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Comparative characterization of a recombinant *Volvariella volvacea* endoglucanase I (EG1) with its truncated catalytic core (EG1-CM), and their impact on the bio-treatment of cellulose-based fabrics

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Abstract

Recombinant *Volvariella volvacea* endoglucanase 1 (EG1) and its catalytic module (EG1-CM) were obtained by expression in *Pichia pastoris*, purified by two-step chromatography, and the catalytic activities and binding capacities were compared. EG1 and EG1-CM exhibited very similar specific activities towards the soluble substrates carboxymethyl cellulose, lichenan and mannan, and insoluble H_3PO_4 acid-swollen cellulose, whereas the specific activities of EG1-CM towards the insoluble substrates α-cellulose, Avicel and filter paper were approximately 58, 43 and 38%, respectively compared to EG1. No increase in reducing sugar release was detected in the reaction mixture supernatants after 50 h exposure of filter paper, Avicel or α-cellulose to EG1-CM, whereas increases in the total reducing sugar equivalents (i.e. reducing sugar released into solution together with new reducing ends generated in the cellulosic substrates) in reaction mixtures were observed after 1 h. In reaction mixtures containing EG1, soluble reducing sugar equivalents were detected in supernatants after 3 h incubation with the insoluble cellulosic substrates. EG1-CM did not adsorb to Avicel, and the binding capacities of EG1-CM towards filter paper and H_3PO_4 acid-swollen cellulose were 27.9–33.3% and 29.6–60.6%, respectively of values obtained with EG1 within the range of total added protein. In enzymatic deinking experiments, the ink removal rate in EG1-CM-treated samples was only slightly higher (∼8%), than that of untreated controls, whereas that of the EG1-treated samples was 100% higher. Bio-stoning of denim with EG1-CM resulted in increases of 48% and 40% in weight loss and indigo dye removal, respectively compared with untreated controls. These increases were considerably lower than the corresponding values of 219% and 133% obtained when samples were treated with EG1.

Keywords: Catalytic module (CM); Cellulose binding module (CBM); Endoglucanase 1; *Volvariella volvacea*; Deinking

1. Introduction

Cellulases have a wide range of applications in biotechnology including stonewashing of denim garments in the textile industry and bio-deinking of waste paper in the pulp and paper industry (Bhat, 2000; Jeffries et al., 1994). These benefits are the result of the hydrolytic action of a soluble enzyme on an insoluble substrate in a controlled and desired manner. Therefore, a good understanding of the impact of cellulase action in these applications is essential.

Most cellulases consist of a catalytic module (CM) connected by a glycosylated linker to a cellulose-binding module (CBM) (Linder and Teeri, 1997). It is believed that the first step in enzymic hydrolysis of cellulose requires the binding of a soluble free enzyme onto an insoluble substrate. Binding to cellulose is mediated by the CBM, which retains its function even when separated from the remainder of the protein (Coutinho et al., 1993; Tomme et al., 1995; Linder and Teeri, 1997). Although enzyme adsorption plays an important role in cellulose degradation, the precise function of CBMs in cellulase-based bio-treatments of fibres such as bio-stoning and bio-deinking has never been clearly defined (Sarisodsuk et al., 1997; Buchert and Heikinheimo, 1998; Lenting and Warmoeskerken, 2001a). The presence of a CBM allowed for the targeting of the enzyme onto the garment and facilitated its involvement in fiber surface

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modification. However, there is also evidence that the presence of CBMs is not essential to endoglucanase activity at high levels of mechanical agitation similar to the process conditions used in the textile industry (Azevedo et al., 2000). Cellulases, both bacterial (*Cellumonas fimi*) and fungal (*H. insolens*), from which the native CBM has been deleted generally decrease indigo staining levels and cause less back-staining than do the intact enzymes (Andreaus et al., 2000).

The impact of different components of cellulases in bio-stoning has been evaluated by several authors. Endoglucanases are known to be key enzymes in bio-stoning and bio-finishing applications (Kumar et al., 1997; Heikinheimo et al., 2000) whereas acid cellulases, mainly from *T. reesei*, are used commercially in bio-stoning due to their low price and their high activity on denim material. Neutral cellulases with a broad pH profile and not affected by the increase in pH that occurs during denim washing due to alkali released from the garment (Miettinen-Oinonen et al., 2004) are preferred. Thus, novel cellulases that are active at neutral pH values and have good denim finishing properties, including low back-staining, are increasingly sought by the textile industry.

Endoglucanase I (EG1), a neutral endoglucanase from the fungus *Volvariella volvacea*, has been shown to have considerable application potential in bio-stoning and bio-deinking. In view of the contradictions in the scientific literature relating to the importance of CBMs in these processes, we have undertaken a comparative study of the biochemical properties and the mode of action of intact EG1 and its truncated catalytic core EG1-CM on various cellulosic substrates in order to clarify the role of CBMs in hydrolysis and their impact on the bio-treatment of cellulose-based fabrics.

2. Materials and methods

2.1. Bacterial and yeast strains, and growth media

Pichia pastoris, strain KM71H, was used for expression of the EG1 catalytic module, and *Escherichia coli* DH5α was used for plasmid construction and propagation. Yeast growth media were prepared according to the *P. pastoris* expression system manual (Invitrogen, Carlsbad, CA).

2.2. Construction of the expression plasmid

The gene fragment encoding the EG1 catalytic module (EG1-CM) along with the linker was amplified by PCR from a plasmid pBluescript-eg1 containing the full-length *V. volvacea eg1* using the following oligonucleotides: 5′-GAAGCTGCAGGCGCCGGACCTACGACAACC-3′ and 5′-TTGTTCTAGATTACACGAATGGTTTCAAAGCC-3′, and Pfu polymerase (Stratagene, La Jolla, CA). After digestion, the fragment was ligated at the Pst1/Xba1 sites of the *Pichia* expression vector pPICZαB to yield the construct, pPICZαB-EG1-CM. The gene insert was confirmed by dideoxy DNA sequencing. Transformation and expression of EG1-CM in *P. pastoris* was carried out according to the manufacturer's protocol (Invitrogen).

2.3. Expression and purification of recombinant EG1-CM

The expression construct pPICZαB-EG1-CM was linearized by Sac I and transformed into *P. pastoris* strain KM71H (his$^-$) by electroporation according to the Invitrogen manual.

Screening for the most efficient EG1-producing transformants under the methanol-inducible AOX1 promoter was carried out according to the Invitrogen manual. Transformants were grown in 25 ml of BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 100 mM potassium phosphate, pH 6.0) in 250 ml flasks at 30 °C and 250 rpm for 16–24 h after which time the cell density reached an OD_{600} value (obtained through dilution and back-calculation) of between 2 and 6. Yeast cells were harvested and re-suspended in 3 ml of BMMY medium (BMGY medium but containing 0.5% methanol instead of glycerol) in 25 ml flasks. Following an additional 2 days induction with 0.5% of methanol at 30 °C and 250 rpm, endoglucanase activity in the supernatant was determined using carboxymethylcellulose (CMC) as the substrate. The clone exhibiting the highest level of EG1-CM expression was selected for time-course and scale-up expression studies. For time-course studies, the transformed yeast cells were grown in 100 ml BMGY medium in 1 L flasks incubated at 30 °C and 250 rpm. After the OD_{600} value reached ∼5, cells were harvested by centrifugation and re-suspended in 15 ml of BMMY medium in 250 ml flasks and the cultures incubated under the same conditions for a further 6 days. Methanol was added to the medium every 24 h to a final concentration of 0.5% in order to maintain the induction. Every 24 h, 0.5 ml of culture was removed from flasks and, after separating yeast cells by centrifugation, EG activity in the supernatants was determined. Scaled-up expression of EG1-CM was carried out in 1 L flasks containing 100 ml BMMY medium under the same conditions.

For purification of recombinant EG1-CM, supernatants from scaled-up cultures were concentrated and dialyzed against 10 mM sodium acetate buffer (pH 4.0). The supernatant was applied to a CM-Sepharose column (2.6 cm × 20 cm) pre-equilibrated with the same buffer (pH 4.0), and the bound recombinant EG1-CM was then eluted with a linear gradient of NaCl (0–2 M) in the same buffer. Fractions containing EG activity were pooled, concentrated, applied to a Sephacryl S100-HR gel filtration column (1.5 cm × 80 cm) (Pharmacia) and eluted with the same buffer. The purity of recombinant EG1-CM and EG1 was analyzed by 10% SDS-PAGE combined with Coomassie blue staining.

2.4. Comparative characterization of recombinant EG1 with EG1-CM

Recombinant EG1 was obtained as described previously (Ding et al., 2002). The substrate specificities of recombinant EG1 and EG1-CM were determined towards CMC (Sigma), H_3PO_4 acid-swollen cellulose, crystalline cellulose (Avicel, Sigmacell 20), filter paper (Whatman), oat spelts xylan (Sigma), birchwood xylan (Sigma), barley β-glucan (Sigma), α-cellulose (prepared from Masson pine (*Pinus massoniana*) bleached kraft pulp according to TAPPI T 203 om 93), chitosan (Fluka), lichenan (Sigma), mannan (Sigma), arabinogalactan (Sigma),