXE is an accessory enzyme essential for the complete mineralization of many xylans (Cybinski et al., 1999). In contrast to the considerable data relating to xylanases and xylosidases, there is relatively little available information on AXEs. In this report, the isolation of a new acetyl xylan esterase gene from *V. volvacea* is described.
Acetyl xylan esterase have been assigned to families 1, 2, 3, 4, 5, 6, and 7 of the 14 carbohydrate esterase families identified by Henrissat et al. (2004). Previous reports indicated that substrate discrimination, including backbone specificity, is different among the AXEs (Biely et al., 1997; Degrassi et al., 2000). VvAXE1 from V. volvacea has a modular structure containing a catalytic module at the N-terminus belonging to family 1 of the carbohydrate esterases, a linker rich in serines and threonines, and a family 1 C-terminal CBD. Owing to the high degree of variation present in AXEs, as shown by our alignment (Fig. 1), the amino acid residues conserved in all proteins are indicated by dots. The amino acid residues absent in proteins are indicated by dashes.

**Fig. 1.** Comparison of the deduced amino acid sequence of Vvaxe1 with esterases from other fungi. AJ291496, Penicillium funiculosum cinnaamoyl esterase B (faeB); AB167976, Aspergillus oryzae acetyl xylan esterase (AoxaA); AF529173, Penicillium purpururogenum acetyl xylan esterase I (axel); AF331757, Aspergillus ficuum acetyl xylan esterase; XM-742365: Aspergillus fumigatus Af293 acetyl xylan esterase. The amino acid residues conserved in all proteins are indicated by dots. The amino acid residues absent in proteins are indicated by dashes.
amino acid sequence similarity between VvAXE1 and both cinnamoyl esterase and AXE, Vvaxe1 was expressed in Pichia pastoris in order to assess the catalytic activity of the enzyme towards different substrates.

The main function of CBDs in cellulases is to bind the enzyme-catalytic domains to the substrate, which increases the longevity and intimacy of contact. However, even

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**Table 1.** Substrate specificity of purified reVvAXE1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (IU μmol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylated xylan</td>
<td>14705</td>
</tr>
<tr>
<td>Xylose tetra-acetate</td>
<td>52450</td>
</tr>
<tr>
<td>Methylumbelliferyl acetate</td>
<td>24733</td>
</tr>
<tr>
<td>p-Nitrophenyl acetate</td>
<td>0</td>
</tr>
<tr>
<td>α-Naphthyl acetate</td>
<td>961</td>
</tr>
</tbody>
</table>

Values are the means of triplicate determinations.

**Table 2.** Binding of reVvAXE1 to insoluble substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Remaining activity (%)</th>
<th>Remaining protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Xylan</td>
<td>92.2</td>
<td>98.8</td>
</tr>
<tr>
<td>Avicel</td>
<td>77.3</td>
<td>84.3</td>
</tr>
<tr>
<td>H₃PO₄ acid-swollen cellulose</td>
<td>12</td>
<td>22.9</td>
</tr>
</tbody>
</table>

Values are the percentage enzyme activity and reVvAXE1 protein remaining in the supernatant after incubation of the enzyme with the insoluble substrate. H₃PO₄ acid-swollen was prepared by the treatment of Avicel with phosphoric acid as described by Wood (1988).
though there is an overall functional similarity between all CBDS, their specificities and affinities are not always identical, even among members of the same family (Linder et al., 1995; Mattinen et al., 1997). There are a few sequences of AXEs with similar modular structures from other fungi. The AXE1 from Penicillium purpurogenum bound strongly to crystalline cellulose (type 50), but not to xylan (Gordillo et al., 2006). In this paper, reVVAXE1 also had no affinity with xylan, but it exhibited a higher binding activity toward \( \text{H}_3\text{PO}_4 \)-acid-swollen cellulose than Avicel, indicating it was bound strongly to xylan, but it exhibited a higher binding activity toward \( \text{H}_3\text{PO}_4 \)-acid-swollen cellulose than Avicel, indicating it was contributing to glycosylation, because there are two potential N-linked glycosylation sites on the VvAXE1 sequence. However, the molecular mass of the deglycosylated band was still higher than the value (39990 Da) calculated from the deduced amino acid sequence, suggesting that other protein modifications such as O-glycosylation were also present.

ReVVAXE1 resembled other reported AXEs in exhibiting \( \beta \)-xylosidase activity towards 4-methylumbelliferyl acetate and \( \alpha \)-naphthyl acetate (Ferreira et al., 1993; Halgasova et al., 1994). Interestingly, ReVVAXE1 showed no detectable activity on generic esterase substrates including nitrophenyl acetate as other family 1 AXE had (Chung et al., 2002); this observation is similar to the acetyl xylan esterases from Streptomyces lividans and Clostridium thermocellum, which belong to carbohydrate esterase family CE4, and both of them showed no activity towards nitrophenyl acetate (Taylor et al., 2006). As the reVVAXE1 has overglycosylated, the influence of glycosylation on the substrate specificities needs to be further understood.

ReVVAXE1 also showed high activity towards sugar-based substrates such as \( \beta \)-D-xylose tetraacetate and acetylated xylan, thereby confirming that it is a true AXE. Further studies on the specificity and regulation of VvAXE1 are underway in order to better understand the role of the enzyme in substrate colonization and fruiting in \( V. \) volvacea.

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**References**


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