Insect Biochemistry and Molecular Biology 43 (2013) 970–981

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Deciphering the synergism of endogenous glycoside hydrolase families 1 and 9 from *Coptotermes gestroi*

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ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form
24 July 2013
Accepted 25 July 2013

Keywords:
Termite
Glycoside hydrolase
Molecular docking
Coptotermes gestroi
Synergistic interaction

ABSTRACT

Termites can degrade up to 90% of the lignocellulose they ingest using a repertoire of endogenous and symbiotic degrading enzymes. Termites have been shown to secrete two main glycoside hydrolases, which are GH1 (EC 3.2.1.21) and GH9 (EC 3.2.1.4) members. However, the molecular mechanism for lignocellulose degradation by these enzymes remains poorly understood. The present study was conducted to understand the synergistic relationship between GH9 (*Cg*EG1) and GH1 (*Cg*BG1) from *Coptotermes gestroi*, which is considered the major urban pest of São Paulo State in Brazil. The goal of this work was to decipher the mode of operation of *Cg*EG1 and *Cg*BG1 through a comprehensive biochemical analysis and molecular docking studies. There was outstanding degree of synergy in degrading glucose polymers for the production of glucose as a result of the endo-β-1,4-glucosidase and exo-β-1,4-glucosidase degradation capability of *Cg*EG1 in concert with the high catalytic performance of *Cg*BG1, which rapidly converts the oligomers into glucose. Our data not only provide an increased comprehension regarding the synergistic mechanism of these two enzymes for cellulose saccharification but also give insight about the role of these two enzymes in termite biology, which can provide the foundation for the development of a number of important applied research topics, such as the control of termites as pests as well as the development of technologies for lignocellulose-to-bioproduct applications.

1. Introduction

Termites are considered the smallest bioreactor in the world (Brune, 1998). The termite's digestome can degrade up to 90% of the lignocellulosic biomass ingested (Katsumata et al., 2007) using a repertoire of endogenous and symbiotic degrading enzymes, accessory proteins, and putative ligninases (Chandrasekharaiah et al., 2011; Coy et al., 2010; Hongoh, 2011; Tartar et al., 2009; Todaka et al., 2010, 2007; Warnecke et al., 2007). The termite gut is generally divided in three compartments: foregut, midgut and hindgut. After the termites ingest lignocellulose material, the biomass is reduced to about 20 μm by their mandibles (Fujita et al., 2010), then further exposing the material to the degradation of endogenous enzymes, such as endo-β-1,4-glucosidase (GH9), β-glucosidase (GH1) (Brune and Ohkuma, 2011; Ohkuma, 2003; Ohkuma et al., 2008), laccases and esterases in the foregut (Coy et al., 2010; Sethi et al., 2013; Wheeler et al., 2010). These

Abbreviations: APTS, 8-aminopyreno-1,3,6-trisulfonic acid; BIN, integral sugarcane bagasse; CD, circular dichroism; CMC, carboxymethyl cellulose; CZE, capillary zone electrophoresis; DNS, 3,5-dinitrosalicylic acid; DS, degree of synergism; GH, glycoside hydrolase; GOD, glucose oxidase; IMAC, immobilized metal affinity chromatography; PASB, phosphoric acid pretreated sugarcane bagasse; *p*-NP, *p*-nitrophenyl.

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http://dx.doi.org/10.1016/j.ibmb.2013.07.007

enzymes promote biomass depolymerization together with a structure called pro-ventricle or gizzard, which further reduce the particle size to 10 μm (Fujita et al., 2010). Then the material reaches the midgut, where some glucose can be assimilated, and later, the hindgut or fermentative chamber, populated by protozoan symbionts that express several cellulases from families GH5, GH7, GH45 and hemicellulases from families GH10 and GH11 (Brune and Ohkuma, 2011; Ohkuma, 2003; Ohkuma et al., 2008).

The endo-β-1,4-glucosidase (GH9) and β-glucosidase (GH1) enzymes from termite salivary glands are the most characterized enzymes from the termite digestome (Cantarel et al., 2009; Ni et al., 2005, 2007; Scharf et al., 2011; Scharf et al., 2010; Uchima et al., 2011; 2012, 2013; Zhang et al., 2012, 2010, 2009, 2011, 2010, 2009; Zhou et al., 2010; Jeng et al., 2011; Khademi et al., 2002). However, the molecular mechanisms involved in the synergistic lignocellulose degradation by these enzymes remain poorly understood (Scharf et al., 2011; Zhang et al., 2010).

Plant feedstocks have been implicated as a promising renewable alternative to petroleum and natural gas, mainly because lignocellulosic resources have the most favorable net energy ratio to maintain environmental sustainability (Hoogwijk et al., 2005). However, the precise breakdown of lignocellulosic biomass is a complex and lengthy process. The current technological schemes for bioconversion of plant cell wall polysaccharides into simple sugars rely on first reducing biomass recalcitrance through a pretreatment step and then, enzymatic cocktails are used where the main components are glycoside hydrolases (GHs). Fermentable saccharides can feed several bioprocesses, such as bioethanol production (Soccol et al., 2010), or the manufacture of bioplastics (Rincones et al., 2009).

The concerted activity of at least three enzymes are needed for cellulose breakdown, endo-β-glucanase, exo-β-glucanase (in some cases, termed cellobiohydrolase), and β-glucosidase (van den Brink and de Vries, 2011). According to several studies (Converse and Optekar, 1993; Murashima et al., 2002; Woodward et al., 1988), the fungal cellobiohydrolases are key enzymes for efficient cellulose saccharification. Additionally, two main types of exocellulases (GH7 and GH6) work together processively from the reducing end and the nonreducing end of cellulose, respectively (Lynd and Zhang, 2002). In contrast, termites can efficiently metabolize lignocellulose without secreting a "true" endogenous cellobiohydrolase, which is commonly found in fungal secretomes. For instance, the lower termite *Coptotermes gestroi*, considered the major urban pest in Brazil (Jenkins et al., 2007), can hydrolyze almost all types of glycoside bonds found in natural polysaccharides (Franco Cairo et al., 2011), but it secretes only two main GHs, a GH9 (*Cg*EG1) and a GH1 (*Cg*BG1). These two enzymes, according to RNA expression studies, are upregulated in work caste suggesting a response to the digestion of lignocellulosic materials (Leonardo et al., 2011), once this caste is responsible for colony feeding (Barsotti and Costa-Leonardo, 2005).

This study aims to gain a thorough understanding of the synergistic relationship between *Cg*EG1 and *Cg*BG1 from *C. gestroi*. The mechanism of action of these enzymes was comprehensively evaluated through enzymatic assays with 8-aminopyreno-1,3,6-trisulfonic acid (APTS)-labeled oligosaccharides and computational docking. The biochemical parameters including substrate specificity, kinetics, thermal unfolding and conformational stability, after the recombinant expression of genes coding for the endo-β-1,4-glucosidase (GH9) and β-glucosidase (GH1) from *C. gestroi*, were pursued in the present work. Our data not only provide an increased comprehension regarding the synergistic mechanism of these two enzymes for cellulose saccharification but also give insight about the role of these two enzymes in termite biology. Investigating this aspect of termite biology may facilitate important applied research topics such as various lignocellulose-to-bioproduct applications.

2. Materials and methods

2.1. Termites

Specimens of *C. gestroi* were collected from field colonies with traps of corrugated cardboard and from incipient colonies reared in the Termite Laboratory of the Biology Department, UNESP, Rio Claro, São Paulo, Brazil (22° 23′S, 47° 31′W). Termites were kept at 25 ± 2 °C. Worker termites were manually selected and frozen at − 80 °C.

2.2. Sequences and phylogenetic analyses

The ORF Finder tool (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to predict the mature protein sequences from the gene sequences of endo-β-1,4-glucosidase (GH9) named *Cg*EG1 and β-glucosidase (GH1) named *Cg*BG1. The protein sequences were analyzed for physical and biochemical parameters by ProtParam tool on the EXPASy server and the signal peptide was predicted by CBS server using SignalP 3.0 (Bendtsen et al., 2004).

2.3. RNA extraction, cDNA construction and gene cloning

About 50 worker termites were utilized for total RNA isolation using TRIzol reagent (Invitrogen). RNA was purified with an RNeasy® Mini Kit (Qiagen). An aliquot of 2 μg of total RNA were reverse transcribed utilizing SuperScript II Reverse Transcriptase (Invitrogen) and the primer oligo dT [5′-GGCGGCCGCACAACTTTG TACAAGAAAGTTGGGT(T)19-3′]. The cDNA was used as a template for the touchdown PCR method for cloning the enzymes. The full-length sequence of *Cg*EG1, excluding the signal peptide, was amplified using Phusion *pfu* DNA polymerase (following guide protocol instructions) with the primers EG1F 5′ − ATATA**GCTAGC**GC TTACGACTACAAGACAGTACTG − 3′ and EG1R 5′ − ATATA**GGATCC** TTAAACGCCCAACGTGACGAGAG − 3′. The PCR was conduced in two rounds under following parameters. First round: denaturation at 98 °C for 2 min; followed for 10 cycles of denaturation at 98 °C for 30 s; annealing at 60 °C for 45 s; extension for 2 min at 72 °C. The annealing temperature was reduced 1 °C for cycler. Second round: denaturation at 98 °C for 30 s; annealing at 50 °C for 30 s; extension for 2 min at 72 °C during 35 cycles and final extension of 10 min.

The full-length sequence of *Cg*BG1, excluding the signal peptide, was amplified with the same polymerase and the following primers: BG1F 5′ − ATATA**GCTAGC**GATGACGTCGATAACGAGACC CTT G − 3′ and BG1R 5′ − ATATA**GGATCC**TTAGTCTCGGAAGCGCTCT GGAATC − 3′. First round: denaturation at 98 °C for 2 min; followed for 20 cycles of denaturation at 98 °C for 30 s; annealing at 65 °C for 45 s; extension for 2 min at 72 °C, The annealing temperature was reduced 1 °C for cycler. Second round: denaturation at 98 °C for 30 s; annealing at 48 °C for 30 s; extension for 2 min at 72 °C during 35 cycles and final extension of 10 min. These primers included restriction sites for *Nhe*I and *BamH*1 (indicated in bold) in forward and reverse primers respectively, for cloning the gene in the correct reading frame into a pET28a expression vector with an N-terminally fused 6 × His-tag.

The amplicons were purified from a 1% agarose gel by electrophoresis with 0.3% crystal violet as dye using the Illustra GFX PCR DNA − Gel Band Purification kit (GE Healthcare). The purified amplicons were subjected to double digestion with *Nhe*I and *BamH*1 (BioLabs). Following a new purification, the amplicons were ligated into a pET28a vector (Novagen) at the corresponding restriction sites using T4 Ligase (Promega). The recombinant pET28a