Xylan-specific carbohydrate-binding module belonging to family 6 enhances the catalytic performance of a GH11 endo-xylanase

Zaira B. Hoffmam[1], Letícia M. Zanphorlin[1], Junio Cota[1], José A. Diogo[1], Gabriela B. Almeida[1], André R.L. Damásio[1], Fabio Squina[1], Mario T. Murakami[2] and Roberto Ruller[1]

[1] Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais, Campinas, São Paulo, Brazil
[2] Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais, Campinas, São Paulo, Brazil

Xylanases catalyze the hydrolysis of β-1,4-linked xylosyl moieties from xylan chains, one of the most abundant hemicellulosic polysaccharides found in plant cell walls. These enzymes can exist either as single catalytic domains or as modular proteins composed of one or more carbohydrate-binding modules (CBMs) appended to the catalytic core. However, the molecular mechanisms governing the synergistic effects between catalytic domains and their CBMs are not fully understood. Thus, the goal of this study was to evaluate the functional effects of the fusion of a CBM belonging to family 6, which exhibits high affinity to xylan, with the GH11 xylanase from *Bacillus subtilis*, which does not have a CBM in its wild-type form. The wild-type enzyme (BsXyl11) and the chimeric protein (BsXyl11-CBM6) were heterologously produced in *Escherichia coli* and purified to homogeneity for biochemical characterization. The molecular fusion did not alter the pH and temperature dependence, but kinetic data revealed an increase of 65% in the catalytic efficiency of the chimeric enzyme. Furthermore, the BsXyl11-CBM6 chimera was used to supplement the commercial cocktail Accellerase® 1500 and improved the reducing sugar release by 17% from pretreated sugarcane bagasse. These results indicate that CBM6 can be used as a molecular tool to enhance the catalytic performance of endo-xylanases (GH11) and provide a new strategy for the development of optimized biocatalysts for biotechnological applications.

Introduction

Endo-β-1,4-xylanases (EC 3.2.1.8) catalyze the cleavage of glycosidic bonds between β-1,4-xylopyranosyl residues in xylan chains. The action of endo-β-1,4-xylanases on xylan produces xylooligomers, which can be broken down further by β-1,4-xylosidases (EC 3.2.1.37) into xylose [1,2]. Xylose and arabinoxylan are, respectively, the main monosaccharide and polysaccharide found in the hemicellulosic fraction of sugarcane [3]. Annually in Brazil, more than 200 million tons of sugarcane bagasse are generated from first-generation ethanol mills. Although 45% of sugarcane bagasse is composed of cellulose, hemicellulose remains an important fraction of bagasse polysaccharides, constituting on average 27% of this raw material [4,5]. On the biorefinery concept, the sugars from bagasse, if hydrolyzed to its primary monosaccharides, can be fully used to produce biofuels and organic acids, making the chemical industry eco-sustainable and renewable [6]. Despite efforts, the development of an efficient process with low-cost enzymatic hydrolysis for biomass saccharification remains the major challenge [7,8].

For depolymerization of lignocellulosic material, a broad arsenal of glycosyl hydrolases (cellulases and hemicellulases) and accessory enzymes, involving different modes of action and synergistic cooperation between them, are required [9]. In particular,

Corresponding author. Ruller, R. (roberto.ruller@bioetanol.org.br)

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endo-β-1,4-xylanases are key enzymes in the saccharification process of hemicellulosic polysaccharides due to their ability to release xylooligosaccharides from xylan polymers [10]. Moreover, endo-β-1,4-xylanases have several industrial uses, such as in food processing, animal feed and kraft pulp biobleaching [11]. These endolytic enzymes are present in several GH families according to the CAZy classification [12,13], with great predominance of the GH10 and GH11 families. Typically, GH10 family members exhibit high molecular weight (around 40–60 kDa) and low p*I*, while GH11 enzymes are characterized by a smaller catalytic domain (around 20–30 kDa) and higher p*I* values [1]. Both families catalyze xylan hydrolysis through the retaining mechanism [10]. These enzymes can either be found in nature as single catalytic domains or fused to one or more additional modules, catalytic or non-catalytic [14]. The non-catalytic modules, commonly referred to as carbohydrate-binding modules (CBMs), are known to enhance the catalytic performance of some enzymes, but the mechanisms associated with this effect still remain unclear. Some studies suggest that CBMs increase the concentration of enzymes on the substrate surface, altering the interactions between the enzyme and the polysaccharide [10,15]. To date, there are 71 families of CBMs cataloged in the CAZy database [16] (see http://www.cazy.org/Carbohydrate-Binding-Modules.html) and this number is growing continuously, as also observed for the glycosyl hydrolase superfamily, indicating our still limited knowledge regarding nature's strategies for carbohydrate synthesis, modification and degradation. For instance, the binding specificity of CBMs from families 1, 10 and 20 are very similar; all bind to highly crystalline cellulosic polysaccharides [16,17], whereas the specificity of others families, for example 6 and 35, can be variable [14].

Previous studies have shown that the enzymatic activity of certain cellulases was improved by fusion with CBMs [18,19]. However, there are few reports describing the modulation of xylanase activity by CBMs [15]. In this work we investigated the effect of a xylan-specific CBM belonging to the family 6 (CBM6) on the enzymatic performance of a mesophilic xylanase from the GH11 family. Interestingly, the novel chimeric enzyme (BsXyl11-CBM6) had a higher catalytic efficiency (65%) and also was able to enhance a multi-enzyme commercial preparation, indicating it to be a biotechnologically-relevant biocatalyst aiming at plant cell-wall deconstruction.

Materials and methods

Molecular cloning and chimera design

The BsXyl11-CBM6 chimera was constructed using the CBM6 from the Cthe_1963 gene, a multi-domain enzyme from *Clostridium thermocellum*. A linker of 78 base pairs from *Thermotoga petrophila* RKU-1 (NCBI-GI: 148270033) was used to connect the two modules. The chimeric gene was built by PCR overlap [20] using the genomes from *Bacillus subtilis* 168 and *Clostridium thermocellum* as template for PCR amplification of BsXyl11 (NCBI-GI: 16078944) and Cthe_1963 CBM6 (NCBI-GI: 125974464), respectively. The PCR assays were performed as recommended by the manufacturer of the Phusion High-Fidelity DNA polymerase (New England BioLabs Inc., USA). Figure 1 shows more details about the primers and templates used in the PCR experiments for chimera construction. Further, the chimeric gene was cloned into the pET28a vector (Merck) for expression and the sequence was confirmed by Sanger DNA sequencing (3500xL Genetic Analyzer, Applied Biosystems, USA).

Heterologous expression and purification

For heterologous expression, a single colony of *E. coli* BL21 pRARE2 harboring the pET28a/BsXyl11-CBM6 plasmid was inoculated in liquid LB broth (500 ml) and expression and purification steps were performed as previously reported [21]. Protein concentration was determined by absorbance at 280 nm using molar extinction coefficient calculated from the amino acid composition (supplementary material, Table S1).

Biochemical characterization and kinetic parameters

The xylanase activity was assayed against 0.5% *(w/v)* of beechwood xylan, oat spelt xylan and rye arabinoxylan (Megazyme, Ireland). All assays were performed with purified enzymes at a final concentration of 0.017 μM. This concentration was used with reference to the enzymatic activity curve with increasing concentrations of enzyme in order to ensure that there was no substrate limitation (Fig. S1, supplementary material) [11]. The apparent optimum pH was determined at 50°C using beechwood xylan as substrate in 100 mM citrate–phosphate buffer plus added glycine 0.1 M, in the range of pH 3.0–10.0. The apparent optimum temperature was determined using beechwood xylan as substrate in the same buffer at pH 6.0 in the temperature range from 25 to 75°C. The xylanase activity assays were performed according to the methodology described by Bailey [22] adapted to 96-well reaction plates. All assays were conducted in 100 μl reactions using an automated liquid handling robot (liquid handler epMotion – Eppendorf Corp., USA). At the end of incubation period at an appropriate temperature for each experiment, 100 μL of DNS reagent was added and boiled at 99°C for 15 min. In order to quantify the reducing sugar that was released, the absorbance was measured at 540 nm and the value was compared to a standard xylose curve. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute [23]. The kinetic parameters were determined using 50 mM sodium phosphate buffer (pH 6.0) at 50°C. The activities of BsXyl11 and BsXyl11-CBM6 enzymes were measured in 12 different concentrations of beechwood xylan from 0.25 to 24 mg/ml. Data analysis was performed in the GraphPad Prism 5 software adjusting the non-linear fit of Michaelis–Menten. All the assays were performed in triplicate.

Capillary zone electrophoresis of oligosaccharides (CZE)

The hydrolysis of beechwood xylan (0.5%) was carried out in 50 mM sodium phosphate buffer (pH 6.0) at 50°C using BsXyl11 and BsXyl11-CBM6 purified enzymes at final concentration of 0.017 μM. The hydrolysis products were derivatized with 8-aminopyreno-1,3,6-trisulfonic acid (APTS) by reductive amination [24]. The CZE analyses were performed in a P/ACE MQD instrument (Beckman Coulter, USA) equipped with laser-induced fluorescence detector. The experimental conditions and data analysis were performed as previously described [21].

Sugarcane bagasse hydrolysis and cocktail supplementation

50 mg of sugarcane bagasse pretreated by steam explosion and delignified using a standardized method [25,26] were incubated

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