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journal homepage: www.elsevier.com/locate/funbio

Expression of novel β-glucanase Cel12A from *Stachybotrys atra* in bacterial and fungal hosts

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ARTICLE INFO

Article history:
Received 25 February 2011
Received in revised form
12 January 2012
Accepted 17 January 2012
Available online 25 January 2012
Corresponding Editor:
Simon V. Avery

Keywords:
Aspergillus
β-glucanase
GH12
Stachybotrys

ABSTRACT

β-glucanase Cel12A from *Stachybotrys atra* has been cloned and expressed in *Aspergillus niger*. The purified enzyme showed high activity of β-1,3-1,4-mixed glucans, was also active on carboxymethylcellulose (CMC), while it did not hydrolyze crystalline cellulose or β-1,3 glucans as laminarin. Cel12A showed a marked substrate preference for β-1,3-1,4 glucans, showing maximum activity on barley β-glucans (27.69 U mg^{-1}) while the activity on CMC was much lower (0.51 U mg^{-1}). Analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focussing (IEF), and zymography showed the recombinant enzyme has apparent molecular weight of 24 kDa and a pI of 8.2. Optimal temperature and pH for enzyme activity were 50 °C and pH 6.5. Thin layer chromatography analysis showed that major hydrolysis products from barley β-glucan and lichean were 3-O-β-cellotriosyl-D-glucose and 3-O-β-cellobiosyl-D-glucose, while glucose and cellobiose were released in smaller amounts. The amino acid sequence deduced from *cel12A* revealed that it is a single domain enzyme belonging to the GH12 family, a family that contains several endoglucanases with substrate preference for β-1,3-1,4 glucans. We believe that *S. atra* Cel12A should be considered as a lichenase-like or nontypical endoglucanase.

Introduction

β-1,3-1,4 Glucans are polysaccharides found in the cell walls of higher plants and particularly abundant in the endosperm of cereals as barley, rye, rice, and wheat. They are linear polymers of about 1200 glucose units linked by β-1,3 and β-1,4-glycosidic bonds. The ratio between these two linkages in the β-glucan molecule varies depending on the plant source, β-1,3-glycosidic bonds accounting for about 25–30 % of the linkages (Parrish *et al.* 1960). β-1,3-1,4-glucanases or lichenases (EC 3.2.1.73) hydrolyze the β-1,4-glycosidic bonds adjacent to β-1,3-glycosidic bonds in mixed linked glucans as β-glucan and lichenan, but cannot split the β-1,4-glycosidic bonds in carboxymethylcellulose (CMC) (Parrish *et al.* 1960; Bielecki & Galas 1991; Wolf *et al.* 1995). They produce 3-O-β-cellotriosyl-D-glucose and 3-O-β-cellobiosyl-D-glucose as the major hydrolysis products from β-glucan and lichenan (Anderson & Stone 1975; Planas 2000). Most of the well characterized lichenases are of bacterial origin and belong to family 16 of glycosyl hydrolases (GH16) (Carbohydrate-Active enZYmes [CAZY] database, Henrissat & Davies 1997).

Endoglucanases or endo-1,4-β-glucanases (3.2.1.4) are key enzymes for the hydrolysis of cellulose, the most abundant polymer in the earth. They randomly cleave the internal β-1,4-glycosidic bonds in cellulose chains. Endoglucanases are grouped into several GH families based on amino acid sequence similarities (CAZY). Endoglucanases of family GH12 have been classified into four subfamilies attending to

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doi:10.1016/j.funbio.2012.01.004

sequence homology (Goedegebuur et al. 2002). Two these subfamilies (12-1 and 12-2) are only comprised by fungal enzymes, all of them devoid of carbohydrate binding modules (CBM), subfamily 12-3 contains mainly *Streptomyces* enzymes containing a CBM, and a fourth subfamily, 12-4, is comprised by enzymes from thermophiles. GH12 endoglucanases differ in substrate specificity, from endoglucanases that typically show high activity on CMC to enzymes that show a remarkable preference for mixed β-glucans. However, analysis of the biochemical properties of more GH12 enzymes is to be required to ascertain the molecular traits related to substrate specificity and to understand the role of these enzymes in cellulose depolymerization.

Cellulases, including members of the GH12 family, are widely used in a number of industrial applications, such as detergent, paper, textile, food, and for bioethanol (Clarkson et al. 1992; Lange 1993; Bayer et al. 1994; Béguin & Aubert 1994; Maurer 1997; Bhat 2000; Margeot et al. 2009). On their side, lichenases have more specific applications, such as in the brewing industry to facilitate the filtration process and to reduce gels and haze formation in the finished products (Bamforth 1982), and in animal foodstuffs to improve the β-glucan digestibility (Dierick 1989).

Stachybotrys atra is a cellulolytic microorganism that is frequently isolated from agricultural wastes and cellulosic materials (Abdelmallek 1994). The production of toxins by several strains of this microorganism has probably determined the lack of in-depth studies of cellulose degradation by this fungus. We have previously identified the nontoxigenic strain *S. atra* BP-A, from a rotten cellulose rag, that exhibits a powerful enzyme system for the degradation of cellulosic materials (Picart et al. 2008). We report here the cloning and expression of one of these enzymes, Cel12A, in the host *Aspergillus niger*. The enzyme has been purified and characterized showing preference for the hydrolysis of β-1,4 bonds in mixed glucans. The results contribute to the understanding of the role of family GH12 enzymes in the hydrolysis of biomass.

Materials and methods

Bacterial strains and plasmids

Stachybotrys atra BP-A was cultured as described (Picart et al. 2008). *Escherichia coli* BL21(DE3) and pET28a were used as host strain and plasmid vector, respectively to express *cel12A*. *Escherichia coli* BL21(DE3)/pET28a-cel12A was cultured in 50 μg ml^{-1} kanamycin supplemented LB at 37 °C. Plasmid DNA was isolated with QIAprep spin miniprep kit isolation columns (Qiagen). *Aspergillus niger* var. *awamori* AP4 (Berka & Barnett 1989) and pRAXdes2 (US2007/0173431A1) were used as fungal host strain and plasmid vector to express *cel12A*. *Aspergillus niger*/pRAXdes2-cel12A was cultured in Robosoy medium at 37 °C. Its composition per litre is: Tryptone 12 g, Soyton 8 g, $(NH_4)_2SO_4$ 15 g, $NaH_2PO_4 \cdot H_2O$ 12.1 g, $Na_2HPO_4 \cdot 2H_2O$ 2.19 g, 5 ml 20 % $MgSO_4 \cdot 7H_2O$, 10 ml 10 % Tween 80, 300 ml 50 % maltose, 50 ml 1 M phosphate buffer pH 5.8.

Cloning of cel12A *in* Escherichia coli

Chromosomal DNA from *Stachybotrys atra* was isolated according to Möller & Peltola (2001). Degenerated primers Atra12FWD (5′-CGARCTNATGATHTGG-3′) and Atra12BWD (5′-GTAAANGGYTCRGTRCC-3′) were designed according to Goedegebuur et al. (2002) and used to amplify an internal fragment of *cel12A* gene from *S. atra*. The full-length sequence of *cel12A* was determined by PCR based genome walking from the fragment amplified by the procedures described by Clon-Tech Laboratories, using the Universal genome walker kit (Protocol PT 3042-1). The sequence was submitted to GenBank with the accession number AM180511. After unravelling the full-length sequence of the gene, cDNA was obtained by fusion PCR using chromosomal DNA as a template. A first PCR step with specific fusion primers was performed to amplify the fragments of the gene corresponding to exons in three separated PCR amplifications. The purified three fragments of this first PCR step were used as a template in a second PCR amplification with the external *cel12A* primers. The specific fusion primers were:

Pint1Fwd
5′CGGAGATTATGAGCTTATGATCTGGCTTGGACGCCTTGG CAATGTCTA3′;
Pint1Bwd
5′TAGACATTGCCAAGGCGTCCAAGCCAGATCATAAGCTCA TAATCTCGG3′;
Pint2Fwd
5′ATGGCTCAGTGCCGAATTGGTAGGTGATGAGGTACTGCG AGTCGGCAG3′;
Pint2Bwd
5′CTGCCGACTCGCAGTACCTCATCACCTACCAATTCGGCA CTGAGCCAT3′.

The external primers were: **GH12outerFwd** 5′CCCATATGC AGTCGCTTTGC3′ and **GH12outerBwd** 5′GAAAGCTTTAGTTG TTTTGTTGGGC3′. They contained *Nde*I or *Hind*III restriction sites, respectively, and were designed to amplify *cel12A* devoid of its signal peptide and linked to a *N*-terminal His tail from pET28a expression vector. Amplified *cel12A* cDNA was doubly digested with *Nde*I and *Hind*III, and ligated to plasmid pET28a, previously digested with the same enzymes. The plasmid obtained was transformed in *E. coli* BL21(DE3) as described (Ausubel et al. 1999). Recombinant strain *E. coli*/pET28a-cel12A was selected from LB agar plates supplemented with 0.5 % lichenan, which after the colony growth were stained with 0.1 % Congo Red and distained with NaCl to visualize clear halos of lichenan degradation. The recombinant strain selected was cultured in 0.2 L of LB supplemented with 50 μg ml^{-1} kanamycin at 37 °C up to 0.5 OD_{600}. Then, 1 mM isopropyl-β-D-thiogalactoside (IPTG, Merck) was added, the temperature was adjusted down to 16 °C and the culture was further incubated for 16 h. Cells were collected by centrifugation, suspended in 20 ml of binding buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazol, pH 8.0) and disrupted by sonication. Cel12A was purified from clarified supernatants by affinity chromatography to a Ni^{+}-trapped column.

A similar construction to express the exonic sequence of *cel12A* with its signal peptide was constructed using the