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Gene cloning, heterologous expression and characterization of a *Coprinopsis cinerea* endo-β-1,3(4)-glucanase

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ABSTRACT

A gene coding endo-β-1,3(4)-glucanase (ENG16A) was cloned from *Coprinopsis cinerea* and heterologously expressed in *Pichia pastoris*. ENG16A only acts on substrates containing β-1,3 glycosidic bonds but not on substrates containing only β-1,4- or β-1,6-glycosidic bonds. Interestingly, compared to the activity of this enzyme towards carboxymethyl (CM)-pachyman containing only β-1,3-glycosidic bonds, its activity towards barley β-glucan containing both β-1,3-glycosidic and β-1,4-glycosidic bonds was increased by 64.72 %,, its activity towards laminarin containing both β-1,3-glycosidic and β-1,6-glycosidic bonds was decreased by 50.83 %. In addition, ENG16A has a higher Km value and Vmax for barley β-glucan than laminarin, which may be related to the fact that barley β-glucan contains mainly β-1,4-glycosidic bonds mixed with a few β-1,3-glycosidic bonds, whereas laminarin mainly contains β-1,3-glycosidic bonds with a few β-1,6-branched glucose residues. The adjacent β-1,4-glycosidic bond promotes ENG16A to hydrolyse β-1,3-glycosidic bonds, leading to an increased Vmax; the nearby β-1,6-glycosidic bonds inhibited its hydrolysis of β-1,3-glycosidic bonds, resulting in a decreased Vmax. This property is suggested to be related to the mechanism that *C. cinerea* uses to degrade and utilize hemicellulose in straw medium and to protect its cell wall during the mycelium growth stage.

Introduction

Coprinopsis cinerea is an ideal model organism with which to study the growth and development of basidiomycete fruiting bodies (Kües 2000). Similar to all other agarics, *C. cinerea* exhibits obvious fruiting body autolysis (Kües 2000; Kawakami *et al.* 2004; Fukuda *et al.* 2008; Sakamoto *et al.* 2012; Tao *et al.* 2013). The autolysis of the fruiting body pileus of *C. cinerea* are mainly resulted from action of various hydrolases which were synthesized and secreted to degrade the cell walls for the release of basidiospores during maturation of fruiting body (Iten 1969; Miyake *et al.* 1980; Hammad *et al.* 1993; Kües 2000; Moore 2003). We recently reported that a group of glucanases with different action mode were isolated from the pileus of mature fruiting body of *C. cinerea* including an endo-β-1,3-glucanase, an exo-β-1,3-glucanase, a β-1,3-glucosidase, as well as a possible β-glucosidase (Zhou *et al.* 2015). These glucanases degrade the β-1,3/1,6-glucans in the cell walls of the

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pileus in a synergistic manner. Furthermore, through mutagenesis, we obtained one mutant of C. *cinerea* in which the pileus does not undergo autolysis (Liu *et al.* 2015). A comparative study of the wild type and non-autolysis mutant of C. *cinerea* revealed that during the processes of pileus opening and hydrolysis, the glucanase activity in the extract of the wild type C. *cinerea* pileus was significantly higher than in the extract of the mutant C. *cinerea* pileus, suggesting that glucanase plays a major role in the autolysis of the C. *cinerea* pileus. When the expression levels of 43 glucanases, which mainly act on β-1,3-glycosidic bonds, were examined in the C. *cinerea* genome, we found that the expression levels of the four glucanases mentioned above that we previously purified and an additional glucanase were significantly higher than the expression levels of other 38 glucanases in the wild type C. *cinerea* in pileus during its opening process and that their expression levels were significantly suppressed in the mutant C. *cinerea* pileus, which does not undergo autolysis, during its opening process. This result suggests that these five glucanase genes may play key roles in the pileus autolysis process of C. *cinerea* (Liu *et al.* 2015).

Apart from the five glucanase genes that may be directly associated with pileus autolysis, there are another 38 functionally similar glucanase genes of the 43 glucanase genes of C. *cinerea* that mainly act on β-1,3-glycosidic bonds. Their expression levels are not significantly different between wild type and mutant C. *cinerea*, and most of them belong to glycoside hydrolase families 2 and 16 (Liu *et al.* 2015). Generally, it is thought that β-glucanase not only plays a role in degrading the cell wall (Fontaine *et al.* 1997; Mouyna *et al.* 2002; Liu *et al.* 2015; Zhou *et al.* 2015) and remodelling cell wall components and structures (Mouyna *et al.* 2000; Cole & Hung 2001; Hung *et al.* 2001; Delgado *et al.* 2003) during the growth, development and morphogenesis of fungi, but also involve in degrading the nutritional components in the culture medium to maintain the energy and nutrition needed for cell growth (Nombela *et al.* 1988; Larriba *et al.* 1993; Fukuda *et al.* 2008). The synthesis of some specific β-glucanases was reported to depend on the induction of some specific polysaccharides in the medium (Jayus *et al.* 2002). To determine wheather those rest of the 38 glucanases play a roles in the degradation of culture medium or the other physiological process during the growth and development of C. *cinerea*, we selected a glucanase from the 38-glucanases, an endo-β-1,3(4)-glucanase (ENG16A) (GenBank accession: XP_001828985), which belongs to glycoside hydrolase family 16, as the study subject. After cloning, the selected gene was heterologously expressed in yeast *Pichia pastoris*, and the properties and possible physiological functions of this enzyme were studied.

Materials and methods

Construction of the expression strain

The mycelia and fruiting bodies of *Coprinopsis cinerea* were cultured in a straw medium containing 88 % dry rice straw, 5 % wheat bran, 3 % corn flour, 2 % fertilizer, 1 % lime powder, 1 % sucrose and water quantum satis, as described by Zhou *et al.* (2015). The total RNA was extracted from the fruiting body of C. *cinerea* using the Spin Column Fungal Total RNA Purification Kit (Bio Basic, Amherst, NY, USA) and the synthesis of the cDNA was carried out using the HiScript II Q RT SuperMix for qPCR kit (+gDNA wiper) (Vazyme, Nanjing, China). The upstream primer *eng16A*-F and downstream primer *eng16A*-R were used to amplify the cDNA of the *eng16A* gene from the total cDNA by PCR, in which the first 72 bp nucleotide sequence encoding a signal peptide of 24 amino acids was removed. The PCR product was purified using the Spin Column Gel Extraction Kit (Takara, Beijing, China) and identified by DNA sequencing.

The *eng16A* cDNA fragment and the plasmid pPICZαA (ThermoFisher Scientific, Grand Island, USA) double digested respectively with EcoRI and NotI were ligated to form the plasmid pPICZαA-*eng16A* following the instruction manual of the ClonExpress® Entry One Step Cloning Kit (Vazyme, Nanjing, China). The plasmid pPICZαA-*eng16A* was transformed into *Escherichia coli* DH5α cells for amplification. After producing an expanded culture of *E. coli* DH5α, the pPICZαA-eng16A plasmid was isolated from *E. coli* cells and singly digested by SacI to linearize it. The linearized plasmid was transformed into *Pichia pastoris* GS115 cells by electroporation. The transformants were allowed to grow on YPDS + Zeocin™ (ThermoFisher Scientific, USA) plates for three days, and single colonies were picked to confirm that the *eng16A* gene was successfully integrated into the genome of *P. pastoris* GS115 at the AOX1 site according to the instructions of the Pichia Expression Kit (ThermoFisher Scientific, Grand Island, USA).

Expression of the recombinant enzyme

The *Pichia pastoris* GS115 cells with the integrated *eng16A* gene were inoculated to a 100 mL flask containing 15 mL of BMGY medium and cultured for 18–20 h at 30 °C with shaking at 220 rpm. When the OD_{600} reached 2–6, the yeast cells were harvested by centrifugation at 1000× *g* for 10 min. The yeast cells were resuspended and transferred to a 500 mL flask containing 100 mL BMMY medium for induction cultivation at 30 °C with shaking at 220 rpm for three days; the medium was supplemented with methanol at 0.5 % of the total volume every 24 h to induce expression.

Purification of the recombinant enzyme

The culture medium was centrifuged at 12 000× *g* at 4 °C to remove yeast cells. The supernatant was dialysed at 4 °C overnight against equilibrium buffer (300 mM NaCl, 50 mM NaH_2PO_4, 10 mM imidazole, 10 mM Tris base, pH 8.0). The Ni-affinity chromatography method was then used to purify the recombinant enzyme from the culture supernatant by the manual of the ProteinIso® Ni-NTA Resin (Transgen, Beijing, China). All of the purification steps were carried out at 4 °C.

Protein analysis of the recombinant enzyme

The purity and molecular weight of the purified recombinant enzyme were determined by SDS-PAGE (Laemmli 1970). The protein concentration of the recombinant enzyme was determined using the Bradford method with Coomassie Brilliant Blue (Bradford 1976), and bovine serum albumin was used as the