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Molecular characterization of a cold-active recombinant xylanase from *Flavobacterium johnsoniae* and its applicability in xylan hydrolysis

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HIGHLIGHTS

- Discovery of new functions of Fn3.
- An ideal model to study interaction between Fn3 and xylanases or related enzymes.
- Potential applications in food additives industries.
- Help elucidate hemicellulose utilization in flavobacteria.

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ABSTRACT

A novel xylanase gene, *xyn10A*, was cloned from *Flavobacterium johsoniae*, overexpressed in a flavobacterial expression system, the recombinant enzyme purified by Ni-affinity chromatography, and enzyme structure and activity analyzed. Xyn10A was found to be a modular xylanase with an Fn3 accessory domain on its N-terminal and a catalytic region on the C-terminal. The optimum pH and temperature for Xyn10A was 8.0 and 30 °C, but Xyn10A retained 50% activity at 4 °C, indicating that Xyn10A is a cold-active xylanase. A Fn3-deletion xylanase had relative activity *ca.* 3.6-fold lower than the wild-type, indicating that Fn3 promotes xylanase activity. The Fn3 region also contributed to stability of the enzyme at elevated temperatures. However, Fn3 did not bind this xylanase to insoluble substrates. The enzyme hydrolyzed xylo-oligosaccharides into xylobiose, and xylose with xylobiose as the main product, confirming that Xyn10A is a strict endo-β-1,4-xylanase. Xyn10A also hydrolyzed birchwood and beechwood xylan to yield mainly xylose, xylobiose and xylotriose.

1. Introduction

Xylanases (EC.3.2.1.8) are diverse and ubiquitously distributed in xylanolytic microorganisms in nature (Collins et al., 2005). Most of the microbial β-1,4-xylanases (those responsible for cleaving internal β-1,4-xylosic linkages in hemicellulose materials) are grouped into 6 glycoside hydrolases (GH) families: GHs 5, 8, 10, 11, 30 and 43 (Shallom and Shoham, 2003). Currently, the best studied β-1,4-xylanases belong to families GH10 and GH11 (Collins et al., 2005). The catalytic mechanisms involved in degradation of xylan by family GH10 and GH11 have been extensively examined. Due to the increasing number of genome/metagenome sequences available, more and more "novel" β-1,4-xylanases are being discovered by genome mining methods (Kim et al., 2009, 2012).

Like various modular glycoside hydrolases (e.g., microbial cellulase, chitinase, and many others), xylanases are frequently found to be multidomain proteins, with modular structures comprising catalytic and additional ancillary modules (Collins et al., 2005; McBride et al., 2009). The most investigated ancillary structures in xylanases are carbohydrate-binding modules (CBMs) which help either increase the stability of the enzyme or direct it to insoluble substrates (Guillen et al., 2010). Furthermore, xylanases carry multiple modules with homology to those from eukaryote proteins such as Fibronectin types 3 domain (Fn3) (Kim et al., 2009), PKD1 (polycystic kidney disease 1) (Xie et al., 2007), and immunoglobulin-like domains (Xie et al., 2007) in addition to CBMs. However, most of these modules' functions remain a mystery. Among

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the above ancillary modules, Fn3-like sequences draw a lot of interests in bacterial carbohydrases because of their widespread occurrence and critical functions (Kataeva et al., 2002; Kim et al., 2009). Unfortunately, only limited studies have been conducted with xylanases carrying Fn3-like domains. Recently, Kim et al. reported a novel modular xylanase (xylK1) carrying Fn3 that was located between catalytic (GH10) and CBD domains from *Cellulosimicrobium* sp. strain HY-13 (Kim et al., 2009). Deletion of the Fn3 domain in XylK1 greatly decreased catalytic hydrolysis of xylosic substrates, indicating that it functioned critically in xylanase activity rather than as a "long linker" region. They further suggested that Fn3 bound insoluble substrates (Kim et al., 2009). However, due to multiple modular structures appearing in one enzyme, it remained unclear whether loss of Fn3 domain affected the functions of the accessory domains such as CBD(s), or catalytic modules, or both. A further investigation is therefore warranted to elucidate the roles of Fn3 in modular xylanases. The ideal model for such studies will be a simple modular xylanase carrying Fn3 as the single accessory domain.

More and more attention has been drawn to cold-active xylanases because of their high catalytic activity at low temperatures and their inherently broad substrate specificity relative to their thermophilic counterparts (Georlette et al., 2002). These properties allow the use of cold-active xylanase in different applications of the textile, food industries, bioremediation and investigation of proteins' cold-active mechanisms (Collins et al., 2005, 2006; Georlette et al., 2002; Shallom and Shoham, 2003). For example, psychrophilic xylanases from *Pseudoalteromonas haloplanktis* TAH3A (XPH), *Flavobacterium* sp. MSY-2 (rXFH) and unknown bacterial origin (rXyn8) effectively improved the dough properties and final bread volume (up to 28%) (Dornez et al., 2011). Cold-active xylanases are desired due to their high activity at cool temperatures required for dough resting and also to their specific mode of xylan hydrolysis (Dornez et al., 2011). Bacteria of the Bacteroidetes phylum are attractive for these applications due to their xylanolytic and cellulolytic ability. Xylanases from various Bacteroidetes species have many important properties as potential catalysts for biomass hydrolysis, such as function at a wide range of pH and temperature, efficient conversion of plant biomass, and high tolerance to environmental stressors. Until now, however, few cold-active xylanases have been reported and only one cold active flavobacterial xylanase, Xyn10 from *Flavobacterium* sp. MSY2, was characterized (Lee et al., 2006).

As a model microorganism, *Flavobacterium johnsoniae* has been widely investigated for biopolymer degradation in oligotrophic freshwater environments (Sack et al., 2011) and for its gliding mechanism of motility (McBride et al., 2009). The complete genome sequence of *F. johnsoniae* revealed that it carried many genes predicted to encode degradation enzymes for chitin, starch, cellulose, hemicellulose and pectin (McBride et al., 2009). However, compared to those in other Bacteriodetes, the hemicellulose degradation mechanisms in flavobacteria are understudied. No xylanase from *F. johnsoniae* has been investigated so far despite its efficient and common utilization of hemicellulose substrates and its novel conversion mechanisms (McBride et al., 2009). Our lab group is interested in *Flavobacterium* physiology because the genus is prominent in certain larval mosquito habitats. This Bacteroidetes group represented by *Flavobacterium* is potentially important to the growth of mosquito larvae because it likely serves as a food resource and aids in the transformation of particulate organic matter into useful nutritional items for developing larvae (Kaufman et al., 2008).

The purpose of this study was several-fold. Initially, to explore its potential for degradation of hemicellulose, a xylanase-encoding gene, *xyn10A* (Fj_3886), was cloned and over-expressed in *F. johnsoniae*. The recombinant protein, Xyn10A, was purified and characterized. Secondly, the Xyn10A enzyme was compared with other characterized xylanases, in order to reveal its novel features. Lastly, the modular Fn3 domain was experimentally manipulated to understand its function, particularly in relation to xylanolytic activity and thermal stability.

2. Methods

2.1. Bacterial strains, plasmids, and growth conditions

Escherichia coli JM109 or *E. coli* DH5α was used for cloning. *E. coli* S17 (λ *pir*) was used for conjugation. *E. coli* BL21 (DE3) was used for heterologous expression. *E. coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. Casitone yeast extract (CYE) was used for *Flavobacterium* culture (Chen et al. 2010). Liquid cultures were grown with shaking (ca. 200 rpm) at either 30 °C (*Flavobacterium*) or 37 °C (*E. coli*). For solid media, Bacto-Agar (Difco, Detroit, Michigan) was added to a final concentration of 15 g/l. Antibiotics were used at following concentrations: Ampicillin (Ap) at 100 μg/ml and erythromycin (Em) at 100 μg/ml.

2.2. Recombinant DNA methods

Plasmid DNA was purified with the QIAprep spin miniprep kit (Qiagen, Germantown, MD). DNA ligations, restriction endonuclease digestions and agarose gel electrophoresis were performed according to standard techniques (Sambrook et al., 1989). DNA transfers of *E.coli* were carried out by the calcium chloride or electroporation method and with *Flavobacterium* strains by conjugation as described previously (Chen et al., 2010). PCR amplifications were performed with the Failsafe PCR system (Epicenter technology, Madison, WI). PCR products were separated on 1.0% (wt/vol) agarose gels, and the bands were purified with the QiaQuick gel extraction system (Qiagen). Ligation mixtures were transformed into *E. coli* DH5α (Invitrogen), and transformants were selected on LB agar plates with ampicillin.

The *xyn10A* gene with 6× his tag on its 3′-end was engineered with primers Walker64 (GGATCCTTTAAGAAGGAGATATACATATGAAAAGTAAATTTTTATTAATGCTGATAAGCGTCG) and Walker65 (GCATGCTTAGTGATGGTGATGGTGATGATCTAAACCTTCTAAAAATCCGGTATGTGAAC) using the same methods as described above. The amplicon was inserted into T-easy vector (pSCH601), released with BamHI and SphI and inserted into the same sites on Fj29, leading to the expression plasmid pSCH602. To delete Fn3 region, primers Walker74 (CCCCCGGGGGCAACTGGTGTTTCCAGAATTTCAGCAGCG) and Walker75 (CCCCCGGGGGTTCTAATGGCATTCCCGAAGATCCTACTTTTTTAAAGG), which were designed at the reverse direction with SmaI restriction site on 5′-end, were used to amplify the plasmid pSCH601. The amplicon was digested with SmaI, self-ligated and transformed into *E. coli* strain, leading to pSCH627. The insert in pSCH627 carrying an Fn3-deleted xyn10A modification was released by BamHI and SphI and cloned into Fj29, creating plasmid pSCH643 (Xyn10AΔFn3). Fn3-mutant Xyn10A was created by replacing the putative Fn3 region (Asp36-Thr121) by adding proline and glycine between Ala35 and Gly122 (positions refer to those in WT). pSCH602 and pSCH643 were conjugatively transferred into *F. johnsoniae*, leading to over-expression strains SCH605 (Xyn10A) and SCH645 (Xyn10AΔFn3), respectively.

2.3. Protein production, purification and detection

To express the xylanase genes in *F. johnsoniae*, cells carrying the over-expression construct (SCH605 or SCH645) were grown at 30 °C in CYE medium containing 100 μg/ml Em for 24 h. *F. johnsoniae* cells were stored at −20 °C before further proceeding.